KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMAS I

COLLEGE OF HEALTH SCIENCES

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

DEPARTMENT OF PHARMACEUTICS

ANTIMICROBIAL PRODUCING PROPERTIES OF MICROORGANISMS ISOLATED FROM SOIL, SEA AND THE KAKUM RIVER

BY

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AUGUST, 2016

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DECLARATION

I hereby declare that this dissertation is my own work toward the M. Phil degree 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 in this university or elsewhere, except where due acknowledgement has been made in the text.

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DEDICATION

To my parents Mr. and Mrs. Nyamena for their love, encouragement and financial support throughout my education.

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ABSTRACT

Infectious diseases such as tuberculosis (TB), pneumoniae, diarrhoea and urinary tract infections continue to pose threat to lives of humans and animals as many causative pathogens have developed resistance to the clinically available antibiotics. There is therefore the need to develop new and potent antimicrobial agents for treatment of these infections. The study aimed at investigating the antimicrobial properties of microorganisms isolated from the Ghanaian environment. A total of 24 samples (8 soil samples from Ejisu, Ashanti region, 7 Sea samples and 9 samples from Kakum River in the Cape Coast Metropolis) were collected and cultivated on Humic Acid Vitamin (HAV) and Glycerol Asparagine (GA) agars. The microorganisms isolated (138) were screened for antimicrobial agents production using the cross streaking (CS) and agar well diffusion (AD) methods, and 36 isolates showed zones of inhibition (CS: 2.3±0.3 – 35.3±1.5mm; AD: 15.0±0.6 – 35.3±0.3mm) against the test organisms used. Ethyl acetate extract of metabolites from isolate, $GKSE_1$, showed growth inhibitory activity when assayed and its minimum inhibitory concentrations against the test organisms were 1.563 mg/mL (for *Staphylococcus aureus* and *Salmonella typhi*), 3.125 mg/mL (for *Staphylococcus epidermidis, Shigella dysentriae, Streptococcus pyogenes, Pseudomonas aeruginosa, Escherichia coli,* and *Enterococcus faecalis*) and 6.250 mg/mL (*Klebsiella pneumoniae*, *Salmonella typhorium*, and *Bacillus subtilis*). The bioactive component of the extract was found to be heat stable and also stable in aqueous solution for a period of 12 weeks. The extract was also found to reduce biofilms formed by *P. aeruginosa, E. coli* and *S. aureus* but did not show biosurfactant activity. TLC analysis of the extract of $GKSE_1$, revealed five (5) spots with two regions of the chromatogram showing inhibitory activity against *B. subtilis* and *K. pneumoniae* in a bioautography assay. The isolate $GKSE_1$, was found to be a gram positive cocci, oxidase and catalase negative. Microorganisms isolated from soil, Sea and the Kakum River have the potential of producing antimicrobial agents with the isolate $GKSE_1$ having the potential of producing novel compounds for development into potent antimicrobial agents.

CHAPTER ONE

GRNERAL INTRODUCTION

The lives of both humans and animals are threatened by so many kinds of infectious diseases. An infectious disease is a disease that may be caused by a virus, bacteria, fungi, protozoa or helminth. Examples of infectious diseases include tuberculosis (TB; caused by *Mycobacterium tuberculosis*), cholera (caused by *Vibrio cholerae*), candidiasis (caused by species of *Candida*), malaria (caused by *Plasmodium species*), Trypanosomiasis (caused by *Trypanosoma species*), schistosomiasis (caused by *Schistosoma species*). Examples of viral infections also include Ebola, hepatitis and HIV/AIDS (Lerner and Lerner, 2008).

For the past two decades, there has been emergence of new infections and also reemergence of old infections in both developed and developing countries (Madigan *et al*., 2015; Morse, 1995; Talaro and Talaro, 2001). It was hypothesized in 2003 that 8.8 million people worldwide were living with TB, and laboratory test(s) showed that 3.9 million of people were living with the disease when the hypothesis was tested (Godreuil *et al*., 2007). Again, in 2012, the World Health Organization pointed out that a total of 8.3 million people developed TB out of which 1.3 million infected individuals died (WHO, 2014). It was also estimated that there will be 450,000 new cases of multidrug – resistant TB infections worldwide (WHO, 2014).

Fortunately, most of these microbial infections have been controlled immensely by the application of antimicrobial agents – a process known as chemotherapy. An antimicrobial agent is defined as an agent which in small concentration can kill or inhibit the growth of

microorganisms (Russell, 2004). Though, these agents are produced naturally by microorganisms (Singh *et al*., 2012), there are also synthetic forms. A good number of antimicrobial agents have been isolated from microorganisms belonging to the phylum *Actinobacteria* where species of *Streptomycetes* are the dominant antimicrobial producers (Hirsch *et al*., 1983; Nike *et al.,* 2013; Kumari *et al*., 2013; Sarkar *et al.*, 2014). About 70% of natural antimicrobial agents have been isolated from actinomycetes, and the remaining 30% are products of non – actinomycete bacteria such as *Pseudomonas* and *Bacillus species*, and filamentous fungi (Singh *et al*., 2012; Hays *et al*., 1945). Actinomycin, streptomycin, tetracycline, chloramphenicol, vancomycin and gentamycin are examples of antimicrobial agents obtained from *Actinomycetes* (Sharma, 2014; Abdulkadir and Waliyu, 2012). Some species of *Bacillus* also produce antimicrobial agents such as bacitracin, surfactins, inturinics, pumulin, bacilysin and gramicidin (Abdulkadir and Waliyu, 2012).

Over 12,000 antimicrobial agents have been isolated from microorganisms but only few are being used today in the treatment and management of infections (Singh *et al*., 2012). The high cost of production of some antimicrobial agents have resulted in fewer numbers of such agents on the market (Naine *et al*., 2014; Brumfitt and Hamilton-Miller, 1988).

Exposure and improper use of the available antimicrobial agents have resulted in different forms of antimicrobial resistance and this has contributed to the difficulty in the treatment of infectious diseases caused by these resistant strains of pathogenic organisms (Costelloe *et al*., 2010; Houben *et al*., 1999). One mechanism of antimicrobial resistance is biofilms formed by microorganisms (Drenkard, 2003). In microbial biofilm formation,

microorganisms adhere themselves tightly to surfaces and then produce polymeric substances around themselves which slow or completely prevent antimicrobial agents and chemical biocides from getting to infecting cells, hence rendering such agents ineffective.

In order to meet the growing challenges of antimicrobial resistance among pathogenic organisms, there is the need to search for new and potent natural antimicrobial agent that is less or non – toxic and has the ability to overcome the mechanisms of microbial resistance.

The Ghanaian environment provides a diverse array of microorganisms that have not been exploited for antimicrobial agents production. This study therefore sought to investigate microorganisms from Ghanaian environments for their antimicrobial agent producing properties.

1.1 Aim of Study

The aim of this study was to investigate the antimicrobial agent producing properties of microorganisms isolated from Soil, Sea and the Kakum River.

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1.2 Specific Objectives

- To collect soil, water and sediments samples from selected environments in Ghana for the isolation of microorganisms.
- To cultivate and isolate microorganisms from the various samples collected.

- To screen the isolated microorganisms for antimicrobial metabolite producing properties
- To select the microbial isolate with the most potent antimicrobial activity for bulk fermentation and extraction of its metabolite
- To assay the extract for antimicrobial activity and other bioassays such as antibiofilm and biosurfactant activities.
- To perform TLC and bioautography analysis of the extract.
- To characterize the most potent antimicrobial agent producing microorganism.

CHAPTER TWO

LITERATURE REVIEW

2.1 Antimicrobial Agents

Antimicrobial agents are agents that either kill or inhibit the growth of microorganisms. They have different chemical structure and spectrum of antimicrobial activity. Some antimicrobial agents act by damaging the cytoplasmic membranes of bacteria cells (Shai, 2002), some also bind to DNA or prevent bacteria DNA synthesis (Marchand *et a*l., 2006) whiles others inhibit specific bacterial metabolic processes (Breukink *et al*, 1999). Traditionally, antimicrobial agents are classified based on their mechanism of action, chemical structure and their spectrum of activity (Schwalbe *et al*., 2007).

2.2 Sources of Antimicrobial Agents

Over the past years, antimicrobial agents have been obtained from two main sources: synthetic and natural.

Synthetic agents are those that are produced in the laboratory through chemical reactions only. In 1935, Domagk synthesized red azo dyes which were shown to have an antimicrobial activity. These agents were classified as Sulfonamides (Denyer, 2004). Diaminopyrimidine derivatives such as trimethoprim and tetroxoprim are also synthetic antimicrobial agents. Trimethoprim was shown to have an antimicrobial activity when a diaminopyrimidine was modified by the addition of three methoxy groups whiles tetroxoprim was produced when a diaminopyrimidine was modified by the addition of

3,5–dimethoxy-4-methoxyethoxybenzyl (Denyer, 2004). Monobactams such as aztreonam was produced synthetically (Tortora *et al*., 2010). Oxazolidinones are also synthetic antibiotics with good activity against gram – positive bacteria (Shinabarger *et at*., 1997).

Among the synthetic antimicrobials are also the semisynthetic ones. The semisynthetic antimicrobial agents are natural agents that have been chemically modified by the addition of extra chemical groups to either increase their potency or to make them less susceptible to inactivation by pathogens hence enhancing their efficacy (Prescott *et al.*, 2002; Madigan *et al.,* 2015). Tigecyclin belonging to Glycylcyclines class of antimicrobial agents is a semisynthetic antimicrobial agent with broad – spectrum antimicrobial activity and was developed by Wyeth. A bulky side chain was added to the position 9 of minocycline molecule (Schwalbe *et al.,* 2007). Azithromycin is a semisynthetic compound that contains a 15-membered structure. Amikacin and Netilimicin are semisynthetic derivatives of gentamicin. Ampicillin, carbenicillin, oxacillin and methicillin are all semisynthetic penicillin (Tortora *et al*., 2010).

Microorganisms predominantly actinomycetes have been the main source of naturally occurring antimicrobial agents (Pandey *et al*., 2008). The agents are obtained when the organisms secrete their metabolic product into an appropriate fermentation medium. *Penicillium notatum* was the first antimicrobial producing organism that produced the very useful antimicrobial agent, Penicillin, and it was discovered by Alexander Fleming in 1928 (Sykes, 2001)**.** Streptomycin, the first aminoglycoside, was discovered from *Streptomyces griseus* in 1944 by Selman Waksman. Chloramphenicol was also isolated from

Streptomyces venezuelae from soil samples collected in Venezuela by John Ehrlich and his colleagues in 1947. In 1948, Giuseppi Brotzu also isolated Cephalothin from *Cephalosporium acremonium* from water samples obtained off the Sardinian coast. In this same year (1948), Benjamin Duggar discovered Chlortetracycline (Aureomycin), from fermentation of *Streptomyces aureofaciens* while studying soil samples for antimicrobial properties. Oxytetracycline (Tobramycin) was isolated from *Streptomyces rimosus* in 1950. In 1952, James McGuire and his colleagues isolated Erythromycin from the metabolic products of *Streptomyces erythreus*. Vancomycin was isolated from *Streptomyces orientalis* by McCormick in 1956. Gentamicin was isolated from *Micromonospora purpurea* by Weinstein in 1963. In the early 1980s, Eli Lilly Company derived Daptomycin (Cubicin) from the fermentation process of *Streptomyces roseosporus* (Schwalbe *et al.,* 2007). Neomycin was produced from *Streptomyces fradiae*. Kanamycin, Tobramycin, and Paromomycin are all naturally occurring compounds that were isolated from other Streptomyces species. Bacitracin was also isolated from *Bacillus subtilis* which was isolated from a wound of a patient. *Paenibacillus polymyxa* also produced the agent, Polymyxin. Griseofulvin was also obtained from *Penicillium griseofulvum*. Just recently, Kwaku Kyeremeh and his colleagues isolated Butremycin from a *Micromonospora* species isolated from the Butre river in the western region of Ghana (Kyeremeh *et al.*, 2014).

2.3 Factors Affecting Antimicrobial Producing Microorganisms

Antimicrobial producing organisms like all other microbes are affected by a number of factors including temperature, pH, sources of carbon and nitrogen, salt, oxygen and carbon

dioxide concentrations (Hogg, 2005). Antimicrobial agents are highly produced when these factors are in their right levels.

2.3.1 Temperature

Microorganisms are able to grow over a wide range of temperatures. The maximum and minimum temperatures over which growth is possible for a particular organism determine the range of temperatures required by that organism. At lower temperatures, enzymatic activities decrease, lipids harden and there is a loss of membrane fluidity hence slowing the growth of microorganisms. Temperatures above the optimum temperature cause a denaturation of enzymes resulting in a decrease in the rate of growth of organisms. Generally, an optimum temperature for a particular organism is closer to its maximum temperature than the minimum temperature (Hogg, 2005).

2.3.2 pH

The growth of microorganisms is highly influenced by the pH of their surroundings. Generally, the range of pH for fungi species is relatively greater than that of bacterial species. Many microorganisms grow best at pH closer to neutrality; however, many bacteria prefer slightly alkaline conditions but relatively few can tolerate acidic environment. Fungi on the other hand prefer slightly acidic environment. Above and below the optimum pH values, the three dimensional structures of proteins (enzymes) are altered hence decreasing the growth rate of organisms. In a laboratory growth medium, the desired pH is achieved by the use of appropriate buffer systems. Phosphate buffer has been the most widely used buffer system in microbiology laboratories to maintain a desired pH (Hogg, 2005).

2.3.3 Nutrition

Microbial growth medium provides microbes with both macronutrients and micronutrients. The macronutrients include carbon, nitrogen, hydrogen, oxygen, sulphur and phosphorus. Among these nutrients, carbon and nitrogen are the most required nutrients and are obtained from many organic and inorganic sources. Carbon is obtained from a monosaccharide, disaccharide, polysaccharide, CO_2 and most other hydrocarbons. Nitrogen can also be obtained from NH_3, NO_3^-, N_2, and nitrogenous organic compounds. Carbon is needed to provide energy to microbial cells and also synthesize proteins, carbohydrates, nucleic acids and lipids whiles nitrogen is required for the synthesis of proteins and nucleic acids.

Micronutrients which are all ions of metals serve as cofactors for enzymatic activities and they are needed only in trace amount. Example of such elements include magnesium (Mg^{2+}), potassium (K^+), sodium (Na^+), calcium (Ca^{2+}) and iron ($Fe^{2+/3+}$).

2.4 Isolation Medium for Antimicrobial Producing Organisms

Several growth medium have been used to isolate antimicrobial agent producing organisms especially actinomycetes. Starch casein agar was used by Wellington and Cross in 1983 and had the composition (g/L); starch (10), casein powder (1), agar (15), and sea water 50% at pH of 7.2±0.2 to isolate antimicobial producing organisms. Actinomycetes isolation agar (AIA) is made up of (g/L) sodium caseinate (2), L - asparagine (0.10), sodium propionate (4), di-potassium phosphate (0.5), magnesium sulphate (0.1), ferrous sulphate (0.001), and agar (15). Balagurunathan and Subramanian (1992) introduced the Kuster's

agar (g/L: glycerol 10, casein 0.3, KNO_3 3, K_2HPO_4 2, NaCl 2, $MgSO_4$ 0.05, $CaCo_3$·0.02, $FeSO_4$ 0.01, agar 16 and 50% sea water; at pH 7 ± 0.1), Bennett agar compose of (g/M) Yeast extract (1), Beef extract (1), Casein enzymatic hydrolysate (2), Dextrose (10), and Agar (15) at pH of 7.3±0.2). Humic acid vitamin agar consists of (g/L): 1 humic acid, 1.7, KCl, 0.5, Na_2HPO_4, 0.5, $MgSO_4$, 0.02, $CaCO_3$,

0.01, $FeSO_4$, 1 mL VB stock solution, 10 agar, and 1000mL distilled water, pH 7.2). Conti, et al., (2012) used the Modified leaf agar (g/L: *S. verticillata* leaf extract 100; glucose 15, peptone 15, agar 15) to isolate antimicrobial producing microbes. Asparagine–glucose medium was introduced by Smith in 1943. Complex Humic acid Vitamin agar consists of (g/L): 0.5 humic acid, 10 agar, 1000 mL soil leaching juice, 1 mL VB stock solution, pH 7.2; Hayakawa, M. and Nonomura, 1987), Zhang'Starch Soil Extract Agar (g/L: 5g soluble starch, 1g KNO_3, 1000 mL soil extracts , 10 g agar, pH7.2),

Gause`s No.1 medium compose of (g/L: 20 soluble starch, 1 KNO_3, 0.5, NaCl, 0.5, K_2HPO_4, 0.5, $MgSO_4$, 0.01g $FeSO_4$, 10g agar, 1000 mL distilled water, pH 7.2;), Soil extracts agar (5g peptone, 3g beef extract, 1000 mL soil extracts, 10g agar, pH7.2). Glycerol – asparagine agar compose of 1 g asparagine, 10 g glycerol, 1 g K_2HPO_4, 1 mL trace salt solution, 10 g agar, 1000 mL distilled water, at a pH of 7.2. Yeast Extract – Malt Extract Agar also known as ISP-2 medium (g/L Yeast Extract 4.0 g, Malt Extract 10.0 g, Dextrose 4.0 g, Agar 20.0 g. (Shirling and Gottlieb, 1966). Inorganic Salts-Starch Agar as is also referred ISP Medium 4 (Soluble Starch 10.0 g, Dipotassium Phosphate 1.0 g, Magnesium Sulfate USP 1.0 g, Sodium Chloride 1.0 g, Ammonium Sulfate 2.0 g,

Calcium Carbonate 2.0 g, Ferrous Sulfate 1.0 mg, Manganous Chloride 1.0 mg, Zinc Sulfate 1.0 mg and Agar 20 g). Glycerol bouillon agar medium (2.0% glycerol, 1.0% Polypeptone, 0.5 % meat extract, 0.3 % CaCO3 and 2.0% agar; Shomura *et al*., 1979).

2.6 Fermentation and Extraction Procedures

Microbial fermentation is a metabolic process in which microbes especially bacteria and fungi (yeast) converts sugar to acids, gases or alcohol. Microorganisms having the ability to produce antimicrobial agents are cultivated in a fermentation medium that provides carbon and nitrogen sources to be utilized by the organisms for their normal metabolic activities. Glucose and soybean meal has been used as carbon and nitrogen sources respectively. (Kumar *et al.,* 2011; Mohan *et al.,* 2013). Other sources of carbon include lactose, maltose, glycerol, fructose, yeast extract, malt extract, and starch. Nitrogen sources also include peptone, tryptone, beef extract, asparagine and casein (Hirsch and Christense, 1983; Kumari *et al.,* 2013). The medium also contains either a complex or simple salt. After sterilizing the fermentation medium it is inoculated with an appropriate inoculum of the desired microorganism. The medium together with the inoculated organism is incubated at a specified temperature until maximum production of active metabolite. The medium is agitated intermittently to ensure a homogeneous mixing of all the three phases of fermentation – thus liquid phase which is made up of the culture/broth media, solid phase which is made up of cells and any debris that may be present, and the gaseous phase usually oxygen and carbon dioxide (Tortora *et al.,* 2010).

After fermentation is complete, the metabolites are harvested. This is preceded by separation of the cell mass and debris from the dissolved metabolites by filtration or centrifugation. The metabolites are then extracted with an appropriate solvent followed by evaporation of the solvent leaving the extract. The solvent system should be able to produce two distinct phases – organic and aqueous – for easily separation.

Another way to get the metabolite is to freeze – dry the entire broth culture after which the powdered product is mixed with an organic solvent to extract the metabolite (Falkinham III *et al.,* 2009). Kyeremeh *et al.,* (2009), extracted metabolites from *Micromonospora* species K310 by adding Diaion HP-20 resin (50 g/L) to a 3–week broth culture and then incubated again for another one week. The metabolites were adsorbed in the Diaion HP-20 resin and were then extracted with methanol and chloroform.

2.5 Antimicrobial Assay Methods

Several methods have been used to determine the antimicrobial activities of agents. These methods include cross streaking method, agar plug method, agar diffusion methods, and microbroth and macrobroth dilution methods.

2.5.1 Cross Streaking Method

This method is used to screen microorganisms for their antimicrobial activity by streaking a loopful of an isolate to be screened once at the center of an agar plate and then incubating the plate at a specified temperature for a number of days, usually one week. The period of

incubation allows the organism to secret their metabolites that diffuse into the agar. Afterwards, test organisms are streaked perpendicular to the already growing isolate and then reincubated for 24 hours after which the plate is observed for zone of inhibition (Mohan, 2013; Ceylan *et al.,* 2008).

2.5.2 Agar Plug Method

This screening method also involves growing the isolates on an appropriate agar plates and then incubating it for a number of days to enable diffusion of any metabolites into the medium. Agar plugs are then cut out from the cultures with a sterile cork borer (Shomur *et al*., 1979). The microbial growth on the plugs is either removed with a sterile knife or it is maintained on the plug. These plugs are then placed on agar plates previously seeded with test organisms and then incubated for 24 hours after which it is observed for zones of inhibitions (Silambarasan *et al*., 2012; Conti *et al*., 2012).

2.5.3 Agar Well Diffusion Method

This method is used to screen isolates for antimicrobial metabolite production and also determine the efficacy of antimicrobial agents. In the case of screening isolates for antimicrobial metabolite production, the isolate is first grown in a broth medium for a number of days to allow the organisms to produces their metabolites into the medium. The broth culture is either filtered or centrifuged to separate the microbial cells and other debris from the filtrate or supernatant that contains the metabolites. Wells are then created on agar

plates seeded with test organisms (1x108 CFU/mL). The wells are filled with the filtrate or supernatant, left on the bench for about 30 minutes or more to enable diffusion of the metabolites into the agar and then incubated at 37 ^{O}C for 24 hours after which the plate is observed for zones of inhibition. In the case of efficacy determination, wells of seeded agar plates are filled with an extract or pure compounds which are also incubated and observed for zones of inhibition. All zones of inhibitions are measured in millimeters (Kumari *et al*., 2013; Tawiah *et al*., 2012; Schwalbe *et al*., 2007).

2.5.4 Disk Diffusion Method

The disk diffusion method also known as the Kirby – Bauer method is used to screen agents for their antimicrobial activities and also to determine the efficacy of antimicrobial agents. In this method a sterile filter paper (6 mm) is saturated with the agent to be tested. The filter paper is then air dried, placed on a seeded agar plate (1x108 CFU/mL) and then incubated at 37 ^{O}C for 24 hours. The plates are observed for zones of inhibition and are measured in millimeters (Das *et al*., 2010; Schwalbe *et al*., 2007; Baris *et al*., 2006).

2.5.5 Macrobroth Dilution Method

This assay is performed to determine the Minimal Inhibitory Concentration (MIC) of antimicrobial agents using glass test tubes filled with a sterile broth medium of volume that is greater than 1 mL. In this assay, two test tubes containing broth medium are used as control to check the sterility of the media (Schwalbe *et al.,* 2007).

2.5.6 Microbroth Dilution Method

The Microbroth dilution method is similar to the Macrobroth dilution method for determining the MIC of antimicrobial agents but the former utilizes small volumes of reagent and also allows large numbers of organisms to be tested at a relatively faster rate. It utilizes microtiter plastic plates containing 96 wells (Schwalbe *et al.,* 2007).

2.5.7 Minimum Bactericidal Concentration (MBC) Determination

The minimum bactericidal concentration is the lowest concentration of an antimicrobial agent that kills the majority of a bacterial inoculum. MBC is determined following a minimum inhibitory concentration test by spreading 50 µL of the culture suspension that showed inhibitory activity on an appropriate agar plate or by streaking a loopful of the suspension on an agar plate (Schwalbe *et al.,* 2007).

2.5.8 Bioautography Methods

Bioautography is an effective and inexpensive method used to detect a bioactive agent(s) in a mixture of compounds on a developed paper or thin layer chromatography (TLC) plate. Goodall and Levi introduced paper chromatography based bioautography in 1946, to estimate the purity of penicillin (Goodall and Levi, 1946). Later, Fisher and Nicolaus and their colleagues introduced thin layer chromatography based bioautography (Fisher and Lautner, 1961; Nicolaus *et at.*, 1961). Basically, there are three bioautographic methods;

contact bioautography or agar diffusion, direct TLC bioautographic detection and immersion or agar overlay bioautography.

In contact bioautography method, a developed TLC plate or paper is placed onto an inoculated agar plate with the plate or paper surface facing down for a specified period of time. This enables the antimicrobial agent on the chromatogram to diffuse into the agar. The chromatogram is then removed from the agar plate and it is incubated at a specified temperature for a period of time. After incubation, the agar plate is observed for zones of inhibition by direct observation or by spraying with a tetrazolium salt. Any zone of inhibition on the agar surface corresponds to a spot on the chromatographic plate and it is an indicative of antimicrobial substance. The incubation period for contact bioautography is usually 16 – 24 hours but it can be reduced to 5 – 6 hours by spraying with 2,6 – dichlorophenol – indophenol or 2,3,5-tetrazoliumchloride to visualize the clear zones (Shahat *et al.,* 2008; Khurram *et al.,* 2009; Mehrabani *et al., 2013*).

With the direct bioautographic detection, the developed TLC plate is dipped into or sprayed with a suspension of the test organisms (Suleimana *et al.,* 2013). The bioautogram is then incubated at 25 ^{O}C for 48 hours under humid conditions after which it is visualized by spraying with a tetrazolium salt. The sprayed bioautogram is then incubated at 25 ^{O}C for 24 hours or 37 ^{O}C for 3 – 4 hours. Zone(s) of inhibition is detected as a clear zone as against a purple background (Silva *et al.,* 2005; Dilika, *et al.,* 1997; Runyoro *et al.,* 2006).

The immersion or agar overlay bioautography on the other hand is a combination of both the direct and contact bioautography. A chromatogram is immersed in a molten seeded agar medium and allowed to solidify. The bioautogram is kept at 4 ^{O}C for an hour or more to

allow diffusion of any bioactive compound to diffuse into the agar after which it is incubated for 24 hours. The bioautogram is observed for zones of inhibition by the eluted spots when the plate is sprayed with a tetrazolium salt (Harborne, 1973; Patil, *et al.,* 2013; Marston, 2011).

2.7 Microbial Biofilms

Bacteria in their natural environment commonly grow; attach themselves to solid surfaces and envelope themselves in a matrix containing polysaccharide, proteins and amino acids (Drenkard, 2003; Donlan, 2002; Hogg, 2005). Examples of surfaces to which microbes preferably attach themselves to include soil particles, stone, metals, glasses and plants (Vinten *et al.,* 2011; Kokare, 2009). In clinical settings, surfaces include artificial implants, endotracheal tubes, urinary catheters, contact lenses, central venous catheters, needleless connectors, intrauterine devices, mechanical heart valves, pacemakers, peritoneal dialysis catheters, prosthetic joints, tympanostomy tubes, and voice prostheses (Donlan, 2001). Epithelial surfaces such as lung and intestinal mucosa are also suitable for pathogens (Knezevic and Petrovic, 2008; Denyer *et al*., 2004). Bacteria attached to a substrate in this way are described as sessile, and are said to exhibit the biofilm or microcolony mode of growth (Denyer *et al.,* 2004). Biofilm formation is enhanced and maintained by; (1) the presence of bacteria fimbriae that serve as an adhesins, allowing organisms to attach themselves to solid surfaces and (2) the production of extracellular polysaccharides/polymeric substances (EPS) that provides a gummy exterior to the cells. As cluster of cells of one or more layers thick, the organisms accumulate moisture and nutrients that support their growth (Talaro and Talaro, 2002; Madigan *et al*., 2015).

Generally, there are four stages in biofilm formation. These are: (1) a reversible attachment of planktonic cells, (2) an irreversible attachment of the same cells, (3) cell growth and production polysaccharide, and (4) development of a tenacious and nearly impenetrable mature biofilm. In the early stages of biofilm formation, the attachment of organisms to surfaces triggers biofilm specific genes that encode proteins responsible to produce cell surface polysaccharides. Molecularly, cyclic di-guanosine monophosphate (c-di-GMP) also triggers biofilm formation in most bacteria (Madigan *et al*., 2015).

2.8 Economic Importance of Biofilms

Biofilms have economic importance in both clinical and industrial settings. Clinically, biofilms may slow or completely prevent penetration of antimicrobial agents and chemical biocides, hence reducing their efficacy. Resistance to components of the host immune system is also an important characteristic of biofilms and it is the major cause of unmanageable infections that usually leads to chronic infection (Brooun *et al*., 2000; Fux *et al*., 2005; Drenkard, 2003). Biofilm – associated organisms differ from their planktonic (freely suspended) forms with respect to the genes that are transcribed and expressed (Donlan, 2002). This together with slow growth rate in biofilms may account for their high resistance to antimicrobials and the host defense system (Madigan *et al*., 2015).

Biofilms on medical devices may compose of a single species or multiple species of organisms, depending on the device and its duration of use in the patient (Donlan, 2001). For example, single species may initially colonize urinary catheter, but longer exposures lead to multispecies biofilms (Stickler, 1996). The most encountered organisms that form

biofilm on medical devices include, coagulase negative *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas earugenosa*, E*nterococcus species* and *Candida albicans* (Kokare, 2009).

Biofilms are found virtually in all aquatic ecosystems that can support microbial growth such as industrial, natural or potable water piping system (Denyer *et al*., 1993; Sutherland, 2001; Donlan, 2002). It sometimes becomes hard to eradicate biofilms in industrial systems where surface growth (fouling) by microorganisms may impair important processes (Madigan *et al*., 2015).

2.9 Antibiofilm Agents

In the 17th century, the tree bark infusion of *Cinchona calisaya*, *C. officinalis* and *C. succirubra* were used purposely for treating infections involving biofilm in South America (Skogman, 2012). Penicillin was also used until penicillin – resistant infection was discovered (Greenwood, 2008). Currently biofilm associated infections are widely managed with antimicrobial agents even though their purpose of usage are being prevented.

2.10 Antibiofilm Methods

A number of methods have been developed to assess the antibiofilm activity of agents. Such methods include; microtiter plate method, fluorometric method and magnetic method among others.

2.10.1 Microtiter Plate Method

This quantitative method is similar to the microbroth dilution method (section 2.8.4). The biofilm forming organism under study together with an antibiofilm agent are dispensed into a 96 – well microtiter plate and then incubated for a period of time. After the incubation, planktonic cells in the wells are washed with physiological solution and the plate is dried. The wells are then stained with a crystal violet (0.1 – 1%) (Merritt *et al*., 2005). The plate is then allowed to stand for some time to enable the crystal violet to stain both viable and dead cells in the well. Crystal violet is a basic dye which has the ability to bind to negatively charged surface molecules in an extracellular matrix. After staining with crystal violet, the wells are washed and dried again. The crystal violet is solubilized in a solvent, usually, ethanol – water (70:30) or acetic acid – water (30:70) and the antibiofilm activity is determined quantitatively by measuring the absorbance of the wells at a specified wavelength (Sabaeifard *et al*., 2014; O'Toole, 2011; Christensen *et al*., 1985).

Another commonly used stain is safranin that is able to detect extracellular substances such as EPS that is present in biofilm matrix. Safranin is commonly used to quantify biofilm mass and can be evaluated qualitatively using light microscopy at a magnification of ×140. The relative amount of biofilm that is formed can be quantified by an optical density measurement at a wavelength of 490 nm (Chaignon *et al*., 2007; Lembke *et al.*, 2006; Zodrow *et al*., 2012).

Bacterial viability in static biofilm can also be quantified by determining the metabolic activity using tetrazolium salt derivatives such as MTT, XTT, and TTC. After the addition of the salt to the wells, optical densities can be determined at a wavelength of 560 nm to determine the absorbance of the formazan produced by the tetrazolium salts metabolism,

and at 700 nm to determine the absorbance of the tetrazolium salt (Welch *et al*., 2012; Cady *et al*., 2012).

2.10.2 Fluorometric Method

This method has been showed to be more sensitive and specific (Burton *et al.,* 2007). In this method, a resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide) which is a blue redox dye is reduced by metabolically active cells to pink florescent product – resorufin. The florescence signals are then measured at an excitation wavelength between 530 – 560 nm and an emission wavelength at 590 nm (Bueno, 2014). It is necessary to optimize dye incubation time due to metabolic differences between microorganisms.

2.10.3 High Content Screening (HCS) Method

This method makes use of florescent dyes such as SYTO – 9 and propidium iodide to measure bacterial adhesion as well as biofilm formation and removal. HCS method quantifies total adhered cells and allows one to measure viability of adhered cells and biofilm alteration when biofilms are treated with antimicrobial compounds (Peng *et al.,* 2010; Francolini *et al.,* 2004).

2.10.4 Magnetic Method

Magnetic method using BioFilm Ring Test is also an advanced approach in static antibiofilm screening to evaluate the ability of bacteria to form biofilms in the presence of an antibiofilm agent. This method is carried out in a modified 96 – microtiter plate of

magnetic beads attached to bacterial cells. It has the ability to evaluate the bacterial biofilm formation without washing and staining the plates (Chavant *et al*., 2007; Badel *et al*., 2008).

2.10.5 Kadouri Drip – Fed Biofilm System

This method is used to study the effect of antibiofilm compounds on resident cells. A constant flow of culture medium is applied onto the system to maintain bacterial growth for a long period of time and also enable one to obtain a lot of biomass that can be monitored by direct observation using an inverted microscope (Jurgens *et al*., 2008). Kadouri drip – biofilm system makes it possible to study and characterize biofilms in reproducible way, and to follow the different processes of biofilm formation (Bueno, 2014). A 6 – well plate is modified by pushing two heated needles through the lid of the plate such that it enters directly above one side of a well. Growth medium and the antibiofilm agent to be tested are dispensed in the plate and then inoculated with the test organism under study. The needles are connected to a peristaltic pump equipped with Marprene manifold tubing such that one needle supplies the plate with fresh medium whiles waste and planktonic cells exit through the other needle. Biofilm formation is then monitored by visualizing the bottom of each well using an inverted microscope (Merritt *et al*., 2011).

2.11 Biosurfactant

Biosurfactants are biomolecules produced by microorganisms as by – product of their metabolic activities (Noudeh *et al*., 2010). These agents are either produced on microbial cell surfaces or are secreted extracellulary and contain both hydrophobic and hydrophilic

moieties (Sarafin *et al*., 2014). Chemically, a biosurfactant can belong to a group of compounds such as phospholipids, fatty acids, lipopeptides, glycolipids and polysaccharide – protein complexes. These agents are desirable alternative to synthetic agents since they are less toxic, persistent, stable, effective in enhancing bioremediation, biodegradable, environmentally accepted, mild production conditions, higher selectivity, lower critical micelle concentration and better activity at extreme temperature, pH and salinity (Cai *et al*., 2014; Das and Mukherjee, 2007; Sarafin *et al.,* 2014). Biosurfactants like synthetic surfactants have the ability to decrease the surface tension of liquids at the air – liquid interfaces. They are also able to decrease the surface tension between immiscible liquids at the liquid – liquid interfaces (Hassanshahian, 2014). They are able to increase the surface area of cells and also enhance the penetrating ability of substances in cells. Biosurfactants increases the bioavailability of water – insoluble hydrophobic substrates to cells (Hamzah *et al*., 2013).

Biosurfactant producers predominantly dwell on hydrophobic substrates such as petroleum products for the production of biosurfactants using the *de novo* pathway of synthesis (Ward, 2010; Syldatk and Wagner, 1987). On the hydrophobic substrate, biosurfactant producers produce biosurfactant to mediate the solubilization of the substrate to be able to utilize them (Hassanshahian, 2014).

2.11.1 Sources of Biosurfactant

Unlike synthetic surfactant, biosurfactants are extensively isolated from diversity of microorganisms (bacteria and fungi). Hydrocarbon contaminated environments (soil and aquatic) has been the best source of complex biosurfactant producers producing different compounds that can degrade hydrocarbons (Kavitha *et al*., 2013). A Bacillus species

isolated from a marine environment was shown to produce a lipopeptide surfactant than has the potential of enhancing oil recovery (Yakimov *et al*., 1995). The biosurfactant Ochrosin (4 – dimethylaminobenzaldehyde) was also isolated from *Ochrobactrum sp* (Kumar *et al*., 2014). *Pseudomonas aeruginosa* has also been reported for the production of the biosurfactant rhamnolipids whiles *Bacillus subtilis* is also known for the production of surfactin (Cameotra and Makkar 2004; Pornsunthorntawee *et al*. 2008). Monoglyceride are aslo produced from glycerol – tallow mixture using *Pseudomonas fluorescens* lipase treatments (Gharaei – Fathabad, 2011) Other microorganisms such as *Acinetobacter calcoaceticus* produced Emulsan and vesicles, *Arthrobacter* species, *Candida bombicola* and *C. apicola* produced sophorolipids, and *Rhodococcus erithropolis* produced Trehalose lipids (Das *et al*., 2008; Reis *et al*., 2013;).

2.11.2 Applications of Biosurfactant

Biosurfactants have broad spectrum of potential applications in food production, pharmaceutical and cosmetic industries, agriculture, mining and the oil industry (Pastewski *et al*., 2006). In food production, biosurfactants are used to stabilized fat and prevent spattering of cooking oil and fats. They are also used for controlling consistency in ice cream formulations and bakery (Kosaric, 2001). The antiadhesive biosurfactant, rhamnolipids, are used to inhibit and disrupt biofilms formation by bacteria in food contact surfaces. Biofilms present on surfaces are potential sources of contamination that lead to food spoilage and disease transmission (Hood and Zottola, 1995; Kamal – Alahmad, 2015). Rhamnolipids are used as food additives to agglutinate fat globules, improve shelf – life

and texture of starch containing products, stabilize aeration and modify rheological properties of wheat dough (Kachholz and Schlingmann, 1987).

In cosmetic and pharmaceutical industry, biosurfactants are used as foaming agents, emulsifiers, solubilizers, cleansers, wetting and antimicrobial agents and mediators of enzymatic action. They are also present in bath products, lipsticks and toothpaste (Gharaei – Fathabad, 2011).

In agriculture, biosurfactants and biosurfactant producing microorganisms enhance the biodegradation of hydrocarbon pollutants in soil to improve the quality of soil for farming. Indirectly, biosurfactants promote plant growth as some have antimicrobial activity that inhibits the growth of plant pathogens. Hydrocarbons are utilized as carbon source by soil inhabiting microbes and this accounts for the biological removal of biosurfactants from agricultural soil (Lima *et al*., 2011; Takenaka *et al*., 2007).

In petroleum exploration, production, oil recovery and processing, oilfield emulsions are formed at different stages. The emulsion formed is then de–emulsified in order to recover oil from the emulsion. Traditionally, de–emulsification is achieved by centrifugation and heat, electrical and/or chemical treatment (Manning and Thompson, 1995). The use of biosurfactant as de–emulsifiers has now replaced the use of chemical de–emulsifiers. *Acinetobacter* and *Pseudomonas* species are the main sources of de–emulsifiers (Glycolipids, glycoproteins, phospholipids and polysaccharides) that act by displacing the emulsifiers from the oil at the oil – water interface. After the de–emulsification process, the biosurfactant is disposed in the aqueous phase and it is separated from the oil phase (Mukherjee *et al.*, 2006).

Microbial enhanced oil recovery (MEOR) is often applied on an oil field to recover oil. In this application, desired indigenous or injected biosurfactant producing microorganisms are exploited in oil recovery by injecting a nutrient with the microorganisms into an oil field that mobilizes oil into wells. This process is followed by reservoir repressurization, interfacial surface tension reduction and reduction of oil viscosity that enhances the movement of additional oil to the producing wells (Singh *et al*., 2008). MEOR is a potent practice to recover oil, especially from reservoirs with low permeability or crude oil with very high viscosity. Nevertheless, this application is dependent on the physico – chemical conditions of soil and the characteristics of rocks formed. Again, the characteristics of oil that has been recovered from the wells influence the application of MEOR (Reis *et al*., 2013).

Biourfactants helps in recovering oil from petroleum tank bottom sludges and also facilitating heavy crude oil transport through pipelines (Reis *et al*., 2013).

2.12 Screening for Biosurfactant Production by Microorganisms

Microorganisms are screen for their ability to produce biosurfactant using methods such as the drop collapse method, oil displacement method, surface tension determination, emulsification activity among others

2.12.1 Haemolytic Activity

The microorganisms to be screened are streaked on 5% blood agar plate and then incubated at 30 ^{O}C for 24 – 72 hours. Haemolytic activity is observed by the presence of clear zones

around the colonies. These zones can then be measured and recorded. The type of haemolysis (α, β or γ) can also be determined (Carrillo *et al*., 1996; Sneha *et al*., 2012)

2.12.2 Drop Collapse Test

In this method, 100 µL of cell free supernatant is pipetted onto 96 – well microtiter plate lid. Ten microliters of oil is added onto the surface of the solution on the lid and then observed after one minute. If the drop of oil on the solution becomes flat 1 min after adding the oil, the result is taken to be positive. If the drops remained beaded, the result is recorded as negative (Hassanshahian, 2014).

Alternatively, 100 µL of oil is added onto the lid of 96 – well microtiter plate lid and then equilibrated for a specified number of hours at room temperature. Ten microliters of cell free supernatant is added to the surface of the oil and then observed after 1 minute.

Biosurfactant – containing solutions gives flat drops, whereas those that gives rounded drops indicates the lack of biosurfactant in the solution (Saminathan and Rajendran 2014; Viramontes – Ramos *et al*., 2010).

2.12.3 Oil Spreading/Displacement Test

This method is used to determine the surface activity of a cell free supernatant by dropping the solution on a thin layer of oil on water surface (Rodrigues *et al.,* 2006). Briefly, 50 mL of water is added to a clean petri dish and about 100 µL of oil is dropped on the water surface to form a thin layer on top of the water. About 10 µL of the cell free supernatant is

then dropped on the oil surface. The presence of a halo or clear zones indicates that the supernatant contains a biosurfactant. The diameter of the halo can be measured, recorded and it allows evaluation of the surface tension reduction ability of the biosurfactant (Chandran and Das, 2010; Hassanshahian, 2014; Hamzah *et al*., 2013).

2.12.4 Surface Tension Determination

The surface tension of cell free broth or a biosurfactant containing solution is determined at room temperature using a tensiometer (Radhakrishnan *et al*., 2011; Abouseoud *et al*., 2007). The operation of the tensiometer is dependent on the manufacturer's instructions.

2.12.5 Emulsification Activity

In this activity, a cell free supernatant is mixed well with equal volume of a hydrocarbon (oil). The mixture is then left to stand undisturbed for 24 hours after which the length of the emulsified layer is measured to determine the emulsification index (E_{24}) using the equation; $E^{24} = \frac{\text{Length of emulsified layer}}{\text{Total length of mixture}}$ x 100% (Cooper and Goldenberg, 1987; Tabatabaee *et al.,* 2005; Satpute *et al*., 2008).

2.12.6 Bacterial Adhesion to Hydrocarbon

Bacterial adhesion to hydrocarbon method is used to assess microbial cell surface hydrophobicity (Chandran and Das, 2010). Microbial cells are harvested from 7 days and 14 days broth cultures by centrifugation and then washed with a phosphate, urea and magnesium sulphate (PUM) buffer. A suspension of the organism is prepared and its optical

density (A_o) is determined after which a specified volume of a hydrocarbon is added to it. The mixture is then vortexed and allowed to stand for about 10 minutes after which the optical density (A_1) of the aqueous phase is measured. The extent of hydrophobicity is calculated from the formula $1 - (\frac{A_0 - A_1}{A_0}) \times 100\%$ (Rosenberg *et al*., 1980; Saminathan and Rajendran, 2014).

2.12.7 Microtiter Plate Method

The microtiter plate method is a qualitative method of determining the surface activity of individual organisms to be screen for their ability to produce biosurfactant. Wells of 96 – microtiter plates are filled with about 100 µL of supernatant of broth cultures of the isolates to be tested. Using a backing paper with a grid, the plate is then viewed and if a biosurfactant is present in the supernatant, the concave surface distorts the image of the grid. Pure water in microtiter plate well has a flat surface but when surfactant is present, it appears concave, taking the shape of the wells (Saminathan and Rajendran, 2014; Femi – Ola *et al*., 2015).

2.12.8 Penetration Method

A hydrophobic paste consisting of oil and silica gel is prepared and about 150 µL aliquot is placed into wells of 96 microtiter plate. The paste is then covered with about 10 µL of oil. About 90 µL of supernatant of a broth culture is stained with 10 µL of a red staining solution and then transferred to the surface of the paste in the well. The presence of

biosurfactant in the supernatant will cause the hydrophilic liquid to break through the oil film barrier into the paste. The Silica will then penetrate the hydrophilic phase and the upper phase will change into clear red to cloudy white within 15 minutes (Saminathan and Rajendran, 2014; Nishanthi *et al.,* 2010).

2.12.9 Foam Formation Method

This method is conducted using broth cultures of organisms (Jazeh *et al*., 2012). Isolates are grown in a suitable liquid growth medium in a flask. The flask is then incubated at 37 ^{O}C in an incubator for a specified number of days. Formation of foam is detected and the duration of foam stability, foam height and foam shape in a graduated cylinder is observed (Chayabutra *et al*., 2001; Dehghan – Noudeh *et al*., 2003).

2.12.10 Methylene Blue Agar Method

This method is used to detect anionic biosurfactant, specifically, rhamnolipids. Microbial isolates are streaked on mineral salt agar plate supplemented with 2% carbon source, cetyltrimethlammonium bromide (0.5 mg/mL) and methylene blue (0.2 mg/mL). The plate is then incubated at 37 ^{O}C for 24 – 48 hours. The appearance of a deep dark blue halo around the culture as against a light blue colour is considered as positive for biosurfactant production (Satpute *et al*., 2008). This dark blue halo is as a result of precipitation of insoluble ion pair that is formed with cationic substances (Rahman *et al*., 2010).

CHAPTER THREE

MATERIALS AND METHODOLOGY

3.1 Sample Collection

A total of 24 samples consisting of soil, river water and sediments and sea water and sea sediments were collected and transported to the laboratory. The soil samples were collected from two different locations (6º 43' 1" N, 1º 28' 34" W and 6º 43' 1" N, 1º 28' 30" W) at Ejisu Zongo in the Ejisu – Juaben Municipality, Ashanti Region, Ghana. At each location, 4 different samples were collected at depth 2 – 15 cm with sterile spoons into sterile plastic containers and were tightly covered with sterile lids.

The river samples were collected from three different locations along the Kakum River in Cape Coast, Central Region, Ghana; Kakumdo (5º 8' 49" N, 1º 17' 15" W), Kwaprow (5º 7' 34" N, 1º 18' 10" W) and Etre (5º 5' 53" N, 1º 19' 19" W). At each location, 200 mL of fresh water was collected into a sterile bottle by completely immersing the bottle in the water and gently opening the lid to fill the bottle. It was then covered tightly and brought

to the surfaced (Tawiah *et al.,* 2012). Two river sediments were also collected from each sample location with sterile spoons into sterile plastic containers which were then tightly covered (Gebreyohannesa *et al.,* 2013).

The Sea samples were also collected from two different locations, Etre (5º 5' 50" N, 1º 19' 12" W) and Oyster Bay (5º 5' 39" N, 1º 19' 51" W) in Central region, Ghana. A bottle of Sea water was collected at each location following the same method used for collecting the fresh water samples as described above. At Oyster bay, two sea sediment samples were collected from the bottom of the Sea with a sterile spoon into sterile plastic containers. The same method was repeated at Etre where three Sea sediments were

collected.

A  B 

Plate 3.1: Sample site at Oyster bay (A) and Etre beach (B)

3.2 Sample Preparation and Cultivation

One gram each of the soil samples were separately suspended in 10 mL of sterile Normal Saline (0.9% NaCl) and allowed to stand for about 30 minutes after which 1 mL was withdrawn and diluted 2 times. One milliliter each of the diluted suspensions were

withdrawn and separately inoculated into 30 mL of Humic Acid Vitamin (HAV) agar. The HAV agar was previously melted and stabilized at 45 ^{O}C. The inoculation was done by transferring 1 mL of the diluted suspensions into a sterile petri dish and the stabilized agar was poured onto it and thoroughly mixed before it solidified.

The sediment samples were also similarly suspended in normal saline and diluted two times before they were inoculated into HAV agar.

For the Kakum River and sea water samples, 1 mL of each was taken and separately inoculated into 30 mL of the HAV agar as described above. All the plates were incubated inverted at 34 ^{O}C for 14 days with daily observation. All the samples were also similarly cultivated in Glycerol Asparagine (GA) agar.

3.3 Isolation of Colonies into Pure Culture

Within the 2 weeks of cultivation, colonies of microorganisms were observed in the culture plates. Morphologically different colonies on the plates were isolated with sterile platinum loop into separate test tubes containing 10 mL of sterile nutrient broth (Oxoid) and then incubated for 24 hours. The isolates were coded (Table 4.1) based on the agar used, type and the source of sample and the order in which they were isolated from the plate.

The isolates were streaked on solid Nutrient Agar plates with a sterile platinum loop and then incubated inverted at 37 ^{O}C for 24 hours after which single colonies (pure cultures) were fished out with sterile platinum loop into 5 mL sterile Nutrient Broth which was then

incubated at 37 ^{O}C for 24 hours. The process of fishing out a colony into Nutrient Broth and incubating it was repeated 2 times for each isolate to ensure that pure culture of the isolate was obtained.

3.4 Test Microorganisms

The test microorganisms used in this study are *Bacillus subtilis* NTCC 10073, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 4853, and clinical isolates of *Salmonella typhi*, *Salmonella typhorium*, *Staphylococcus epidermidis*, *Shigella dysentriae*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*, and the fungi *Candida albicans*. These test organisms were taken from the stocks kept in the Pharmaceutical Microbiology laboratory, KNUST.

3.5 Screening of the Isolates for Antimicrobial agent Production

The isolates were screened for their antimicrobial activity against 5 typed bacteria strains, 4 clinical bacteria isolates and a clinical isolate of *Candida albicans* using the cross streaking (CS) method adopted by Mohan *et al*., (2013) with few modifications as well as the agar well diffusion (AD) method adopted by Tawiah *et al*., (2012).

For the cross streaking method, 25 mL sterile Nutrient Agar (Oxoid) which was prepared and stabilized in water bath (New Brunswik Scientific, Model R76, USA) at 45 ^{O}C was transferred into a sterile petri dish and allowed to solidify. A loopful of the isolate was streaked at the center of the plate and then incubated at 35 ^{O}C for 7 days after which 24

hour broth cultures of test organisms were streaked perpendicular to the already growing isolates. The plate was incubated at 37 ^{O}C for 24 hours and observed for zones of growth inhibition of the test organism which were measured and recorded (Table 4.1).

With the agar well diffusion method, the isolates were separately grown in 10 mL Nutrient broth at 35 ^{O}C for seven days. Two milliliter quantities of each broth culture were transferred into a 2.5 mL eppendorf tubes, covered tightly and then centrifuged at a speed of 1400 rpm for 15 minutes to separate cells and other debris from the liquid phase (supernatant). Two loop full of 24 hours broth cultures of test organisms were surface inoculated on Nutrient agar plates. Six wells were created on each plate using sterile cork borer (diameter 12 mm). The wells were then filled with the supernatant and the plates were preincubated at room temperature for 1 hour to allow diffusion of any bioactive metabolite in the supernatant into the agar after which they were incubated at 37 ^{O}C for 24 hours and observed for zones of inhibition; which were measured in millimeters and recorded (Table 4.2). Based on the results of the two screening, the isolate, $GKSE_1$ which exhibited the highest activity was selected for further investigation.

3.6 Incubation Period

Five milliliters of the selected isolate $GKSE_1$, was inoculated into 20 mL of sterile nutrient broth and then incubated for 12 days at 35 ^{O}C. Samples were taken each day and tested against *K. pneumoniae* using the agar well diffusion method as described in section 3.4.0. The mean zones of inhibition were plotted (Figure 4.1) using Graph pad prism version 5.01.

3.7 Fermentation and Extraction of Metabolite of $GKSE_1$

Four of 600 mL bottles each containing 500 mL of sterile Nutrient broth were inoculated with 20 mL of 24 hour broth cultures of the isolate $GKSE_1$. The bottles were then incubated at 35 ^{O}C and were shaken 3 times daily for 7 days (Waites *et al*., 2001).

On day 7, the broth culture was filtered through 125 mm Whatman NO.1 filter paper (pore size = 0.7 µm; GE healthcare UK limited, UK) and a vacuum pump (Diaphragm Vacuum Pump, Vacuubrand GMBH+co., Germany) was used to facilitate the process. The metabolites in the filtrate were extracted four times with ethyl acetate. This was done by vigorously shaking the filtrate – ethyl acetate mixture (2:1) in a 2 L amber Winchester glass bottle for about 1 minute and then transferring the mixture into a 1 L separating funnel. The funnel was allowed to stand for 1 hour to enable good separation of the aqueous (bottom layer) and the organic phases (upper layer). The organic layer was collected into a preweighed evaporating dish and the ethyl acetate was evaporated at 45 ^{O}C on a thermostatically controlled water bath (New Brunswik Scientific, Model R76, USA). The weight of the extract was determined and the extract was kept in the fridge at 4^{O} C until it was needed.

Plate 2: (A) Setup for filtration of cultures; (B) Content of separating funnel showing both aqueous (bottom) and organic (upper) phases; (C) Extract in evaporating dish

3.8 Antimicrobial Activity of the Extract

The extract was assayed for its inhibitory activity against test organisms using the agar well diffusion and microbroth dilution methods (sections 2.5.3 and 2.5.6).

3.8.1 Agar Well Diffusion Method

Two – fold dilutions of the extract (between 12.5 – 400 mg/mL) were tested against *B. subtilis*, *E. faecalis*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, *S. epidermidis*, *S.*

pyogenes, *S. typhi*, *S. typhorium*, and *Shigella dysentriae* using the agar well diffusion method as described in section 3.4.0. The test was conducted in triplicate and ciprofloxacin (0.1%) was used as a control. The zones of inhibition were measured and recorded (Table 4.3).

3.8.2 Microbroth Dilution Method.

The Minimum Inhibitory and Bactericidal Concentrations (MIC and MBC) of the extract were determined using the 96 – well microtiter plates (Thermo scientific, USA). Briefly, graded concentration of the extract (50, 25, 12.5, 6.25, 3.13, 1.57, 0.79, 0.40, 0.20, 0.10 and 0.05 mg/mL) were prepared and 70 µL aliquots of the various concentrations were added to the wells of a sterile microtiter plate filled with 100 µL of sterile double strength Nutrient broth. Twenty – four hours broth cultures of the various test organisms were diluted in sterile saline to 10^6 of which 30 µL aliquots were added to the wells. Graded concentrations (20, 10, 5, 2.5, 1.25, 0.63, and 0.3 µg/mL) of ciprofloxacin were used as a control. The plates were incubated at 37 ^{O}C for 24 hours after which 20 µL of 1.25 mg/mL tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Applichem Chemical Synthesis Services, Germany) was added to the wells. Wells that showed purple colour 30 minutes after the addition of the MTT signified growth and those that remained yellowish also signified inhibitory activity.

A loopful of the content of each of the wells that showed inhibitory activities were streaked on separate Nutrient agar plates and incubated at 37^{O} C for 48 hours after which they were

observed for signs of growth. The least concentration that did not show growth was noted and recorded as the MBC.

3.9 Effect of Temperature on the Antimicrobial Activity of the Extract

A concentration of 200 mg/mL of the extract was prepared and distributed into eight screw capped test tubes. Seven of the tubes were labeled with temperatures of 60, 70, 80, 90, 100 and 110 and 121 ^{O}C respectively whilst the last tube was labeled control. The first six tubes were heated in hot water bath at their respective temperatures for 1 hour. The seventh tube (121 ^{O}C) was also autoclaved at 121 ^{O}C for 15 minutes. Aliquots of all the tubes were then taken and tested against *B. subtilis* and *K. pneumoniae* for their antimicrobial activities using the agar well diffusion method (section 3.4.0). The experiment was carried out in triplicate and the resulting zones were measured and recorded (Figure 4.2). Statistical Package for Social Sciences (SPSS), version 16, software was used to determine the statistical difference between the control and the other heat treated groups.

3.10 Effect of Carbon Sources on the Antimicrobial Metabolite Production

The ability of the isolate to utilize different carbon sources to produce the antimicrobial metabolite was assessed. Ten different carbon sources (Xylose, Mannitol, Lactose, Starch, Arabinose, D – fructose, Raffinose, Glucose, Pectin and Sorbose) were used in this assay. Eleven test tubes were each filled with 30 mL Peptone water (Oxoid) and 1% (w/v) of the various carbon sources were incorporated into 10 of the test tubes such that one test tube contained one carbon source. The remaining one test tube served as a control. The tubes were sterilized at 121 ^{O}C for 15 minutes, allowed to cool and 1 mL of 24 hour broth culture of isolate $GKSE_1$ was inoculated into the various tubes. They were then incubated at 35 ^{O}C

for 7 days after which the metabolite produced in each tube was extracted as described in section 3.7.0. The various extracts were then tested against *K*. *pneumoniae* and *B*. *subtilis* using the agar well diffusion method (section 3.7.1). The experiment was carried out in triplicates and a graph of mean zones of inhibition of each test organism was plotted (Figure 4.3) using graph pad prism 5.

3.11 Stability Assessment of Aqueous Solution of Extract

Two hundred milligrams per milliliter concentration of the extract was freshly prepared with sterile distilled water. Its activity was tested on the same day (Day 0) against *K*. *pneumoniae* and *B*. *subtilis* using the agar well diffusion method (section 3.7.1). The remaining extract solution was divided into two equal volumes and dispensed into two screw capped test tubes. One of the tubes was kept at 4 ^{O}C whiles the other tube was kept at room temperature. Their antimicrobial activities were tested weekly for 8 weeks and on weeks 10 and 12 against the above test organisms. Graph pad prism 5 was used to plot a graph (figures 4.4a and 4.4b) and SPSS (version 16) software was used to determine the significant differences between the mean zones of inhibitions obtained on day 0 and the other weeks.

3.12 Thin Layer Chromatography (TLC) Analysis of the Extract

The extract was subjected to TLC analysis as a way of characterizing it. An aqueous solution (100 mg/mL) of the extract was prepared for the analysis. TLC plates made of silica spread on aluminum sheets (Merck, Germany) were cut into 1.5 × 6 cm portions. A

pencil was used to mark two horizontal lines that were 1 and 5.5 cm above the bottom of the plate. The extract solution was spotted on the 1 cm line (baseline) of the TLC plate with the help of a capillary tube. The solvent in the spot was air dried.

After several preliminary trials in solvents such as petroleum ether, chloroform, ethyl acetate and ethanol, chloroform – ethanol (85:15) was selected and used for developing the chromatogram. The plates were observed under UV light at wavelengths of 254 nm and 365 nm and the spots observed were circled with pencil. The plates were then sprayed with p – anisyladehyde, air dried and placed on a hot plate for about 40 seconds to make stained spots more visible. The distances travelled by the spots from the baseline were measured and used to calculate their Retardation Factor (Rf) values

3.13 Bioautography Assay of the Extract

The agar overlay method described by Dewanjee *et al*., (2014) was adopted and used for the Bioautography assay. Briefly, 10 µL aliquots of 100 mg/mL concentration of the extract was spotted on 2 of 2×7 cm TLC plates and were developed as described in section 3.8.0. A test tube containing 20 mL sterile molten nutrient agar was seeded with 100 µL of 24 hour broth cultures of *B. subtilis* (10^5 CFU/mL). One of the developed plates was immersed into the seeded agar with the help of forceps and then transferred into a sterile petri dish. The plate was kept at 4 ^{O}C for 2 hours to allow diffusion of the antimicrobial agent into the agar. The plate was then incubated at 37 ^{O}C for 24 hours after which it was sprayed with 1.25 mg/mL MTT and reincubated for 30 minutes and then observed. Regions/areas with

clear zones indicated inhibition on the plates whereas a purple background indicated growth of microorganism. The experiment was repeated using *K. pneumoniae*.

3.14 Antibiofilm Activity of the Extract

The extract was assessed for antibiofilm activity using crystal violet assay (Drago *et. al.,* 2014; Sabaeifard, *et. al.,* 2014). Briefly, two – fold dilutions (4, 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125mg/mL) were prepared. A sterile round bottom 96 – well microtitre plate was filled with 100 µL of sterile double strength Nutrient broth (Oxoid). Twenty four (24) hour broth culture of *S. aureus* was diluted to 10^2 of which 30 µL portion was added to the wells of the plate. The different concentrations of the extract were also added to the wells of the plate which and then incubated at 37 ^{O}C for 24 hours under static conditions. After incubation, the wells of the plate were washed five times with sterile distilled water (to remove any planktonic organisms in the plate) and dried at 40 ^{O}C for 1 hour (Parrilli *et al*., 2015). The wells of the plate were then stained with 1% crystal violet and left at room temperature for 30 minutes after which it was washed five times with sterile distilled water. The plate was then dried at 40 ^{O}C and the crystal violet stain was solubilized in ethanol – acetone mixture (70:30). The antibiofilm activity was analyzed quantitatively by measuring the optical densities (OD) of the crystal violet at wavelength of 570 nm using a microtiter plate reader (Synergy H1 Hybrid Reader, Biotek). Ethylenediaminetetra – acetic acid (EDTA) was used as a positive control, whereas in the negative control, neither EDTA nor extract was added to the organisms in the wells. The experiment was repeated for *E. coli* and *P. aeruginosa*.

3.15 Biosurfactant Activity Assessment of Isolate $GKSE_1$

Biosurfactant action of the extract obtained from isolate $GKSE_1$ was assayed using tree different techniques: Emulsification test (Cooper and Goldenberg, 1978; Iqbal *et al*., 1995), Oil spreading method (Hassanshahian *et al.,* 2014; Morikova *et al*., 2000; Youssef *et al*. 2004) and Drop collapse test (Jain *et al.,* 1991; Sarafin *et al.,* 2014; Hassanshahian, 2014).

3.15.1 Emulsification Test

The $GKSE_1$ extract was assayed for biosurfactant activity by adding 1 mL of 50 mg/mL extract to 1 mL of light crude oil in a screw capped test tube. The test tube was then agitated vigorously for 2 minutes and left to stand for 20 minutes after which the total length of the mixture in the tube was measured. The tube was left to stand for 24 hours at room temperature and the length of the emulsified layer was measured. The test was carried out in triplicate and the emulsification index (E_{24}) was calculated from the

equation; $E^{24} = \frac{\text{length of emulsified layer}}{\text{total length of mixture}} \times 100\%$.

3.15.2 Oil Spreading/Displacement Test

In this assay, a sterile clean petri dish was filled with 50 mL of sterile water. Hundred microliters of light crude oil was added to the surface of the water in the petri dish after which 10 µL of the extract (50 mg/mL) was added to the surface of the crude oil. Zones of oil displaced were measured and recorded. The test was carried out in triplicate.

3.15.3 Drop Collapse Test

Hundred microliters of the extract (50 mg/mL) was added to the lid of 96 – well microtiter plate. Five microliters of light crude oil was added to the surface of the extract and then observed after 1 minute. A flattened surface of the extract that allows the crude oil to settle on top of it signifies a positive test whiles a concave surface that pushes the crude oil from the top of the extract signifies a negative result.

3.16 Characterization of Isolate $GKSE_1$

Morphological, physiological and a number of biochemical and test were used to characterize isolate $GKSE_1$.

3.16.1 Gram Staining

The isolate was streaked on nutrient agar plate and incubated for 24 hours at 37 ^{O}C. A single colony was picked from the plate and smeared on a clean grease free glass slide (Surgifriend Medicals Middlessex, England). The slide was air dried for about 30 minutes and then heat fixed on a Bunsen burner. Ammonium oxalate crystal violet was added to it for 30 seconds and then washed off with distilled water. Iodine solution was then added for 30 seconds and again washed with distilled water. The slide was then washed with ethanol until the ethanol running down the slide turn colourless and quickly washed with distilled water. The slide was counterstain with safranin for 1 minute, washed with water and air dried. It was then observed under the microscope using oil immersion method.

3.17 Growth at 6.5% NaCl

One percent glucose was added to peptone (from meat) in three separate test tubes. 6.5% (w/v) NaCl was added to each tube and the mixture was dissolved in 10 mL of water and then sterilized at 121 ^{O}C for 15 minutes. Three colonies of the isolate were transferred from an already plated nutrient agar plate into the tubes and incubated at 37 ^{O}C for three days with daily observation. Turbidity in tubes signifies the presence of growth in the medium.

3.18 Growth at 45 ^{O}C

Hundred microliters of 24 hours broth culture of the isolate was inoculated into 10 mL sterile nutrient broth. It was then incubated at 45 ^{O}C for three days with daily observation.

3.19 Growth of Isolate GKSE$_1$ on Different Solid Media

3.19.1 Cetrimide Agar

Sixty milliliters of Cetrimide agar was prepared and distributed into three test tubes (20 mL each). They were then sterilized at 121 ^{O}C for 15 minutes and then stabilized at 45 ^{O}C in a thermostatically controlled water bath. The media were then transferred into sterile petri dishes and allowed to solidify. The isolate was then streaked on the plate and incubated inverted at 37 ^{O}C for three days with daily observation for growth.

3.19.2 Bismuth Sulphite Agar

Bismulth sulphite agar was dissolved in three test tubes each containing 20 mL of water. It was heated on a Bunsen burner flame and then stabilized at 45 ^{O}C. The media were

transferred into sterile petri dishes and allowed to cool. The isolate was streaked on the plate and incubated inverted at 37 ^{O}C for three days with daily observation.

3.19.3 Mannitol Salt Agar

Three test tubes containing 20 mL of dissolved Mannitol salt agar was sterilized in an autoclave at 121 ^{O}C for 15 minutes. It was stabilized in water bath at 45 ^{O}C and then transferred into sterile petri dishes. The media were allowed to solidify in the plates and the isolates were streaked on the plate. The plates were incubated inverted at 37 ^{O}C for three days and was observed daily for growth.

3.19.4 MacConkey Agar

MacConkey agar was also dissolved in 20 mL of distilled water in test tubes. They were sterilized by autoclaving at 121 ^{O}C for 15 minutes and then stabilized in a water bath at 45 ^{O}C. The media were then transferred into sterile petri dishes and then allowed to solidify. A loopful of the isolate was streaked on the plate and incubated inverted at 37 ^{O}C for three days with daily observation.

3.20 Biochemical Identification of the Isolate

3.20.1 Catalase Test

The isolate was incubated on nutrient agar slant at 37 ^{O}C for 24 hours. Two milliliters of H_2O_2 was run down the slope and observed immediately for evolution of gas which indicates catalase activity.

3.20.2 Oxidase Test

Using a loop, a single colony of the isolate was picked from nutrient agar plate and rubbed onto the surface of a disc impregnated with tetraphenyldiamine (Abtek biological Ltd, UK). Colour change to indophenol blue within seconds was an evidence of oxidase activity.

3.20.3 Hydrogen Sulphide (H_2S) Production

Peptone water (Oxoid) was inoculated with a loopful of the isolate, and a filter paper impregnated with lead acetate was inserted between the cup and the tube. The tube was incubated at 37 ^{O}C and examine daily for 7 days for blackening of the paper that indicate H_2S production.

3.20.4 Indole Production

Sterile peptone water (Oxoid) was inoculated with a loopful of the isolate and incubated at 37 ^{O}C for 48 hours after which 0.5 mL Kovacs' reagent was added and mixed well. It was examine after 1minute for indole production. A red colour in the reagent layer indicates indole production.

3.20.5 Methyl Red – Voges–Proskaiier (MR – VP) Reaction

MR – VP broth (Oxoid) was dissolved in 10 mL of water in test tubes and then sterilized. A loopful of the isolate was inoculated into the medium and then incubated at 37 ^{O}C for 2

days. Two drops of methyl red solution was added to the tubes, mixed and examined. A positive MR reaction is shown by the appearance of a red colour at the surface of the tubes.

After completion of the methyl red test, VP test was conducted. Two drops of creatine solution and 1 ml 40% KOH (aqueous solution) were added to the tubes, shaken, sloped, and then examined after 1 and 4 hours. A positive reaction is indicated by an eosin-pink colour.

3.20.6 Nitrate Reduction

Nitrate Broth was prepared and sterilized in 10 ml test tubes and then inoculated lightly with the isolate and incubated for 5 days at 37 ^{O}C. One milliliter of nitrite reagent A followed by 1 mL of reagent B were added to the tubes. The tubes did not show a red colour within 5 minutes so powdered zinc was added and allowed to stand. The presence of red colour indicated that nitrate was still present in the medium, i.e. not reduced by the organism.

3.20.7 Citrate Utilization

Koser's citrate medium was inoculated with a straighten loop of the isolate and incubated at 37 ^{O}C with daily observation for 10 days. In the absence of colour change from light green to light blue or turbidity showed that citrate was not utilized.

3.20.8 Glucose Fermentation/Oxidation

Peptone water was incorporated with 1% glucose in test tubes. A drop of bromocresol purple was then added and a Durham tube was dropped into it. The tube was inoculated

with 100 µL of 24 hour broth culture of the isolate and incubated at 37 ^{O}C for 3 days with daily observation. A changed of the medium from purple to yellow colour indicates that there is fermentation or oxidation of glucose and a space or bubble in the Durham tubes also indicates the production of gas.

3.20.9 Lactose Fermentation/Oxidation

A drop of bromocresol purple was added to lactose broth in a test tube and a Durham tube was dropped into it. The tube was then incubated at 37 ^{O}C for 3 days with daily observation. A changed of the medium from purple to yellow colour indicates that there is fermentation or oxidation of lactose and a space or bubble in the Durham tubes also indicates the production of gas.

CHAPTER FOUR

RESULTS

4.1 Isolation and Screening

A total of 138 microorganisms were isolated: twenty seven (27) from Ejisu soil samples, 68 from the Kakum River samples and 43 from the sea samples.

The isolates were screened for their antimicrobial metabolites production against nine test organisms – *Bacillus subtilis*, *Salmonella typhi*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans* – using the cross streaking and the agar diffusion methods (section 3.5.0). Thirty six (36) isolates produced metabolites that had inhibitory activity with their mean zones of inhibition ranged between 2.3±0.3 – 35.3±1.5 mm (cross streaking) and 15±0.6 – 35.3±0.3 mm (agar well diffusion method; Tables 4.1 – 2).

In the cross streaking method, 31 isolates showed inhibitory activity against *B. subtilis*, 25 against *S. typhi*, 27 against *E. faecalis*, 26 against *S. aureus*, 24 against *S. pyogenes*, 28 against *K. pneumoniae*, 20 against *E. coli*, 16 against *P. aeruginosa* and then 10 against *C. albicans*. In the agar well diffusion method, 11 isolates showed inhibitory activity against *B. subtilis*, 14 against *E. faecalis*, 9 against *S. aureus*, 11 against *P. aeruginosa*, 10 against *E. coli*, and 1 against both *K. pneumoniae* and *C. albicans*. In the agar well diffusion method, no isolate showed inhibitory activity against *S. pyogenes*. Isolate $GKSE_1$ was selected for further investigation since it showed relatively high zones of inhibition (CS:

15.7±0.3 – 29.3±1.8mm; AD: 24±0.0 – 35.3±0.3mm) when compared to the other isolates (Tables 4.1 and 4.2).

Table 4.1: Antimicrobial activity of isolates using the cross streaking method

Sample sources	Isolates	Test microorganisms/Mean zones of inhibition (mm)								
		B. sub	S. typ	E. fae	S. aur	S. pyo	K. pne	E. col	P. aer	C. alb
Soil	$GSSA_4$	22.0±0.6	19.3±0.9	26.7±0.3	-	5.7±0.7	23.3±1.8	15.3±1.5	14.7±1.2	-
	$GSSC_1$	-	-	-	5.3±0.9	-	10.3±0.9	-	5.3±0.3	-
	$GSSC_{14}$	19.7±1.7	30.3±1.7	22.3±0.3	23.0±1.2	20.7±1.8	25.3±0.9	-	14.7±0.7	-
	$HSSA_1$	22.3±0.6	17.7±0.3	25.0±1.2	19.3±0.3	14.3±0.9	23.3±0.9	15.7±1.5	8.3±1.5	-
	$HSSA_{2a}$	16.7±0.6	-	-	16.7±0.3	20.3±0.3	-	-	-	-
	$HSSA_{3a}$	16.0±0.0	-	20.0±0.6	-	-	-	-	-	-
	$HSSA_5$	21.3±0.3	17.3±0.3	-	16.0±0.3	15.3±0.9	20.7±0.3	11.0±1.2	-	-
	$HSSA_6$	15.7±0.7	-	17.7±0.3	13.0±1.2	15.0±0.0	21.3±0.9	-	-	-
	$HSSC_2$	22.0±0.7	10.0±0.6	10.3±0.3	12.0±0.0	11.3±1.5	26.7±0.7	15.7±0.3	28.3±2.0	21.0±1.2
Kakum River-water	$GFWC_{16}$	19.7±0.9					-	12.3±1.5	-	20.7±0.3
	$GFWK_{1a}$	18.3±0.3	31.3±0.9	16.3±0.3	24.3±1.2	27.0±1.2	-	8.0±1.7	-	-
Kakum	$GKSC_3$	20.7±0.3	10.3±1.5	15.3±0.3	-	-	19.3±1.5	6.3±0.3	19.7±0.7	11.7±0.7

River sediment	$GKSE_1$	27.3±0.9	25.3±0.3	20.3±0.9	15.7±0.3	-	29.3±1.8	21.3±0.9	24.3±0.3	-	
	$HKSC_{10}$	10.7±0.3	10.3±0.3	-	15.7±0.3	7.7±0.7	20.3±2.0	-	-	-	
	$HKSC_{2b}$	19.7±0.3	-	25.3±0.9	30.0±1.2	22.0±0.6	27.0±1.2	-	-	-	
	$HKSC_{2c}$	19.7±0.7	16.3±1.5	24.0±0.0	-	-	28.0±1.2	14.3±2.1	-	7.3±0.9	
	$HKSC_{3a}$	-	18.7±0.3	20.7±0.3	13.3±0.3	10.0±0.0	25.3±1.5	-	25.0±1.7	-	
	$HKSE_{10}$	10.7±1.2	10.3±1.5	-	15.0±0.6	7.3±1.5	20.3±1.7	-	-	-	10.3±1.5
	$HKSE_4$	19.3±0.3	15.7±0.7	21.3±0.3	-	-	22.0±1.5	18.0±0.0	5.3±0.3	11.3±1.5	-
	$HKSK_2$	14.7±0.7	-	14.7±0.7	21.7±1.5	-	-	27.0±0.5	-	-	
	$HKSK_{3b}$	22.0±0.0	20.7±0.3	-	10.7±1.5	-	-	22.7±0.6	14.7±0.7	5.7±0.7	
	$HKSK_{4b}$	19.7±0.7	-	5.7±0.7	12.7±0.3	10.3±0.9	21.0±0.0	16.7±0.7	-	-	
	$HKSK_{7c}$	-	18.0±0.0	19.7±0.3	11.3±0.3	11.0±1.7	21.7±0.3	7.7±0.7	13.3±0.3	4.7±0.7	
	$HKSK_{8a}$	11.0±1.2	-	10.3±1.5	-		-	-	-	-	
Sea water	$GSWE_{1b}$	-	3.7±0.3	-	35.3±1.5		25.7±0.3	-	-	-	
	$GSWE_{2a}$	10.3±1.5	20.3±0.9	-	8.7±0.3	10.3±0.9	10.7±0.7	15.7±0.3	9.3±0.9	-	
	$HSWO_{1a}$	3.7±0.3	24.7±0.3	8.3±2.0	25.3±1.7	20.3±0.3	4.3±1.3	2.7±0.7	-	-	
	$HSWO_3$	2.3±0.3	10.7±0.3	13.0±1.6	26.3±0.6	23.0±1.5	25.7±0.3	Sea	22.0±0.6	26.0±1.5	23±0.9
	$HSWO_{11}$										19.0±1.2
-	22.3±0.3			15.3±0.3	22.0±1.2	26.3±1.5	14.7±0.7	17.0±0.5			
sediment	$GSSO_4$	16.7±0.7	18.3±0.9	12.7±0.3	12.3±0.3	21.0±0.6	19.0±1.2	-	-	-	
	$HSSE_1$	15.0±0.0	9.7±0.3	-	-	-	18.3±0.9	-	29.7±0.7	-	
	$HSSE_{2a}$	19.0±0.6	-	16.3±0.9	20.7±0.3	17.0±0.6	-	-	14.7±0.7	-	
	$HSSE_{3a}$	11.7±0.7	-	16.3±0.3	9.0±1.2	-	17.7±0.7	-	-	-	
	$HSSO_2$	19.0±0.0	11.3±0.9	17.7±0.3	-	5.0±1.5	18.0±1.5	-	-	8.3±0.3	
	$HSSO_{4c}$	12.0±1.5	21.0±0.0	19.0±1.2	-	-	22.3±0.3	8.7±0.3	11.0±0.0	4.7±0.3	
	$HSSO_{5a}$	18.7±0.9	11.3±0.9	-	10.7±0.7	15.7±0.3	26.0±1.5	15.3±0.9	25.3±2.0	-	

n=3; B. sub=*B. subtilis*, E. fae=*E. faecalis,* S. typ=*S. typhi*, S. aur=*S. aureus,* S. pyo=*S. pyogenes*, K. pne=*K. ıeumoniae,* E. col=*E. coli*, P. aer=*P. aeruginosa* and C. alb=*C. Albicans*.

Table 4.2: Antimicrobial activity of the isolates using the agar well diffusion method

	Test organisms/Mean zones of inhibition (mm)

Sample sources	Isolates	B. sub	S. typ	E. fae	S. aur	S. pyo	K. pne	E. col	P. aer	C. alb
Soil	$GSSA_4$	-	18.3±0.3	-	-	-	-	-	-	$GSSC_1$ -
		-	-	-	-	17.7±0.3				
	$GSSC_{14}$	-	17.7±1.8	-	-	-	-	-	17.3±0.3	-
	$HSSA_1$	-	-	-	-	-	-	18.3±0.3	-	-
	$HSSA_{2a}$	16.3±0.3	-	-	-	-	-	-	-	-
	$HSSA_{3a}$	-	28.0±0.6	-	-	-	-	-	35.0±0.6	-
	$HSSA_5$	15.0±0.6	-	-	-	-	-	-	-	-
	$HSSA_6$	-	-	-	21.3±0.3	-	-	-	-	-
Kakum Riverwater	$GFWC_{2b}$	-	16.3±0.3	-	-	-	-	-		-
	$GFWK_{1a}$	16.7±0.3	-	-	-	-	-	19.7±0.3	-	-
	$HSSC_2$	-	18.3±0.7	-	-	-	-	-	17.3±0.3	-
Kakum River sediment	$GKSC_3$	24.0±1.2	-	-	-	-	-	-	-	-
	$GKSE_1$	33.3±1.5	24.0±0.0	-	-	-	29.7±0.3	-	35.3±0.3	-
	$HKSC_{10}$	15.3±0.9	-	-	-	-	-	-	-	-
	$HKSC_{2b}$	16.0±0.0	16.0±0.6	-	15.7±0.3	-	-	-	-	-
	$HKSC_{2c}$	16.7±0.7	-	-	-	-	-	-	-	-
	$HKSC_{3a}$	-	-	-	19.7±0.3	-	-	-	24.3±0.3	-
	$HKSE_{10}$	17.3±0.7	-	-	-	-	-	15.3±0.3	-	-
	$HKSE_4$	-	-	-	-	-	-	-	30.0±0.0	-
	$HKSK_2$	-	20.7±0.9	-	-	-	-	-	-	-
	$HKSK_{3b}$	-	-	-	31.0±0.6	-	-	-	-	-

	$HKSK_{4b}$	-	-	-	-	-	-	-	20.3±0.3	
	$HKSK_{7c}$	-	24.3±0.3	-	-	-	-	26.3±0.3	-	-
	$HKSK_{8a}$	-	-	-	16.0±0.0	-	-	-	-	-
Sea water	$GSWE_{1b}$	-	18.3±0.3	-	-	-	-	16.7±0.3	-	-
	$GSWE_{2a}$	-	16.0±0.6	-	-	-	-	-	17.0±0.0	-
	$HSWO_{1a}$	17.0±1.0	-	-	-	-	-	-	-	-
	HSWO3	-	-	-	-	-	-	16.3±0.3	-	-
Sea sediment	$GSSO_{11}$	15.3±0.3	-	-	25.0±0.6	-	-	-	-	
	$GSSO_4$	-	-	-	15.7±0.7	-	-	-	19.7±0.3	-
	$HSSE_1$	-	-	-	16.3±0.3	-	-	18.0±0.0	-	-
	$HSSE_{2a}$	17.7±0.7	-	-	-	-	-	16.3±0.3	16.7±0.3	-
	$HSSE_{3a}$	-	16.3±0.3	-	27.0±0.6	-	-	16.0±0.0	-	-
	$HSSO_2$	-	17.7±0.7	-	-	-	-	17.7±0.3	19.3±0.3	-
	$HSSO_{4c}$	-	-	-	16.7±0.3	-	-	-	-	-
	$HSSO_{5a}$	-	-	-	16.3±0.3	-	-	17.0±0.0	-	-

n=3; B. sub=*B. subtilis*, E. fae=*E. faecalis,* S. typ=*S. typhi*, S. aur=*S. aureus,* S. pyo=*S. pyogenes*, K. pne=*K. pneumoniae,* E. col=*E. coli*, P. aer=*P. aeruginosa* and C. alb=*C. Albicans*.

4.2 Incubation Period

The selected isolate, $GKSE_1$, was incubated for 12 days and its metabolite was tested daily for inhibitory activity against *K. pneumoniae*. Generally, there was an increase in the antimicrobial activity on the first three days and a decrease on day four after which the activity increased on days 5, 7 and 8. A decreased activity was observed on days 9 and 10, and a slight increase in activity on days 11 and 12 (Figure 4.1).

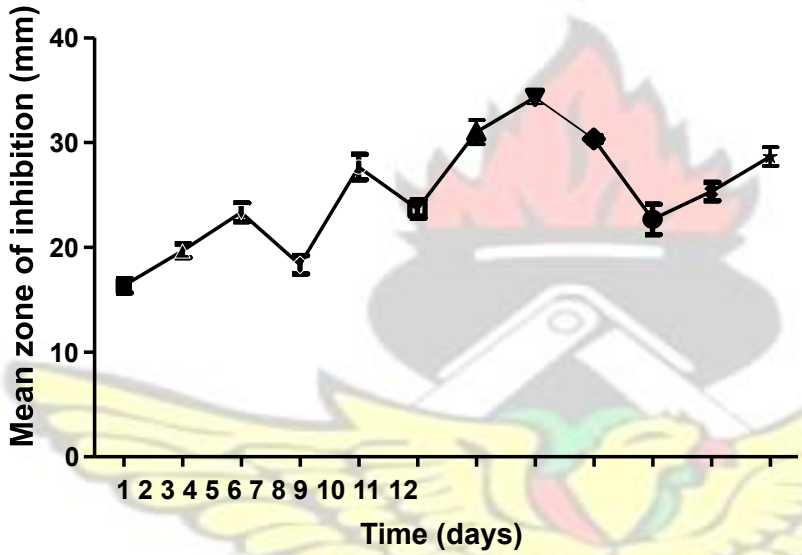


Figure 4.1: Effect of incubation period on the antimicrobial activity of the metabolite of isolate $GKSE_1$. (n=3)

4.3 Extraction and Antimicrobial Activity of the Extract of Isolate $GKSE_1$

The ethyl acetate extract yielded a hygroscopic brownish precipitate. Antimicrobial activity of the extract using the agar well diffusion method showed that the extract has inhibitory activity against all the test microorganisms (Table 4.3). The mean zone of inhibition observed at concentration 400 mg/mL against the test organisms ranged between 19.33±0.22 – 32±1.16 mm. At concentration 200 mg/mL, the mean zone of

inhibition ranged between 16±0.58 – 29.67±0.33 whiles at concentration 100 mg/mL, the mean zone of inhibition was observed to be 12.67±0.33 – 25.33±0.88 mm. At concentrations 50 and 25 mg/mL, the mean zones of inhibition were found to range between 11±0 – 16±0 mm and 10±0 – 13±0.58 mm respectively. The extract was active against *S. epidermidis* at concentration 12.5 mg/mL with a mean zone of inhibition of 10±0 mm whiles the other test organisms were not inhibited at this concentration. The antibacterial activity of the extract decreased with decreasing concentrations. The minimum and maximum mean zones of inhibition of ciprofloxacin (1 mg/mL) against the test organisms were found to be 32.33±0.33 and 48.67±0.67 mm (Table 4.3).

Table 4.3: Antimicrobial activity of the extract and Ciprofloxacin

Test organisms	Concentrations of extract (mg/mL)/Mean zones of inhibition (mm)						Cipro(µg/mL)
	400	200	100	50	25	12.5	1
Bacillus subtilis	20.67±0.33	17.33±0.33	13.00±0.58	12.00±0.58	10.00±0.00	0.00±0.00	40.00±0.00
Escherichia coli	30.33±2.60	28.33±1.67	17.67±1.20	12.67±0.33	10.00±0.00	0.00±0.00	43.33±0.88
Enterococcus faecalis	32.00±1.16	29.67±0.33	25.33±0.88	16.00±0.00	10.00±0.00	0.00±0.00	33.67±0.67
Klebsiella pneumoniae	23.00±2.08	16.00±0.58	12.67±0.33	11.00±0.00	12.33±0.33	0.00±0.00	33.00±0.00
Pseudomonas aeruginosa	20.33±0.67	16.33±0.33	13.00±0.58	11.00±0.00	10.00±0.00	0.00±0.00	32.33±0.33
Staphylococcus aureus	20.67±0.33	16.67±0.33	13.67±0.67	10.67±0.33	10.33±0.33	0.00±0.00	45.33±0.67
Staphylococcus epidermidis	28.67±0.67	24.33±1.33	19.67±0.67	13.67±0.67	13.00±0.58	10.00±0.00	46.67±1.20
Streptococcus pyogenes	24.67±0.88	20.67±0.33	16.33±0.33	12.00±0.57	10.67±0.33	0.00±0.00	28.33±0.67
Shigella dysentriae	21.67±0.67	15.33±0.33	12.67±0.33	11.33±0.33	10.00±0.00	0.00±0.00	48.67±0.67
Salmonella typhi	23.33±0.67	19.67±0.33	16.67±0.67	12.67±0.33	11.33±0.33	0.00±0.00	48.67±0.67
Salmonella typhorium	19.33±0.33	17.33±0.67	15.33±0.88	13.33±0.33	10.33±0.33	0.00±0.00	34.33±0.33

Mean±SEM; n=3; Cipro=ciprofloxacin

4.4 Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) of the Extract and Ciprofloxacin

Six test organisms had their MICs at a concentration of 3.13 mg/mL. Other three test organisms also had their MICs at 6.25 mg/mL whiles two test organisms had their MICs at 1.56 mg/mL (Table 4.4). The extract was bactericidal to *B. subtilis* and *S. typhorium* at concentration 12.5 mg/mL, whiles *S. pyogenes*, *S. aureus*, and *K. pneumoniae* were killed at concentration 6.25 mg/mL. The extract was bactericidal to *S. typhi*, *E. faecalis*, *P. aeruginosa*, *S. epidermidis*, *S. dysentriae* and *E. coli* at concentration 3.13 mg/mL. With ciprofloxacin, *S. dysentriae* had the highest MIC (10 µg/mL) followed by *B. subtilis* (5 µg/mL) whilst *E. coli*, *S. aureus*, *S. typhi* and *S. pyogenes* showed the least MIC (1.25 µg/mL). Ciprofloxacin was bactericidal to *S. dysentriae* at concentration 20 µg/mL, whilst concentration 1.25 µg/mL was bactericidal to *E. faecalis* and *E. coli* (Table 4.4)

Table 4.4: MICs/MBCs of the extract and Ciprofloxacin

Test organisms	Extract (mg/mL)		Cipro (µg/mL)	
	MIC	MBC	MIC	MBC
Bacillus subtilis	6.25	12.50	5.00	10.00
Escherichia coli	3.13	3.13	1.25	1.25
Enterococcus faecalis	3.13	3.13	2.50	1.25
Klebsiella pneumoniae	6.25	6.25	2.50	10.00
Pseudomonas aeruginosa	3.13	3.13	2.50	5.00
Staphylococcus aureus	1.56	6.25	1.25	2.50
Staphylococcus epidermidis	3.13	3.13	2.50	10.00
Shigella dysentriae	3.13	3.13	10.00	20.00
Streptococcus pyogenes	3.13	6.25	1.25	2.50
Salmonella typhi	1.56	3.13	1.25	2.50
Salmonella typhorium	6.25	12.50	2.50	10.00

Cipro = Ciprofloxacin

4.5 Effect of Temperature on the Antimicrobial Activity of the Extract

Statistically, there was no significant difference between the control and the other heat treated groups when their inhibitory activities were tested against *K. pneumoniae* and *B. subtilis* (Figure 4.2).

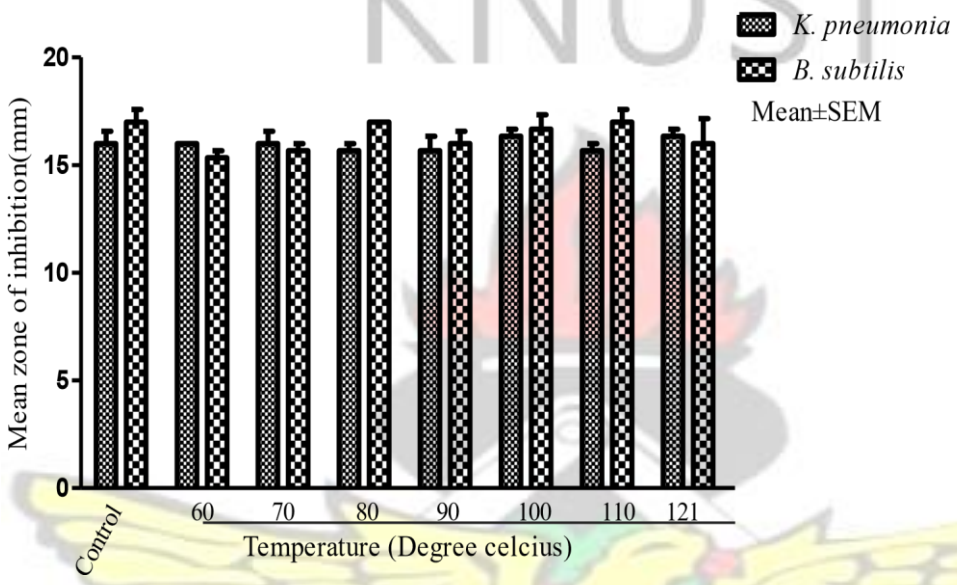


Figure 4.2: Effect of temperature on the antimicrobial activity of the extract

4.6 Effect of Sources of Carbon on Antimicrobial Metabolite Production

Microorganisms are known to utilize carbon from different sources. These sources range from simple sugars to complex sugars. The isolate $GKSE_1$ was able to produce an antimicrobial metabolite in a medium containing Xylose, Mannitol, Lactose, Arabinose, D-fructose, Raffinose, Glucose, Pectin and Sorbose as carbon sources. There was a higher activity observed with the medium supplemented with glucose whiles that supplemented with starch did not have any activity against both *K. pneumoniae* and *B. subtilis.* In the

absence of a carbon source, the isolate was able to produce antimicrobial metabolite in a peptone water medium (Figure 4.3).

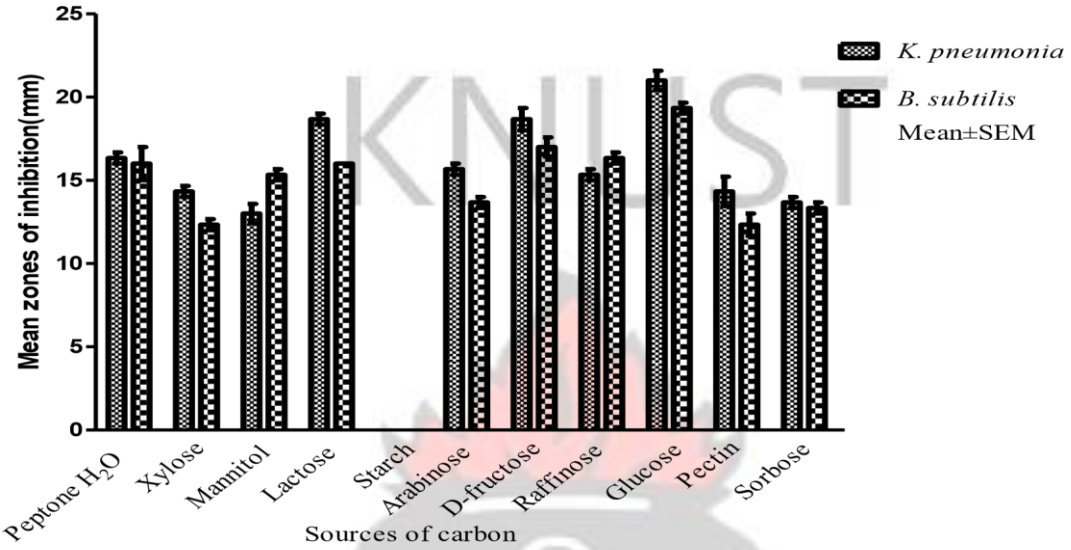


Figure 4.3: Effect of different carbon sources on antimicrobial metabolite production

4.7 Stability Assessment of Aqueous Solution of the Extract

The extract was stable in an aqueous medium for a period of 12 weeks. There was no significant difference in the inhibitory activity of the extract when it was kept at 4 ^{O}C for 12 weeks and was tested against *K. pneumoniae* and *B. subtilis*. The same observation was made when the extract was kept at room temperature (Figure 4.4a and Figure 4.4b).

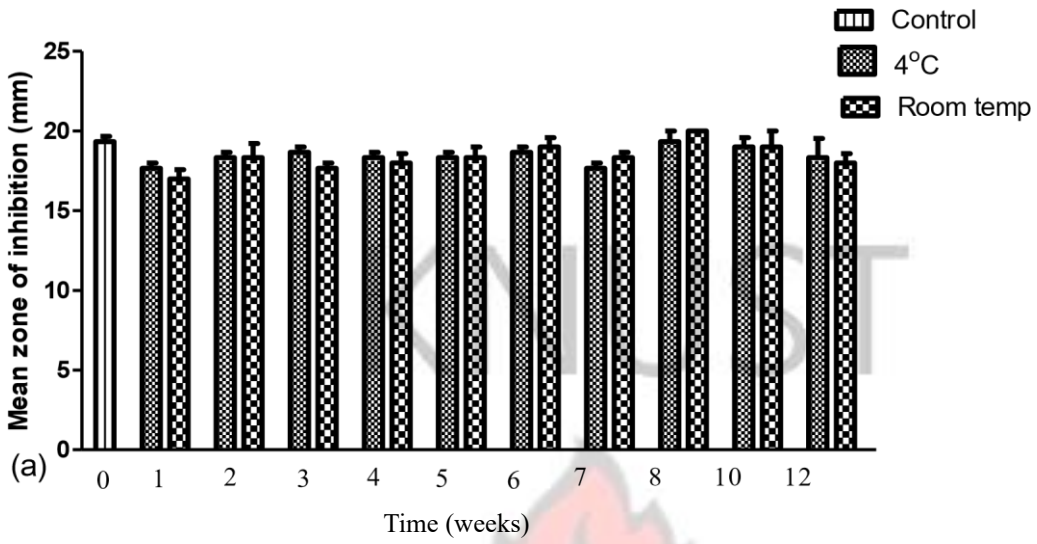


Figure 4.4a: Mean zones of inhibition of aqueous solution of the extract kept for a period of twelve weeks and tested against *B. subtilis*.

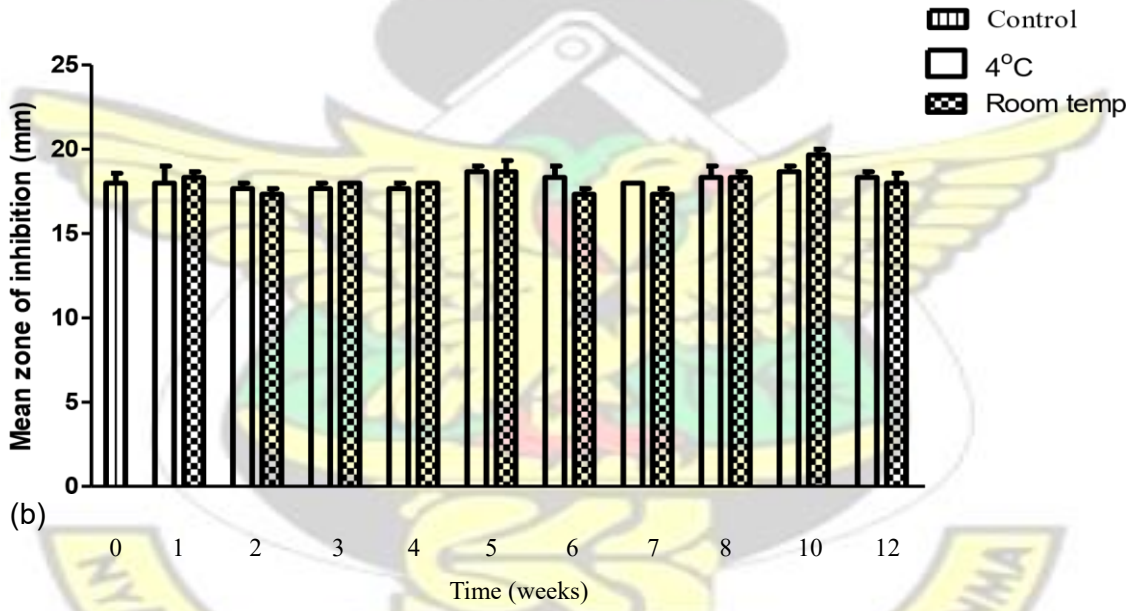


Figure 4.4b: Mean zones of inhibition of aqueous solution of the extract kept for a period of twelve weeks and tested against *K. pneumoniae*.

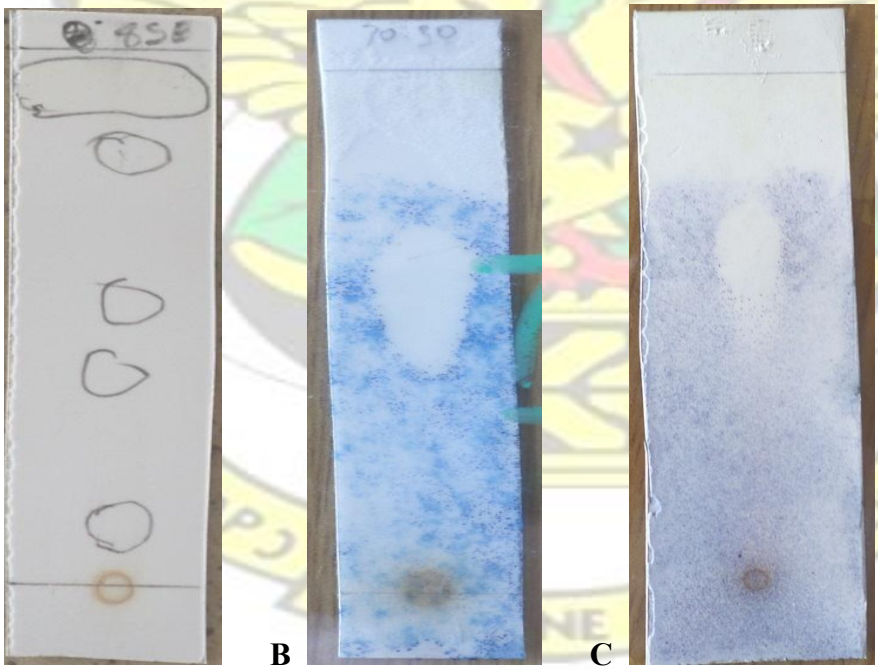
4.8 Thin Layer Chromatography (TLC) Analysis of the Extract

Chloroform – ethanol (85:15) solvent system yielded five spots with their retardation factor (R_f) values being 0.095 (1), 0.405 (2), 0.524 (3) 0.810 (4) and 0.976 (5). Three spots (3, 4 and 5) were seen when the chromatogram was observed under UV light at wavelength 254nm, whiles four spots (1, 2, 4 and 5) were seen at wavelength 365nm.

One spot (5) was stained with *p* – anisyladehyde.

4.9 Bioautography Assay

Two areas on the developed TLC plates showed inhibitory activities against *K. pneumoniae* and *B. subtilis* (Plate 4.1). This is indicated by clear zones against blue/purple background.



A **B** **C**

Plate 4.1: Spots observed and their inhibitory activities. Developed plate (A); zones of inhibition exhibited by *K. pneumoniae* (B), and *B. subtilis* (C).

4.10 Antibiofilm Activity

The extract was able to prevent *S. aureus*, *E. coli* and *P. aeruginosa* biofilm formation when its antibiofilm activity was assessed using the crystal violet assay. There were significant difference ($p<0.05$) between the extract treated groups and the untreated group for all the tested organisms. Similar observation was made for the EDTA treated groups and the untreated groups (Figures 4.5a, 4.5b and 5.5c).

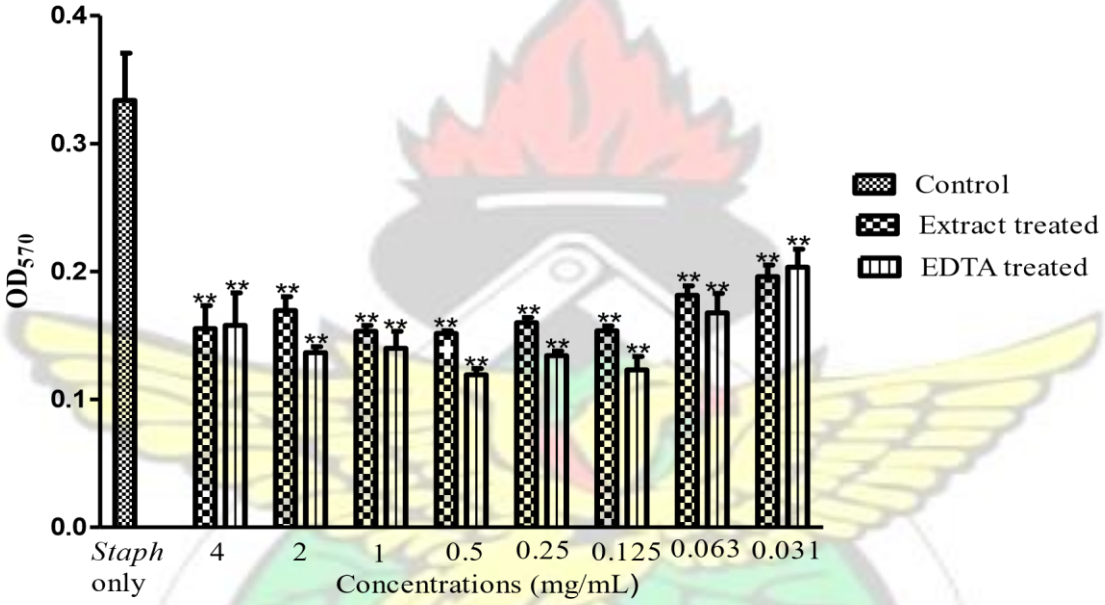


Figure 4.5a: Inhibition of *S. aureus* biofilm formation.
** shows that there is a significant difference between the control and the treated groups.

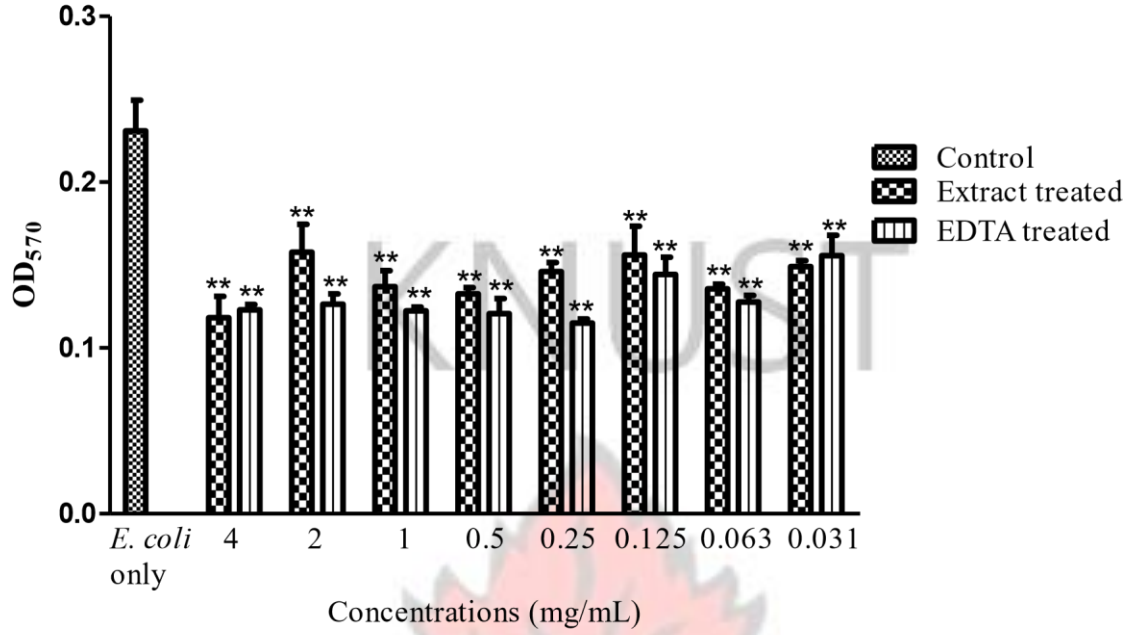


Figure 4.5b: Inhibition of *E. coli* biofilm formation.
** shows that there is a significant difference between the control and the treated groups.

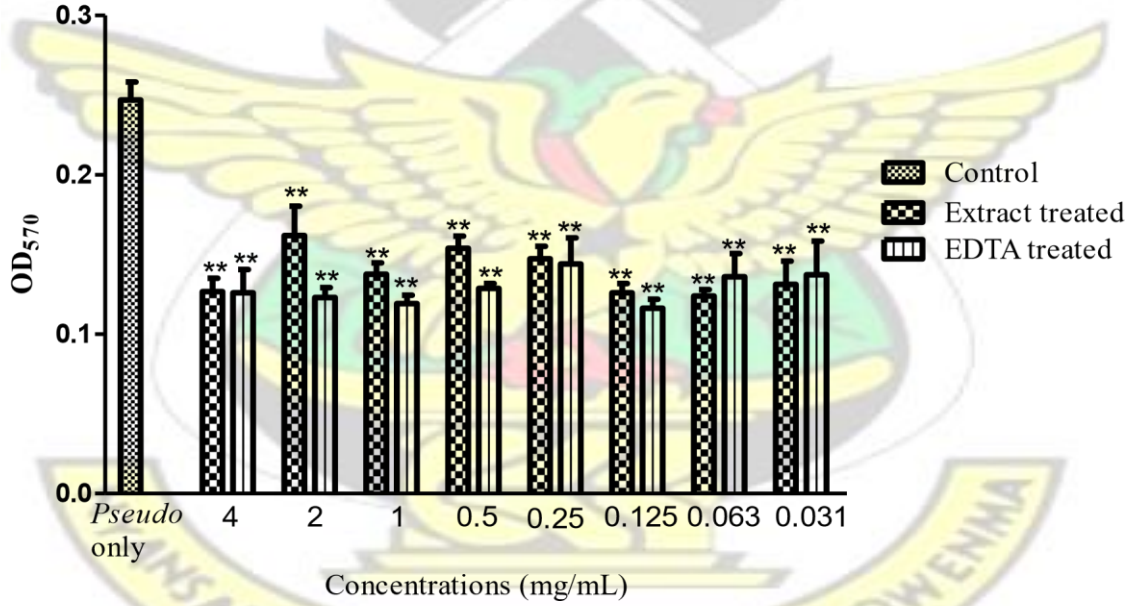


Figure 4.5c: Inhibition of *P. aeruginosa* biofilm formation.
** shows that there is a significant difference between the control and the treated groups.

4.11 Biosurfactant Activity Assessment of the Isolate GKSE$_1$

There was no emulsification activity when 50 mg/mL of the extract was mixed with light crude oil. In the oil displacement test, the observed hallow or zone was determined to be

9.67±0.88 mm, indicating a biosurfactant activity. There was no surface interaction between the extract and the light crude oil in the drop collapse test (Table 4.6 and Plate 4.2).

Table 4.5: Biosurfactant producing ability of the isolate $GKSE_1$

Test	Observation	Inference/Remarks
Emulsification index (%)	0.00	No emulsification
Oil displacement (mm)	9.67±0.88	Little activity
Drop collapse	–	No surface interaction

– = negative test; Values = Mean ± SEM

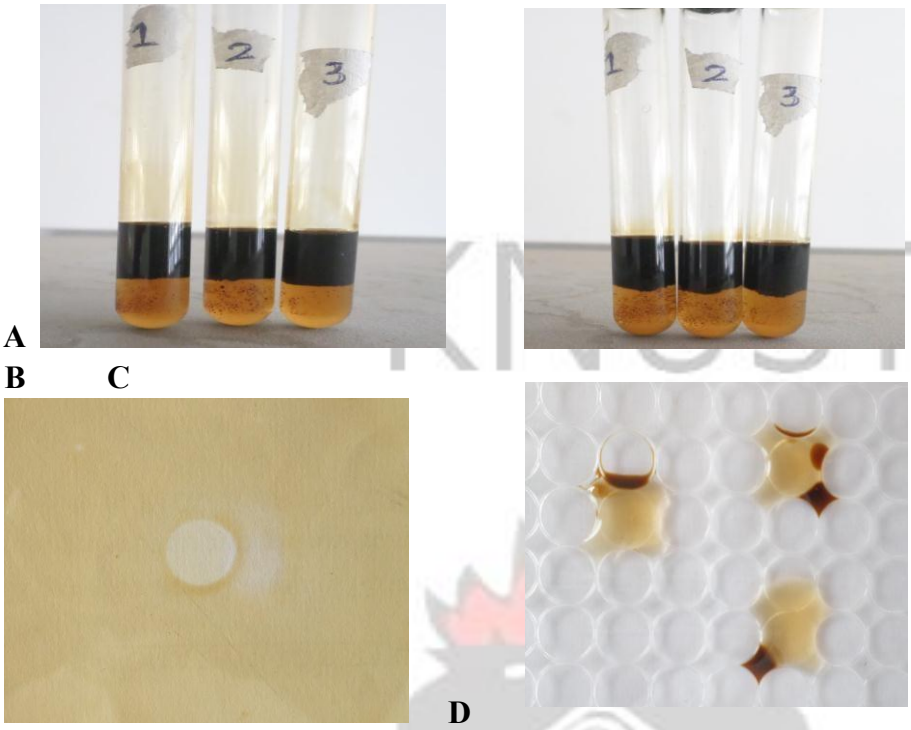


Plate 4.2: Biosurfactant activity of the extract. Emulsification activity; before 24 hours (A), after 24 hours (B); Oil displacement test (C); Drop collapse test (D)

4.12 Characterization of Isolate GKSE$_1$

Gram staining of the isolate showed that the organism is gram positive cocci. It is able to grow at 45 ^{O}C and in 6.5% NaCl medium. It is not able to grow on Mannitol salt, MacConkey, Bismuth sulphide and Cetrimide agar and does not produce indole and H_2S. It does not utilize citrate and is not able to reduce nitrate. It produces acid from glucose on lactose with no gas production.

Table 4.6: Characterization of isolate $GKSE_1$

TEST	OBSEVATION
Gram reaction	+
Shape	Cocci
Growth at 6.5% NaCl	+
Growth at 45 ^{O}C	+
Growth on	-
Cetrimide agar	
Bismuth sulphite agar	-
Mannitol salt agar	-
MacConkey agar	-
Biochemical tests	-
Catalase	
Oxidase	-
H_2S production	-
Indole production	-
MR	+
VP	-
Nitrate reduction	-
Citrate utilization	-
Acid from	
Glucose	+
Lactose	+
Gas from	-
Glucose	
Lactose	-

(+) = Positive activity, (–) = Negative activity

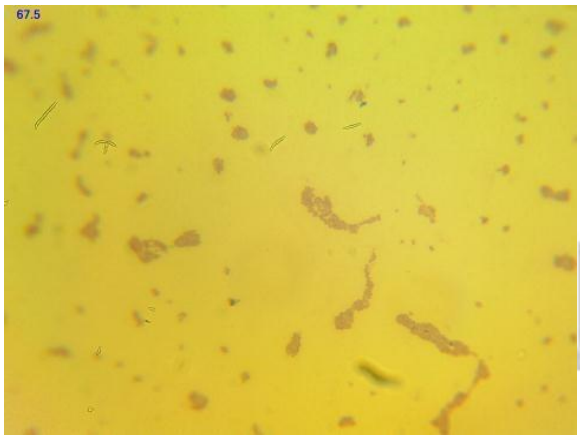


Plate 4.3: Gram reaction of the isolate.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 Discussion

Microorganisms are widely distributed in nature– air, soil and water (Talaro and Talaro, 2002; Sarkar *et al*., 2014). This was evident when the Ejisu soil, Kakum River and the

Sea samples yielded a total of 138 microorganisms after inoculation in growth media. These environments provide the necessary growth conditions that support the growth of microorganisms.

Thirty – six (36) out of the 138 microorganisms isolated were found to produce antimicrobial metabolites when they were screened against nine test organisms (Tables 4.1 – 2). Studies by other people have shown that not all isolated microorganisms screened against test organisms exhibit antimicrobial activity. Out of the 119 microorganisms isolated and screened by Tawiah *et al*., (2012), only 27 showed inhibitory activity against at least one test organism. Out of the 78 marine Actinobacteria isolated by Kumar *et al*., (2011), 22 showed antibacterial activity, 12 had antifungal activity whiles 11 exhibited both antibacterial and antifungal activities. Gebreyohannes *et al*., (2013) also isolated 31 actinomycetes from water and sediments samples of Lake Tana, Ethiopia, and only 13 isolates showed antibacterial activities against at least one test organism. Microorganisms produce antimicrobial metabolites (may be a protein, peptide or hydrocarbon) probably as a defensive mechanism against other organisms that may compete with them for nutrient and site of colonization (Waites *et al*., 2001).

The selected isolate, $GKSE_1$, was found to produce antimicrobial metabolites after 24 hours of incubation. The antimicrobial metabolite production peaked on day 8 of incubation and it was marked by a higher mean zone of inhibition when it was tested against *K. pneumoniae* (Figure 4.1). This observation may suggest that the organisms produced more antimicrobial metabolites, the period during which bioactive metabolite is preferably extracted (Bharti *et al*., 2012).

The ethyl acetate extract obtained from the fermentation of isolate $GKSE_1$ showed varying degrees of antimicrobial activity against all the test organisms used. In the agar well diffusion method, inhibitions were observed at all tested concentrations against all the test microorganisms except at concentration 12.5 mg/mL where the extract showed activity against *S. epidermidis* only (Table 4.3). The Minimum Inhibitory Concentration of the extract was found to range between 1.56 – 6.25 mg/mL and this suggests that some of the test organisms were more susceptible than the others (Table 4.4). Six (6) of the test microorganisms (*E. coli*, *E. faecalis*, *P. aeruginosa*, *S. epidermidis*, *S. dysentriae* and *S. typhi*) were killed at concentration 3.13 mg/mL; 3 others (*K. pneumoniae*, *S. aureus* and *S. pyogenes*) were killed at concentration 6.25 mg/mL whiles *B. subtilis* and *S. typhorium* were killed at concentration 12.50 mg/mL (Table 4.4). Ciprofloxacin being a pure compound was observed to have antimicrobial activity at lower concentrations compared to the extract.

The chemistry of most compounds gets destroyed when they are exposed to high temperatures hence rendering them biologically inactive. When the extract was exposed to different temperatures (60 – 121 ^{O}C) and its antimicrobial activity was tested against *K. pneumoniae* and *B. subtilis* using the agar well diffusion method, there was no significant difference in the mean zones of inhibition obtained (figure 4.2). This means that the tested temperatures did not have any effect on the antimicrobial agents or compounds produced by isolate $GKSE_1$. This finding agrees with that reported by Xiao – Yan *et al*., (2006) where Trichokonins isolated from *Trichoderma koningii* SMF2 was found to be heat resistant. A report by Bharti *et al*., (2012) was in contrast with this current study since they observed a reduction in the antimicrobial activity of an extract obtained from *Burkholderia gladioli*

when the extract was subjected to temperatures of 70 ^{O}C and above. Muiru *et al*., (2007) also reported that extract obtained from *Actinomycetes* species were thermolabile at temperatures above 90 ^{O}C.

Microorganisms utilize different carbon sources for their metabolic activities. These carbon sources may be simple or complex sugars. The isolate $GKSE_1$ was able to produce antimicrobial metabolite when it was cultivated in a medium containing Xylose, Mannitol, Lactose, Arabinose, D – fructose, Raffinose, Glucose, Pectin and Sorbose as carbon sources (Figure 4.3). This may suggest that the sugars were used as carbon sources for the metabolic processes of the organism or they were used as substrate for the production of secondary metabolites of which the antimicrobial agent was one of them. The medium containing glucose favored the production of more antimicrobial

metabolites, and it was evidenced by exhibiting the highest inhibitory activity against *B. subtilis* and *K. pneumoniae* (Figure 4.3). Metabolites produced in the medium containing starch on the other hand did not show any inhibitory activity. This may be attributed to the complexity of starch making it not a suitable carbon source for isolate $GKSE_1$ or substrate for antimicrobial metabolite production.

Since compounds lose their biological activities through degradation when kept in solution for a long period of time, the stability of the extract in this current study was assessed in aqueous solution (Deshpande *et al*., 2004). Contrarily, the extract in this current study exhibited potent antimicrobial activity even after three month of keeping it in aqueous solution irrespective of the temperatures (4 ^{O}C and room temperature) at which it was kept (Figures 4.4a and 4.4b). Work done by other people have shown that the bioactive components of extracts maintain their antimicrobial activities when kept in solution. Muiru

et al., (2007) observed inhibitory activities when extracts from two isolates, CS35 and 28P, were tested for their antimicrobial activities for a period of 5 months.

The isolate $GKSE_1$ appeared to produce more than one secondary metabolite as evidenced in the TLC analysis where five separate spots were observed when a spotted TLC plate was developed in chloroform – ethanol (85:15%) mixture. Two regions on the chromatogram showed inhibitory activity against *B. subtilis* and *K. pneumoniae* in the bioautography assay and this suggests that the isolate produced more than one antimicrobial agent. The bioautography analysis by Bharti *et al*., (2012) showed three spots having inhibitory activities against *S. aureus* whiles two spots had activities against *Candida tropicalis*.

Most microorganisms form biofilms as common means of growth in both biotic and abiotic environments (Sayem *et al*., 2011). The polymeric substance produced in biofilms prevents antimicrobial agents from getting to the cells of target pathogenic organisms hence increasing the resistant forms of microbial strains. The extract when assayed for its antibiofilm property showed that it has the potential to reduce biofilms formation by *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* at all the tested concentrations (0.031 – 4 mg/mL; Figures 4.5a – c). The observed antibiofilm activity of the extract from isolate $GKSE_1$, may be attributed to extracellular compounds that were probably produced as secondary metabolites. Some of these extracellular compounds secreted by microorganisms have been found to be polysaccharide in nature and it is known that polysaccharides interfere with cell–surface interaction between organisms and their substratum – a requirement for biofilm development (Reddy *et al*., 1993; Valle *et al*., 2006; Qin *et al*., 2009; Pihl *et al*., 2010; Jiang *et al*., 2011; O'Toole *et al*., 2000). The antibiofilm

activity of the extract can also be attributed to the antimicrobial property of the compounds produced by the isolate (Fonseca *et al*., 2004; Sayem *et al*., 2011).

Isolate $GKSE_1$ was assayed for its ability to produce biosurfactant. Biosurfactant increases the penetrating ability of agents into cells by decreasing the surface tension of cells. Three different tests – oil displacement, drop collapse and emulsification tests – were used to access biosurfactant activity of the extract. In the oil displacement test, the extract produced a very little hallow (diameter = 9 mm) suggesting the presence of a biosurfactant. Similar result was reported by Hassanshahian, (2014) where one isolate among others produced a hallow of diameter 9 mm in the oil displacement test. There was no emulsified layer in the emulsification test whiles the drop collapse test yielded a negative result (Table 4.5). It may be possible that the isolate produced very little biosurfactant which was not able to significantly emulsify crude oil and also produce significant surface interaction in the drop collapse test (Hassanshahian, 2014). Among the three methods used for the biosurfactant activity, it has been shown that the oil displacement method is more sensitive than the emulsification and drop collapse test and may account for the observed activity in the oil displacement test (Mounira and Abdelhadi, 2015). Microorganisms isolated from an oil contaminated environments have been shown to produce more biosurfactants than those isolated from uncontaminated environments (Ghayyomi *et al*., 2012) and this might be another reason why the isolate $GKES_1$ did not give any positive result in the emulsification and drop collapse tests since it was isolated from an environment (Kakum River) that was not contaminated with oil or a hydrocarbon.

Morphologically and biochemically, isolate $GKSE_1$, is a gram positive cocci and does not grow on Mannitol salt agar, MacConkey agar, Bismuth sulphite agar and Cetrimide agar. Again, it does not produce H_2S and indole but is able to produce acid from glucose and lactose (Table 4.6). When the observed characteristics were compared to characteristics of other organisms in literature, there was no organism with which the isolate perfectly matches so it was not possible to draw conclusion on the identity of isolate $GKSE_1$.

5.2 Conclusion

This study has revealed the presence of antimicrobial producing microorganisms from the Ghanaian environments (soil, Sea and river). Out of the 138 microorganisms isolated and screened, 36 of them showed inhibitory activity against at least two test microorganisms in the cross streaking method and at least one test microorganism in the agar well diffusion method. The ethyl acetate extract of isolate $GKSE_1$, (from the sediment of Kakum River at Etre) showed inhibitory activity (with MIC and MBC ranging between 1.56 – 6.25 mg/mL and 3.13 – 12.50 mg/mL respectively) against *Bacillus subtilis* NTCC 10073, *Salmonella typhi*, *S. typhorium*, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, *S. epidermidis*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 4853 and *S. dysentriae*. The extract was found to be thermostable after autoclaving (at 121 ^{O}C for 15 minutes) and also stable in an aqueous solution for a period of three months. Two regions on the chromatogram were found to have inhibitory activities against *B. subtilis* and *K. pneumoniae* in the bioautography assay. Again, the extract was found to significantly

reduce biofilms formed by *S. aureus*, *E. coli* and *P. aeruginosa* but did not exhibit good biosurfactant activity.

5.3 Recommendation

- The isolate $GKSE_1$ should be identified using molecular methods.
- The bioactive components of the metabolites produced should be isolated, purified, identified and tested in both *in vitro* and *in vivo* studies.
- Toxicological studies of the isolated compound(s) should be done.

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APPENDIX

Table 6.1: Screening of isolates for antimicrobial metabolite production using the cross streaking technique.

ISOLATES	B. sub			S. typ			E. fae			S. aur			S. pyo			K. pne			E. col			P. aer			C. alb		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
$GSSA_4$	23	22	21	21	19	18	26	25	26	-	-	-	7	5	5	20	24	26	18	15	13	13	14	17	-	-	-
$GSSC_1$	-	-	-	-	-	-	-	-	-	4	5	7	-	-	-	13	10	11	-	-	-	6	5	5	-	-	-
$GSSC_{14}$	23	19	17	27	32	32	22	22	23	21	23	25	18	20	24	24	25	27	-	-	-	16	14	14	-	-	-
$HSSA_1$	22	22	23	18	18	17	23	27	25	19	19	20	13	14	16	22	23	25	13	16	18	6	8	11	-	-	-
$HSSA_{2a}$	17	16	17	-	-	-	-	-	-	17	16	17	21	20	20	-	-	-	-	-	-	-	-	-	-	-	-
$HSSA_{3a}$	16	16	16	-	-	-	19	20	21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$HSSA_5$	22	21	21	18	17	17	-	-	-	14	16	18	14	15	17	21	20	21	13	11	9	-	-	-	-	-	-
$HSSA_6$	15	15	17	-	-	-	18	17	18	15	13	11	15	15	15	20	21	23	-	-	-	-	-	-	-	-	-
$HSSC_2$	23	22	21	9	10	11	11	10	10	12	12	12	9	11	14	16	16	18	3	3	5	-	-	-	21	20	21
$GFWC_{2b}$	-	-	-	15	16	16	25	28	32	19	21	23	21	18	20	-	-	-	10	12	15	-	-	-	-	-	-
$GFWK_{1a}$	18	18	19	30	31	33	16	16	17	22	25	26	25	27	29	-	-	-	5	8	11	-	-	-	13	11	11
$GKSC_3$	21	20	21	13	10	8	16	15	15	-	-	-	-	-	-	17	19	22	7	6	6	21	19	19	-	-	-
$GKSE_1$	26	27	29	26	25	25	19	20	22	16	15	16	-	-	-	26	30	32	21	23	20	24	24	25	-	-	-
$HKSC_{10}$	11	10	11	10	10	11	-	-	-	14	15	15	7	7	9	17	20	24	-	-	-	-	-	-	-	-	-
$HKSC_{2b}$	20	20	19	-	-	-	27	25	24	28	30	32	21	22	23	29	27	25	-	-	-	-	-	-	-	-	-
$HKSC_{2c}$	19	19	21	14	16	19	24	24	24	-	-	-	-	-	-	29	27	25	11	18	17	-	-	-	6	7	9
$HKSC_{3a}$	-	-	-	18	18	17	21	20	21	13	13	14	10	10	10	23	25	28	-	-	-	22	25	28	-	-	-
$HKSE_{10}$	9	10	13	8	10	13	-	-	-	17	15	16	5	7	10	17	22	22	-	-	-	-	-	-	-	-	-
$HKSE_4$	20	19	19	15	15	17	22	21	21	-	-	-	-	-	-	19	23	24	18	18	18	5	5	6	9	11	14
$HKSK_2$	16	14	14	-	-	-	14	14	16	19	22	24	-	-	-	-	-	-	26	27	28	-	-	-	-	-	-
$HKSK_{3b}$	22	22	22	21	20	21	-	-	-	8	13	11	-	-	-	-	-	-	22	22	24	16	14	14	5	5	7
$HKSK_{4b}$	21	19	19	-	-	-	7	5	5	13	12	13	12	10	9	21	21	21	18	16	16	-	-	-	-	-	-
$HKSK_{7c}$	-	-	-	18	18	18	18	19	19	12	11	11	8	11	14	20	21	21	9	7	7	14	13	13	6	4	4
$HKSK_{8a}$	13	11	9	-	-	-	8	10	13	-	-	-	-	-	-	13	10	8	-	-	-	-	-	-	-	-	-

$GSSO_{11}$	24	27	25	17	19	21	16	15	15	20	22	24	24	26	29	14	14	16	16	17	18	-	-	-	23	22	22
$GSSO_4$	16	18	16	20	18	17	13	12	13	12	12	13	22	21	20	17	19	21	-	-	-	-	-	-	-	-	-
$GSWE_{1b}$	-	-	-	4	3	4	-	-	-	33	35	38	25	26	26	-	-	-	-	-	-	-	-	-	-	-	-
$GSWE_{2a}$	8	10	13	23	20	21	-	-	-	7	8	8	9	10	12	12	10	10	16	15	16	10	10	8	-	-	-
$HSSE_1$	15	15	15	10	9	10	-	-	-	-	-	-	-	-	-	17	18	20	-	-	-	29	29	31	-	-	-
$HSSE_{2a}$	18	19	20	-	-	-	18	16	15	21	20	21	18	17	16	-	-	-	-	-	-	16	14	14	-	-	-
$HSSE_{3a}$	11	11	13	-	-	-	16	16	17	7	9	11	-	-	-	17	17	19	-	-	-	-	-	-	-	-	-
$HSSO_2$	19	19	19	13	11	10	18	17	18	-	-	-	8	4	3	15	19	20	-	-	-	-	-	-	8	8	9
$HSSO_{4c}$	9	14	13	21	21	21	17	19	21	-	-	-	-	-	-	22	22	23	9	8	9	11	11	11	5	4	5
$HSSO_{5a}$	20	17	19	13	11	10	-	-	-	12	10	10	16	15	16	23	27	28	17	15	14	22	25	29	-	-	-
$HSWO_{1a}$	4	3	4	24	24	23	5	8	12	22	27	27	21	20	20	7	3	3	4	2	2	-	-	-	-	-	-
$HSWO_3$	3	2	2	11	10	11	10	14	15	20	24	25	12	10	10	20	22	21	28	26	24	25	26	27	26	25	26

Isolates	B. sub			S. typ			E. fae			S. aur			S. pyo			K. pne			E. col			P. aer			c. albicans		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
$GSSA_4$		-	-	18	18	19	-	-	-															-	-	-	-
				8						-	-	-	-	-	-	-	-	-	-	-	-	-	-				
$GSSC_1$	-	-	-	16	16	16	-	-	-															-	17	1	1
				6						-	-	-	-	-	-	-	-	-	-	-	-	-	-			8	7
$GSSC_{14}$	-	-	-	17	17	21	-	-	-															18	-	-	-
				5						-	-	-	-	-	-	-	-	-	-	-	-	17	17				
$HSSA_1$	-	-	-	-	-	-	-	-	-												1			-	-	-	-
										-	-	-	-	-	-	-	-	-	18	18	9	-	-				
$HSSA_{2a}$	16	16	17	-	-	-	-	-	-															-	-	-	-
										-	-	-	-	-	-	-	-	-	-	-	-	-	-				
$HSSA_{3a}$	-	-	-	27	29	28	-	-	-															36	-	-	-
										-	-	-	-	-	-	-	-	-	-	-	-	34	35				

$HSSA_5$	16	15	14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 6.2: Screening of isolates for antimicrobial metabolite production using the agar well diffusion technique.

$HSSA_6$	-	-
$HSSC_2$	-	-
$GFWC_{2b}$	-	-
$GFWK_{1a}$	15	16
$GKSC_3$	22	24
$GKSE_1$	31	33
$HKSC_{10}$	14	15
$HKSC_{2b}$	16	16
$HKSC_{2c}$	16	16
$HKSC_{3a}$	-	-
$HKSE_{10}$	15	17
$HKSE_4$	-	-
$HKSK_2$	-	-

$HKSK_{3}$
b
$HKSK_{4}$
b
$HKSK_{7}$
c
$HKSK_{8}$
a

-	-	-	-	-	-	-	21	21	22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	17	19	19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	16	16	17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	17	-	-	-
16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	20	-	-	-	-	-	-	-
26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	20	19	20	-	-	-	-	-	-	-
36	24	24	24	-	-	-	-	-	-	-	-	-	30	29	30	-	-	-	35	35	36	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	16	15	17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	16	15	16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	20	19	20	-	-	-	-	-	-	-	-	-	25	24	24	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15	15	16	-	-	-	-	-	-	-
-	22	19	21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	30	32	31	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	20	20	21	-	-	-	-
-	24	24	25	-	-	-	-	-	-	-	-	-	-	-	-	26	26	27	-	-	-	-	-	-	-
-	-	-	-	-	-	-	16	16	16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	27	25	26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

GSSO11 - - - - - 15 1

		5																							
$GSSO_4$	-	-	-	-	-	-	-	-	-	17	15	15	-	-	-	-	-	-	-	-	-	20	19	20	-	-	-
$GSWE_1$	-	-	-	1	18	18	-	-	-										1						-	-	-
b				9						-	-	-	-	-	-	-	-	-	7	17	16	-	-	-			
$GSWE_2$	-	-	-	1	15	16	-	-	-																-	-	-
a				7						-	-	-	-	-	-	-	-	-	-	-	-	17	17	17			
$HSSE_1$	-	-	-	-	-	-	-	-	-										1						-	-	-
										16	16	17	-	-	-	-	-	-	8	18	18	-	-	-			
$HSSE_{2a}$	19	1	17	-	-	-	-	-	-										1						-	-	-
		7								-	-	-	-	-	-	-	-	-	6	17	16	17					
$HSSE_{3a}$	-	-	-	1	16	16	-	-	-										1						-	-	-
				7						26	28	27	-	-	-	-	-	-	6	16	16	-	-	-			
$HSSO_2$	-	-	-	1	17	19	-	-	-										1						-	-	-
				7						-	-	-	-	-	-	-	-	-	8	18	17	18	19	18			
$HSSO_{4c}$	-	-	-	-	-	-	-	-	-	17	16	17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$HSSO_{5a}$	-	-	-	-	-	-	-	-	-										1					-	-	-	-
										17	16	16	-	-	-	-	-	-	7	17	17	-	-				
HSWO	15	1	18	-	-	-	-	-	-															-	-	-	-
1a		8								-	-	-	-	-	-	-	-	-	-	-	-	-	-				
HSWO	-	-	-	-	-	-	-	-	-										1					-	-	-	-
3										-	-	-	-	-	-	-	-	-	6	17	16	-	-				

Table 6.3: Maximum period for incubation

Tests	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12
1	15	19	22	20	27	25	31	35	30	25	25	27
2	17	21	25	17	30	22	33	33	30	20	24	29
3	17	19	23	18	26	24	29	35	31	23	27	30

Test organism= *K. pneumoniae*

Table 6.4: Antimicrobial activity of the extract

Test organisms	Concentrations of extract (mg/mL)/Mean zones of inhibition (mm)																		Ciprofloxacin		
	400			200			100			50			25			12.5			1 mg/mL		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
S. aureus	21	20	21	17	17	16	15	13	13	11	10	11	10	10	10	0	0	0	44	46	46
P. aeruginosa	21	19	21	17	16	16	14	13	12	11	11	11	10	10	10	0	0	0	32	32	33
S. epidermidis	28	28	30	23	23	27	19	19	21	15	13	13	12	13	12	0	0	0	46	45	49
K. pneumoniae	27	20	22	16	15	17	13	12	13	11	11	11	10	10	10	0	0	0	33	33	33
B. subtilis	21	21	20	18	17	17	12	13	14	12	11	13	10	11	10	0	0	0	40	40	40
S. dysenteriae	23	21	21	15	15	16	13	12	13	11	12	11	10	10	10	0	0	0	48	50	48
S. pyogenes	26	25	23	20	21	21	16	16	17	13	12	11	10	10	10	0	0	0	29	29	27
E. faecalis	30	32	34	29	30	30	24	27	25	16	16	16	14	12	13	10	10	10	33	35	33
S. typhi	24	22	24	19	20	20	16	18	16	12	13	13	11	10	11	0	0	0	48	48	50
S. typhorium	20	19	19	18	18	16	17	14	15	13	14	13	11	11	12	0	0	0	35	34	34
E. coli	26	30	35	25	30	30	20	16	17	13	12	13	10	10	11	0	0	0	42	43	45

Table 6.5: Effect of temperature on antimicrobial activity of the extract

Test organisms	Tests	Temperatures (^{O} C)/Zone of inhibitions (mm)							
		50	60	70	80	90	100	110	121
K. pneumoniae	1	15	16	17	16	17	16	16	17
	2	17	16	16	16	15	17	15	16

	3	16	16	15	15	15	16	16	16
B. Subtilis	1	16	15	15	17	15	15	16	14
	2	17	15	16	17	17	17	18	18
	3	18	16	16	17	16	16	17	16

Table 6.6: Effect of carbon source on antimicrobial metabolite production

Carbon source	Test organism/ zones of inhibitions (mm)					
	K. pneumoniae			*B. subtilis*		
	1	2	3	1	2	3
Peptone	16	16	17	18	15	15
Xylose	15	14	14	12	13	12
Mannitol	13	14	12	16	15	15
Lactose	19	18	19	16	16	16
Starch	0	0	0	0	0	0
Arabinose	15	16	16	14	14	13
D-Fructose	20	18	18	18	17	16
Raffinose	15	16	15	16	17	16
Glucose	21	22	20	19	20	19
Pectin	14	13	16	11	13	13

Sorbose	14	13	14	13	14	13

Table 6.7: Stability of the extract in aqueous solution

Test organisms	Condition	Week 0			Week 1			Week 2			Week 3			Week 4			Week 5			Week 6			Week 7			Week 8			Week 10			Week 12		
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
K. pneum	4°C	19	20	19	18	17	18	18	19	18	19	16	20	19	19	18	19	18	18	18	18	19	19	19	18	17	18	18	20	20	18	19	18	20
	Room temp				17	18	16	17	20	18	18	17	19	18	18	17	17	18	19	17	19	19	20	19	18	19	18	18	20	20	20	21	18	18
B. subtilis	4°C	18	17	19	16	19	19	18	17	18	18	17	18	18	17	18	19	18	19	17	19	19	18	18	18	17	19	19	19	18	19	19	18	18
	Room temp				19	18	18	17	17	18	18	18	18	18	18	18	18	20	18	18	17	17	18	17	17	19	18	18	19	20	20	19	17	18

Table 6.8: Antibiofilm activity

		Test organism/Optical density (570)								
Agent	C0ncentrations(mg/mL)	S.aureus			E. coli			p. aeruginosa		
		1	2	3	1	2	3	1	2	3
EXTRACT	4	0.191	0.134	0.141	0.112	0.1	0.143	0.143	0.122	0.115
	2	0.152	0.168	0.189	0.137	0.145	0.191	0.138	0.198	0.15
	1	0.145	0.154	0.161	0.127	0.128	0.156	0.13	0.131	0.152
	0.5	0.148	0.152	0.155	0.13	0.14	0.128	0.163	0.139	0.16
	0.25	0.16	0.167	0.153	0.137	0.156	0.145	0.141	0.138	0.163
	0.125	0.152	0.161	0.148	0.19	0.133	0.145	0.116	0.126	0.136

	0.065	0.185	0.192	0.167	0.132	0.141	0.134	0.119	0.132	0.121
	0.03125	0.214	0.19	0.184	0.142	0.154	0.151	0.14	0.151	0.103
	4	0.204	0.153	0.117	0.129	0.122	0.118	0.11	0.113	0.155
	2	0.144	0.128	0.138	0.139	0.121	0.119	0.12	0.114	0.135
	1	0.166	0.132	0.122	0.12	0.12	0.127	0.111	0.118	0.129
	0.5	0.11	0.122	0.126	0.138	0.117	0.107	0.123	0.129	0.134
EDTA	0.25	0.14	0.135	0.128	0.12	0.112	0.113	0.177	0.13	0.125
	0.125	0.122	0.305	0.342	0.335	0.365	0.133	0.105	0.121	0.123
	0.065	0.188	0.177	0.138	0.13	0.133	0.12	0.124	0.119	0.165
	0.03125	0.205	0.227	0.178	0.151	0.137	0.179	1.403	0.133	0.176

Table 6.9: List of Chemicals and Reagents

Item	Manufacturer/company/place
3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT)	Applichem Chemical Synthesis Services, Germany
Bromocresol purple	Hopkin and Williams Ltd, England
Ciprofloxacin	Sigma Aldrich, Michigan, USA
Creatine solution	BDH Limited, Poole, England
Crystal violet D	
– Glucose	BDH Laboratory Supplies, Poole, England
D (-) Fructose	BDH Chemicals Ltd, Poole, England
D (+) Xylose	The British Drug Houses Ltd, Poole, England
Ethylenediaminetetra – acetic acid (EDTA)	Merck BDH, Poole, UK
Hydrogen peroxide (H_2O_2)	Earnest Chemist Ltd, Tema, Ghana
Iodine solution	
KOH	
Kovac's reagent	Techno Pharmchem, Haryana, India
L (-) Sorbose	The British Drug Houses Ltd, Poole, England
L (+) Arabinose	BDH Chemicals Ltd, Poole, England
Lactose	BDH Chemicals Ltd, Poole, England
Lead acetate	The British Drug Houses Ltd, Poole, England
Mannitol	Hopkin and Williams Ltd, England
Methyl red	BDH Chemicals Ltd, Poole, England
NaCl	BDH Chemicals Ltd, Poole, England
P – anisyladehyde	Sigma Aldrich Deisenhofen, Germany
Pectin	The British Drug Houses Ltd, Poole, England
Raffinose	The British Drug Houses Ltd, Poole, England
Starch	BDH Laboratory Supplies, Poole, England
Sulfanilic acid	Merck Darmstadt, Germany
Zinc powder	May & Baker Ltd, Dagenham, England
α - Naphthylamine	Hopkin and Williams Ltd, England

Table 6.10: List of growth medium

Item	Manufacturer/company
Bismuth sulphite agar	Oxoid Ltd, Basingstoke, UK
Cetrimide agar	Oxoid Ltd, Basingstoke, UK
Glycerol asparagine agar	-
Humic acid vitamin agar	-
Koser's citrate medium	Oxoid Ltd, Basingstoke, UK
Lactose broth	Oxoid Ltd, Basingstoke, UK
MacConkey agar	Oxoid Ltd, Basingstoke, UK
Mannitol salt agar	Oxoid Ltd, Basingstoke, UK
MRVP broth	Oxoid Ltd, Basingstoke, UK
Nitrate broth	E. Merck, Darmstadt, Germany
Nutrient agar	Oxoid Ltd, Basingstoke, UK
Nutrient broth	Oxoid Ltd, Basingstoke, UK
Peptone water	Oxoid Ltd, Basingstoke, UK