Characterization of Antimicrobial Compounds Produced by Bacteria Isolated from Marine Soil.

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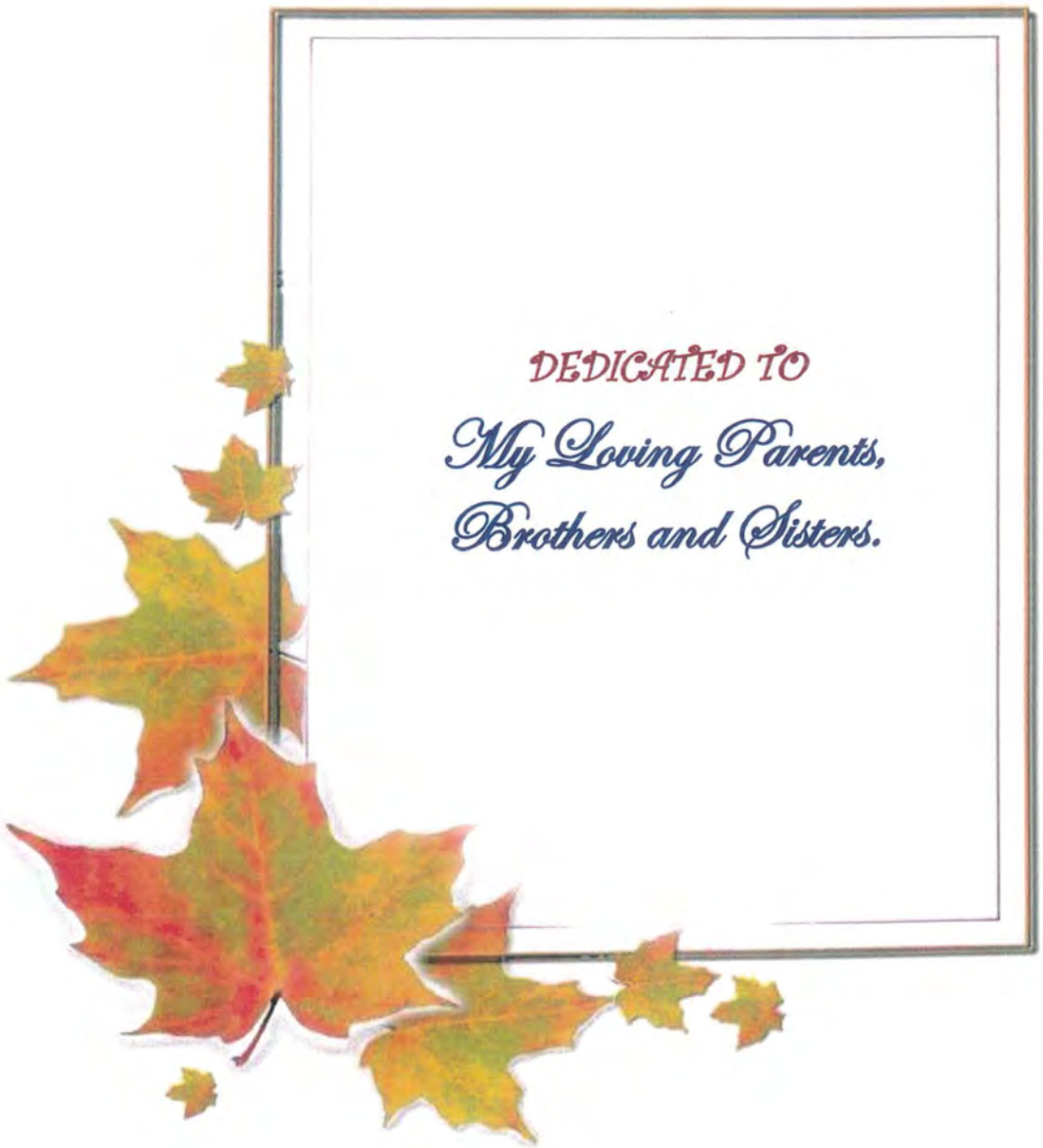
"IN THE NAME OF ALLAH THE MOST BENEFICENT, THE MOST MERCIFUL"

Read! And thy Lord is Most Honorable and Most Benevolent,
Who taught (to write) by pen, He taught man that which he knew not.

(Surah Al-Alaq 30: 3-5)

Al-Quran

DEDICATION



DECLARATION

The material and information contained in this thesis is my original work. I have not previously presented any part of this work elsewhere for any other degree.

FAIZ-UR-RAHMAN

Certificate

This thesis submitted by **Faiz-ur-Rahman** is accepted in its present form by the Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan; as satisfying the thesis requirements for the degree of Master of Philosophy in Microbiology.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
FFBP	First Fungal Bank of Pakistan
TSA	Tryptic Soy Agar
SCA	Starch Casein Agar
MHA	Muller Hington Agar
MDR	Multi Drug Resistant
hrs	Hours
KSS	Karachi Sea Shore
ISP	International Streptomyces Project
LB	Luria Bertani
rpm	Revolution per minute
min	Minutes
O.D	Optical Density
SCB	Starch Casein Broth
TSB	Tryptic Soy Broth
NB	Nutrient Broth
d.H_2O	Distilled Water
NaCl	Sodium Chloride
DMSO	Di methyl sulfoxide
CTAB	Cetyl trimethyl ammonium Bromide
TE	Tris-EDTA (Ethylene diamine Tetra acetic Acid)

SDS	Sodium Dodecyl Sulphate
TBE	Tris Boric acid EDTA
UV	Ultra Violet

LIST OF UNITS

µg	Microgram
µl	Microliter
ml	Milliliter
gm	Gram
mm	Millimeter
°C	Centigrade
%	Percentage
psi	Per Square Inch
L	Liter

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In the name of **ALLAH,** the most gracious, the most merciful. First and foremost praise and thanks to **ALLAH,** the lord, the almighty, the all-knowing and the glorious who bestowed upon me all the blessings and faculties of thinking, searching and learning.

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ABSTRACT

Antibiotics are the secondary metabolites of bacterial or fungus origin which inhibit the growth of other microorganisms. In the start it was been isolated from natural sources, due to decrease in the isolation from there it is now prepared synthetically. Several factors involve in the failure of antibiotic therapy. The failure is due to miss use of antibiotics that led to the evolution of multi-drug resistant pathogens like Methicillin resistant *Staphylococcus aureus*, which is important emerging pathogen. There is a large decline in the novel antibiotic production from several decades due to lower interest of pharmaceutical industries in the antibiotics. New strategies to compete with multi-drug resistant pathogens are in progress but there is still increase in demand for novel antibiotics. For this purpose new sites are being explored in the search for novel antibiotics. The objective of this study is to evaluate the antimicrobial potential of marine bacteria against MDR pathogens, optimize culture condition for production and produce antimicrobial from it. Initially the 172 marine isolate were screened from six soil samples for their antimicrobial production ability against ATCC bacteria and FFBP fungi. Then in secondary screening 84 isolates were screened for their activity against multi-drug resistant bacteria and fungi. One highly producer strain KSS 1.4 was selected from these 32 isolates which produce antimicrobial in secondary screening. The strain KSS 1.4 was biochemically characterized and different parameters were optimized for this isolate. The optimized temperature was 37^0C, starch was best carbon source, peptone as best nitrogen source, starch casein as best production medium, initial pH 5 as best pH, 50%:50% sea water and distilled water as best ratio, 2% NaCl presence in the media in case of distilled water were the optimized parameter for production of antimicrobial from isolate KSS 1.4. The optimized solvent for extraction was n-hexane. The results from this study reveal that the marine bacteria have a great potential to be use for antimicrobial production. The study from this environment will led to the discovery of new types of microorganisms and novel metabolites from them which will be of medical and industrial importance.

INTRODUCTION

S.A. Waksman for the first time introduced the term "antibiotic" in 1942. Antibiotics were natural drugs produced by several fungi or bacteria (Aminov, 2010). Antibiotics are the substances which is produced as a secondary metabolites by a microorganism e.g. fungus or bacterium or may be of other origin, which kills another microorganism or it may inhibit other microorganism's growth. Infections caused by pathogenic microorganisms are treated by these antibiotics which are not resistant to these antibiotics, such as bacteria, viruses and fungi (Rudi *et al.*, 2012).

The use of chemical compounds to treat the infections and its development divided into three periods. First detection of alkaloid extracts from china bark and ipecacuanha extracts from the roots of a Brazilian herb *Cephaelis Ipecacuanha* in 1600s in South America (Cui and Su, 2009, Yazdankhah *et al.*, 2013). The extracts proved to be effective against some infections, especially malaria (quinine) and amoebic dysentery (Emetine). This was followed by synthetically making of antimicrobial compounds which was started by Paul Ehrlich (1854 - 1915) in the Germany preparing salvarsan in 1909. It was crowned by Gerhard Domagk (1895-1964) preparing sulfonamides in the 1930s (Aminov, 2010). Finally The Penicillin development was a major breakthrough in the history and development of antibiotics which took place in the late 1920s performed by a researcher who never worked in a team and never under any program.

It was A. Fleming that discovered Penicillin from the mold. The antimicrobial action of mold was observed by researcher before him and also from ancient times (Aminov, 2010). Fleming had the problem with the purification of penicillin so why it was delayed until a team of Oxford headed by H. Florey, a microbiologist and E. Chain, a chemist developed the purification protocol for penicillin (Chain *et al.*, 2005). The development of an effective formulation occurred about a decade later and was due to research now going on in large cooperative team, often a collaboration between universities and resourceful manufacturing. In the latter half of 1940s the hunt for new types of antibiotics, majorly from the soil microbes began. New types of antibiotics were continuously discovered which can be termed as antibiotics golden age (Yazdankhah *et al.*, 2013).

Since time immemorial infections had the dominant place in human disease panorama. Even if one did not know the cause of these diseases, has always tried to fight them. The diversity of microorganisms is incalculable large (Aminov, 2010). The first major microbiologists, such as L. Pasteur (1822-1895) and R. Koch (1843-1910) had a clear notion of the microbes to survive, in a mutual competition. They reported that due competition among microbes they had able to form life-threatening weapons ("antibiosis") directed against competitors. They also reported that those who survived the competition in one way or another, had managed to neutralize the opponent's "weapons", i.e. developed "resistance." These mechanisms would they assumed, be strongest where the microbial diversity was greatest, i.e. in the soil (Yazdankhah *et al.*, 2013). The term "antibiotic" (to life) was coined by Selman Waksman (1888-1973) in 1942. He defined it as a substance produced by microorganisms that all in low concentrations can eradicate or stop other microorganisms to grow (Spring, 1975). In the next two decades, the concept was applied in accordance with the definition. Still used the term, but it now also includes the many "antibiotic" that industry has managed to produce semi-synthetic or synthetic (Walsh, 2003).

The suitable environment for microbial growth is soil, so maximum number of antibiotic producing microorganisms has been isolated from this environment from the start of antibiotic era (Cavalcanti *et al.*, 2006). An important genus of gram positive filamentous bacteria that is found in every environment is *"Streptomyces"* (Rudi *et al.*, 2012). About 80% of the antibiotics using today are produced from the genus *"Streptomyces"* of Actinomycetes family (Watve *et al.*, 2001). This has been used for the isolation of antibiotics very extensively from the terrestrial environments and most of the important antibiotics and other secondary metabolites of medical and industrial importance has been isolated from this genera (Rudi *et al.*, 2012).

It is very alarming situation that besides so much success in the antibiotics and advances in their synthesis and production, still infectious diseases are on the top second in causing death, throughout the world. The bacterial diseases kills about 17 million people every year targeting mainly immunocompromised people such as children and elder people (Watve *et al.*, 2001). As there is problem with the antibiotics that microorganisms develop resistance to these compounds due to

incorrect prescription or over dosage which may lead to super infection. This problem is more common to the broad range antibiotics that are those which is active against several types of microorganisms (Rudi *et al.*, 2012). The remarkable increase in antibiotics resistant bacterial species has increased the interest in search of new types of microorganisms from new sources containing different groups of bacteria, fungus and actinomycetes having the ability for production of potent new antibiotics (Hoda, 2010). Novel and more effective antibiotics development is essential, with the increase in resistant forms of infectious diseases and development of newer diseases and multi-drug resistant pathogens.

Antibiotics currently are acquired from terrestrial life source or synthetically made from intermediates. Ocean signifies almost untouched resource from which novel antibiotics can be discovered. Marine world will be provide with the next generation of antibiotics to the pharmaceutical industry. Marine antibiotics are those antibiotics which are obtained from marine organisms. Scientists have reported various antibiotics from marine bacteria (himalomycins, aplasmomycin, and pelagiomycins), and actinomycetes (marinomycins C and D). Marine environment can be a major source for the discovery of novel antibiotics and new generations of antibiotics (Dushi *et al.*, 2011). Oceans covers 70% of the earth crust and is the source of several new types of microorganisms which can be a source for different novel antibiotics and other secondary metabolites which can be industrially or medically important (Usha *et al.*, 2011).

From the several decades the research in the field of developing new antibiotics has been stopped and antibiotic resistance in the microorganisms has increased. This condition has alarm us to develop and acquire new types of antibiotics and other strategies against these infectious microbes and to overcome the infectious diseases (Yazdankhah *et al.*, 2013).

So this study was carried out to search novel microorganisms from the new sites like marine environment. The microorganisms from these environments will be screened for their ability to produce potent antimicrobials against multi-drug resistant pathogens.

AIM AND OBJECTIVES

The aim of the present study is production and characterization of antimicrobial compounds produced by bacteria isolated from marine soil samples.

The objectives of this study are:

- Screening of the marine isolates for antimicrobial production.
- Isolation of the potent antimicrobial producer strain against multi-drug resistant pathogens.
- Optimization of the culture conditions for antibiotic production.
- Production of antimicrobial from that potent producer strain with optimized culture conditions.
- Screening of antimicrobial compounds for their antimicrobial activity against multi-drug resistant pathogens.

LITERATURE REVIEW

Antibiotics:

Selman Waksman (1888-1973) coined the term "antibiotic" (to life) in 1942 (Spring, 1975). He defined antibiotics as a substance that is produced by microorganisms in low concentrations to inhibit the growth or kill other microorganisms. For two decades, this concept was functional in accordance with the above definition. But now "antibiotic" also includes semi-synthetic or synthetically made substances industrially (Yazdankhah *et al.*, 2013).

The diversity of microorganisms is incalculably large. Already the first chief microbiologists, Robert Koch (1843 - 1910) and Louis Pasteur (1822 - 1895), had a clear idea about the survival of microbes that is in a mutual competition, microbes had to be able to form life-threatening weapons ("antibiosis") directed against competitors, and that those who survived the competition in one way or another, had managed to neutralize the opponent's "weapons", by developing "resistance." They assumed these mechanisms would be strongest where the microbial diversity was utmost, i.e. in the soil (Yazdankhah *et al.*, 2013).

History:

According to Spring (1975) the discovery of compounds that were used to treat the infectious diseases is divided in three periods; Preparation of alkaloid extractor, the development of synthetic means and the discovery of antibiotics.

1. Alkaloids

The beginning of the chemotherapeutic era set to the 1600s when the wife of the Spanish governor of Peru, Part Chinchon, probably was cured of malaria. After she was instructed by the natives to chew her bark extract of Cinchona tree (kina bark - named, Albeit Misspelled, after the mistress) (Greenwood, 2008). Ipecacuanha (Local name of the plant) bush was imported to Europe from Brazil in 1658. In 1817, it succeeded in producing Emetine from the bush rot. Until the early 1900s was Emetine and china bark the most utilized therapeutics against amoebic dysentery and malaria, and they are still available in the medical armamentarium (Greenwood, 2008).

2. Synthetic compounds: The hunt for the "magic bullet"

Efforts for synthetic antimicrobials had its origins in the work of dyes and took a long time almost exclusively in Germany. Paul Ehrlich (1854 - 1915) began this era in which he based on the theory of "selective toxicity" tried to find the "magic bullet". The

term "the magic bullet" has its origin in German folklore (Williams, 2009; Buds, 2007), but Ehrlich defined it as a means to attack pathogenic microbes (or more generally pathogenic cells, including cancer cells) without having any effect on the healthy host cells. It led to the development of the arsenic-containing Salvarsan or Arsfenamin in 1907 (Williams, 2009).

Salvarsan intended treatment of African trypanosomiasis (sleeping sickness). A widespread perception at that time was in fact the only protozoa, not bacteria, could be cured with chemotherapy agents. Salvarsan rapidly proved to be active also against *Treponema pallidum*, the cause of syphilis. The agent soon replaced mercury in the treatment of syphilis, but the side effects proved soon to be too large (Greenwood, 2008).

As part of a research program to find dyes with antimicrobial effect, Fritz Mietzsch (1896 - 1858) and Josef Klarer (1898 - 1953) in 1932 synthesized Prontosil (sulfonamide krysoidin) (Yazdankhah *et al.*, 2013). Gerhard Domagk (1895 - 1964), director of research at IG Farben industries in Germany, proved that this drug in mouse experiments had antibacterial action against hemolytic *streptococcus* (Greenwood, 2008). The reason (believed by French scientists already in 1935) was that the effect was related to the colorless component (sulfanilamide), not to dye krysoidin (Greenwood, 2008). Later the manufacture of multiple sulfonamides, which were used against diseases such as acute pneumonia (often caused by *pneumococci*), urinary tract infections, severe throat infections (most commonly caused by *streptococcus*), gonorrhea, some forms of meningitis and - not least - the dreaded puerperium fever. The success of the sulfonamides were here so dramatically that the death rate from puerperal sepsis practically had reached our time low level already before penicillin was introduced (Greenwood, 2008).

Within 1945 pharmacologist had managed to synthesize several thousand different sulfonamides (Ryan, 1993). Although only a few got practical application, used the remains to treatment in both human and veterinary medicine. In 1939 for his work Domagk received further Nobel Prize in Physiology or Medicine, but had to say it aside by order of Hitler, who had enough of Nobel Prizes after Carl von Ossietzky (1889-1938) received the Peace Prize in 1935 (Ryan, 1993). After the war (in 1947) accepted Domagk it anyway. He had the honor and the medal, but not money (Ryan, 1993).

It was sulfonamides which finally changed the perception that not only protozoa, bacteria were available for "chemotherapy". Probably was sulfonamides therefore a crucial stimulus for efforts to develop antibiotics.

3. Discovery of Antibiotics

The concept of naturally occurring "antibiotic" was formulated by the first pioneers of microbiology at the end of the 1800s. Pyocyanase, isolated from *Bacillus pyocyaneus* (now known as *Pseudomonas aeruginosa*) was the first "antibiotic" as introduced. This too is a dye (a blue pigment, the origin of the "blue plastered" you can see for skin infections with *P. aeruginosa*) (Greenwood, 2008). The substance, discovered in 1888 by Rudolf Emmerich (1852-1914) and Oscar Loew (1844-1941), was proven to have bactericidal activity against microbes that cause diphtheria, typhoid, anthrax and cholera (Yazdankhah *et al.*, 2013). The hope that one here had finally found a "miracle bullet" flourished immediately up but disappeared just as quickly. The drug was too toxic and unstable (Greenwood, 2008).

America René Dubos (1901 - 1982) produced in 1939, by the way before penicillin was adopted, tyrothricin from a bacterium, *Bacillus brevis*, which he found in soil samples. The fabric consisted essentially of two antibiotics gramicidin and tyrocidin. Also these were so toxic that they could only be used topically in the form of ointments (Solberg, 1977). Dubos presented the way his findings at an international conference in New York in September 1939 (at the time of the outbreak of war) where, among others, Selman A. Waksman (1888-1973), Howard Florey (1898-1968) and Alexander Fleming (1881-1955) attended. While A.R. Dubos itself soon lost interest in antibiotics, got his discovery probably great importance to Florey and Waksman further efforts in this area (Greenwood, 2008).

Penicillin was - almost - "the magic bullet"

History will have it that penicillin was discovered by a pure case when a cultivation dish of *staphylococci* that the British scientist Fleming summer 1928 had forgotten incubating, had been contaminated by a mold. Around mold fungus arose a large aseptic zone - the fungus had obviously produced a substance that inhibited the growth of bacteria (Fleming, 1929). This should have been the beginning of the long road to penicillin production. But history should be modified somewhat. Admittedly, a number of coincidences at Flemings side. Perhaps the most important was that the dish was forgotten in a cool period with temperatures <20 ° C (which allowed the fungus to

grow while *staphylococci* were lying dormant), which was then replaced by warmer summer (which inhibited fungal growth but left *staphylococci* grow - with except where it met fungal toxins) (Greenwood, 2008). If the weather had not changed from cold to hot in this period, would penicillin hardly been discovered at the time of Fleming.

Fleming was incidentally not the first discovered mold antibacterial effect. Known is that Joseph Lister (1827 - 1912) ("antiseptic father") in 1871 investigated that mold contaminated urine, did not allow the bacteria to grow (Yazdankhah *et al.*, 2013). In 1874 William Roberts (1830-1899) have observed that cultures of *Penicillium glaucum* was rarely bacterially contaminated and in 1897 Ernest Duchesne (1874-1912) argued in his doctoral work that *Escherichia coli* was eliminated by the same fungus. He also showed that *P. glaucum* in animal studies to protect against typhoid fever and recommended further tests on the basis of these observations, but did not manage even to perform such before he died of tuberculosis (Yazdankhah *et al.*, 2013).

Rather it is probably not entirely coincidental that Fleming, unlike most who must have observed the phenomenon, drew the right conclusions when he rediscovered fungal effect. He had the knack of experience with infections during the First World War already for a decade been looking for funds with antibacterial effect. In 1922, he described so that lysozyme isolated from lacrimal fluid, had such an effect (Fleming, 1921). He was therefore mentally prepared not only on such observation, but also on the potential importance it could get.

Fleming published observation in 1929 and continued even a few years working with penicillin (Solberg, 1977). One of his former students, Cecil Paine (1905 - 1994), should have been the first proved penicillin medical effect when he successfully treated two patients with eye infections, respectively, a *pneumococcal* infection after an injury and gonorrheal infection in a newborn, with crude extract of mushrooms (Zaffiri *et al.*,2012). Without this treatment would the first most likely have lost the eye and the other was blind. Paine did not publish this discovery (and delayed thus probably penicillin research with several years) (Greenwood, 2008), but at a meeting in 1932 shall have mentioned it to Florey, Professor of Pathology at Sheffield University but that it should have caused significant interest (Friedman *et al.*, 1998). However six years later, in 1938, Florey, then at Oxford, together with chemist Ernst Chain (1906-1979), a Russian- German Jew that ran away from Nazi Germany and lived in England, finally started it "penicillin program" that would lead to what we first thought was the

final "miracle bullet". While Florey had knowledge of microbiology and clinical research, was Chains strength to isolate, purify and characterize substances such as penicillin (Greenwood, 2008).

In late 1930 Florey and employees infected eight mice with a lethal dose of *Streptococcus* spp. Four of the mice received injections of penicillin, while the control group were not treated. After ten days, the mice in the control group died, while all of the test group survived. The startling result was published in the prestigious journal *Lancet* in August 1940 (Chain *et al.*, 1940). Fleming, who at this point had given up efforts for penicillin, no Florey and his team today probably would have heard of the many "forgotten" that reached almost but not all the way.

The first documented use of penicillin in the treatment of a human took place in 1941 in England (Abraham *et al.*, 1941). In 1941, the big challenge for Florey was finding pharmaceutical firms that invest in "miracle medicine". The road went to the big pharmaceutical companies in the US, when the European were spending on war what they had available resources (Greenwood, 2008). This move contributed to research that changed character. From largely to have been dominated by private researcher, only rely on their own (intellectual and material) resources, it was now far more dependent on teamwork, often in major cooperation projects between Universities and industry, and to the audience as a demanding third on the sidelines. Penicillin quickly became a publicly accepted "miracle"(Levy, 2001). In the times of World War II, Penicillin had been adopted by the Allies as early as the beginning of the war (Bud, 2007). The horrors of war had created a huge demand for good news. The time was ripe for a "miracle" - and penicillin was cast. In 1945 Nobel Prize was awarded to Alexander Fleming, Howard Florey and Ernst Chain in Physiology or Medicine (Levy, 2001). The importance of penicillin was in the early postwar years raised to almost mythical dimensions. It should be able to help against virtually all diseases, including cancer. But miracle limitations soon became apparent. Not helped against colds and certainly not against cancer (Bud, 2007). And although it worked against a range of microbial, particularly against gram-positive bacteria, it seemed not to all, not usually against most gram-negative bacteria. It also turned out that some patients developed severe allergic reactions to penicillin (Greenwood, 2008). Large pharmaceutical companies began to invest large to find new types of antibiotics. The success of penicillin led to several

classes of antibiotics were discovered and marketed, in a period that can be termed as antibiotics golden age (Yazdankhah *et al.*, 2013).

The first antibiotic against tuberculosis

The microbiologists had, as mentioned, long been recognized that the soil contains microorganisms that produce substances with antimicrobial properties. In 1943 Waksman and his group examined, in collaboration with the pharmaceutical company Merck, 10000 microbial for eventual production of antibacterial substances. Of these, ten cultures considered particularly promising, and in 1944 could finally Waksman pupil Albert Schatz (1920-2005) after great effort (Ryan, 1993) isolating an aminoglycoside from the bacterium *Streptomyces griseus.* Therefore, it had the name streptomycin (Saga *et al.*, 2009). This is the first antibiotic that proved to be effective also against *Mycobacterium tuberculosis* (Ryan, 1993). Another substance known as para-amino-salisylsyre (PAS) was developed by a Danish –Swedish Jorgen Lehmann (1898-1989) and was effective against this bacteria (Yazdankhah *et al.*, 2013).

Isoniazid and rifampicin , the other anti-tuberculosis drugs entered the market, respectively in 1952 and 1963. Already in 1912, isoniazid synthesized by the German University in Prague (Yazdankhah *et al.*, 2013), without being aware of its great potential for the treatment of tuberculosis. In the end, 40 years later scientists found at Hoffmann-La Roche and ER Squibb & Sons in USA and Bayer West Germany simultaneously and independently that isoniazid also was effective against tubercle bacteria (Greenwood, 2008; Ryan, 1993). It soon became clear that combination therapy with streptomycin , para-amino-salisylsyre and isoniazid reduced the development of resistance to each individual preparations.

In 1957, more than ten years after Schatz discovered streptomycin from a *Streptomyces*. Later two Italian researchers, Piero Sensi (b. 1920) and Maria Theresa Timbales (1925-1969) in the pharmaceutical company Lepetit in Milan discovered a new bacterium, *Streptomyces mediteranei,* which produced a new class of antibiotics, rifamycin B. In 1963, rifampicin, a semisynthetic product of a modified form of rifamycin B, marketed by the pharmaceutical company Ciba (Ryan, 1993).

Chloramphenicol, the first broad spectrum antibiotic

The beginning of novel antibiotics search, which started in the second half of the 1940s, was largely grounded on soil microbes. Microbiologist Paul Burkholder

(1903-1972) at Yale University examined microorganisms isolated from soil from different parts of the world. In 1947 he received a microbe from Caracas in Venezuela that he could prove produced substances that inhibited the growing gram negative and gram positive bacteria (Greenwood, 2008). This microbe were sent to the firm Davies Park for further investigations. The current microbe was later named *Streptomyces venezuelae*, after its origin, while the substance itself was named klormycetin - later renamed chloramphenicol (Yazdankhah *et al.*, 2013). The first clinical trial was conducted in La Paz in Bolivia in connection with a typhus epidemic. Park Davies sent what they had of the new drug chloramphenicol. All 22 patients who received chloramphenicol survived while 40 of 50 patients those not given chloramphenicol died (Levy, 2001).

Chloramphenicol had the property that went into the cerebrospinal fluid in both the normal and inflamed meninges. Therefore, it was quickly taken into use for the treatment of meningitis, also in the neonatal period. In the 1950s and 60s it was common in many places, especially in the US, giving infants antibiotic prophylaxis for a few days after birth, and chloramphenicol quickly came into use also for this indication (Feder, 1986). Eventually, it appeared more and more cases of a new syndrome - "the gray baby syndrome." The children were cyanotic, and many died (Feder, 1986). The cause proved to be the use of chloramphenicol as newborns have a reduced ability to eliminate. The children were quite simply poisoned (Mulhall *et al.*, 1983).

During the 1950s there came a series of articles (Feder, 1986) on severe bone marrow depression following the use of chloramphenicol , and the agent got in many places a bad reputation, which led to greatly reduced use. Most bone marrow depressions proved to be reversible, and severe aplastic anemia using chloramphenicol receive providentially rare. It has now been long established that the appropriate dose of agent may be administered to patients in all age groups (Feder, 1986). The drug is cheap and has significant broad spectrum antibacterial activity. It is still used extensively in many developing countries, both against diarrhea, pneumonia and meningitis.

Tetracyclines

At the same time as Burkholder discovered chloramphenicol, Benjamin Duggar (1872 - 1956) at Lederle Laboratories in Pearl River, New York, examined the

bacterium *Streptomyces aureofaciens* , which spun off a yellowish substance with antimicrobial properties (Yazdankhah *et al.*, 2013). The agent was first named aureomycin, but was later renamed klortetrasyklin . It had broad activity against a variety of bacteria, including intracellular, was less toxic than chloramphenicol and had the advantage - like chloramphenicol - that it could be taken orally. Due to its action against gram negative, gram positive and intracellular bacterial pathogens and low production costs, use is still made, particularly in developing countries.

Cephalosporins, penicillin similar antibiotics with broad-spectrum activity

In 1945 the Italian hygienists Giuseppe Brotsu (1895-1975) discovered antibacterial activity of extracts produced by fungi that he had isolated from a sewage outlet on the coast of Sardinia. The fungus was later named *Cephalosporium acremonium* (Levy, 2001).

It took a long time before he managed to purify active substances from the fungus, which he sent to Florey and his colleagues in Oxford (Greenwood, 2008). It was proven more stable derivatives with a wide antibacterial spectrum - including derivatives cephalothine and kefaloridin. They were introduced to the market in 1964. Today, there are five different "generations" of cephalosporins on the market.

Other β-lactams

β-lactam antibiotics include many different derivatives including penicillins, cephalosporins, carbapenems and monobactams (Saga *et al*, 2009). Penicillins were initially particularly effective against aerobic gram-positive bacteria like *staphylococcus and streptococcus*. When *S. aureus* isolates were resistant to benzyl-penicillin because they produce penicillinase, methicillin were prepared in 1960, which is penicillinase resistant (Saga *et al.*, 2009). In addition there was also prepared new β-lactams which had a different or broader effect spectrum than the original penicillin. Ampicillin (1961) (Saga *et al.*, 2009) and amoxicillin (1971) (Saga *et al.*, 2009) have action against gram negative and gram positive bacteria, piperacillin (1976) (Saga *et al.*, 2009) acting against even the otherwise resistant *Pseudomonas aeruginosa*. Carbapenems (1980) (Saga *et al.*, 2009) are active against gram positive and gram negative aerobic bacteria. Monobactams (1980) (Saga *et al.*, 2009) are the class of β-lactams which are exclusively active against gram negative bacteria (Saga *et al.*, 2009).

Other antibiotics

Successes with sulfonamides, penicillins, cephalosporins, streptomycin , chloramphenicol and klortetrasyklin led to continued major focus of the pharmaceutical industry and led to the discoveries of neomycin (1949) (Chopra, 2001), erythromycin (1952), kanamycin (1957) (Chopra, 2001), vancomycin (1956) (Chopra, 2001), lincomycin (1962) (Chopra, 2001), fusidic acid (1962) (Chopra, 2001) and gentamicin (1963) (Chopra, 2001), all of soil microorganisms (Chopra, 2001). Bacitracin was discovered in 1943, named after a culture of *Bacillus* isolated from a seven year old patient (1943) (Yazdankhah *et al.*, 2013). The synthetic antimicrobial agent nalidixin was developed in 1962 (Mah, 2003) and trimethoprim in 1968 (Darrell, 1968). The preparation of most classes of antibiotics continued by synthesizing antibiotics with broader antibacterial spectra and higher activity (Greenwood, 2008).

Arrested development

The period referred to antibiotics golden age ended in the mid-1960s (Yazdankhah *et al.*, 2013), and after 1987 have not discovered any antibiotic with new mechanisms of action (Yazdankhah *et al.*, 2013). Almost all antibiotics used today belong to classes that were discovered before 1970 (Yazdankhah *et al.*, 2013). The pharmaceutical industry has in fact longer prioritized other drugs, not only because antibiotics characteristically given in short courses and thus provide less economic return than funds for long-term treatment, but also because microbes inevitable development of resistance makes it difficult to predict which antimicrobial mechanisms should be focused on (Yazdankhah *et al.*, 2013). Moreover, also the change of development methods to explain the current situation with the lack of production of new antibiotics. In the 1990s, went one namely of a gene based development strategy, which has not been successful (Yazdankhah *et al.*, 2013). Furthermore, although each new antibiotic has been met with a corresponding development of resistance in microbes (Unemo *et al.*, 2011). Finally led the development of ever new antibiotics in the first decades after World War II that the resistance problems were largely swept under the carpet. The combination of increasing antibiotic resistance and lack of development of new types of antibiotics creates a future scenario that will require a global effort to oppose (Yazdankhah *et al.*, 2013).

Soil: A rich source for antimicrobials

Soil is a main and rich source of antimicrobials and most of antibiotics are produced from soil dowelling microorganisms the leading in which is the genus *Streptomyces*. The great microbiologists Louis Pasteur and Robert Koch had clearly stated that the antibiotic producing microorganisms are mainly found in the environment where microorganism's diversity is high, i.e. in the soil (Yazdankhah *et al.*, 2013). Initially all the antibiotic producing microorganisms leading in which are bacterial isolates were isolated from soil. America René Dubos (1901 - 1982) produced in 1939, by the way before penicillin was adopted, tyrothricin from a bacterium, *Bacillus brevis*, which he found in soil samples (Solberg, 1977). The discoveries of neomycin (1949), erythromycin (1952), kanamycin (1957), vancomycin (1956), lincomycin (1962), fusidic acid (1962) and gentamicin (1963), all of soil microorganisms (Chopra, 2001).

This was the golden age of antibiotic. Still there are many reports from different regions of the world where antimicrobial producing microorganisms are isolated from the soil. The research has also being diverged from terrestrial soil to marine soil. Antibiotic producing streptomyces from Yemen's soil has been isolated (Ahmad, 2003). A hundred bacteria were isolated from six samples of Egypt, 20 of which having antagonistic effect on several fungus pathogens of plants and humans (Gebreel *et al.*, 2008). Soil samples studied in India from stressed environment, isolating bacterial isolates also showed antimicrobial activity (Singh *et al.*, 2009). Soil samples from Al-Madina Al-Monawwara, Saudi Arabia has been reported having antimicrobials producing bacteria and fungi active against pathogens of dermatological diseases (H.M Ahmed Sheikh, 2010). Marine soil sample from Royapuram sea shore, India (Valli *et al.*, 2012), soil sample from the Andra Pradesh sea coast, India (M.V. Arasu *et al.*, 2013), an actinomycetes T-4 isolated from the soil samples of Taif city, Saudi Arabia (H.M Atta, 2012), a *streptomyces* strain isolated from marine soil sample from coastal areas of Tamil Nadu, India (Usha *et al.*, 2013), actinomycetes from various soil samples i.e. agricultural, barren and forest, isolated from Punjab, India (Salam *et al.*, 2014) all have antimicrobial activity against different pathogenic microorganisms. Hence it can be concluded that soil is still a rich source for antimicrobials active against pathogenic bacteria and fungi of various diseases.

The most important genera for antibiotics: The "Streptomyces"

The history of *Streptomyces* derived antibiotics began with the finding of streptothricin in the year 1942, and within the following two years discovery of streptomycin occurred, scientists in that era got deepened to pursuit for antibiotics inside the genus. (Rudi *et al.*, 2012).

About 23,000 of reported bioactive (secondary) metabolites are made by these microorganisms and above 10,000 signifying 45% of entirely discovered bioactive microbial metabolites of all these are formed only by *actinomycetes*. About 7600 of these bioactive compounds are formed by Streptomyces species. Several of which are effective antibiotics, making streptomycetes the most important antibiotic-producing organisms and is being explored by the pharmaceutical industry (Valli *et al.*, 2012).

Mostly antibiotics production is species specific, and *Streptomyces* species produce antibiotics against different microorganisms competing for same space even microorganism belonging to same genus. Symbiotic relationship between plant and *Streptomyces* species have important role in antibiotics production. *Streptomyces* species protect the plant from other pathogenic microorganisms and plant produces the substances which permits the growth of *Streptomyces* (Rudi *et al.*, 2012). Several single molecule antibiotics can change the expression of many genes which have not any relation with stress responses. (Chater *et al.*, 2010).

Antibiotics mechanism of action:

The molecular mechanisms of the action of these antibiotics and their targets are well understood and well-known. Antibiotics are even classified on basis of their mechanism of action and the cellular function they target to inhibit the growth of pathogenic bacteria (Kohanski *et al.*, 2010). Antibiotics action is complex process starting with physical contact of molecules and the cellular target involving many changes in the targeted functions like biochemical, structural and molecular etc. Multiple targets of antibiotics for inhibition of bacterial growth are: 1) Cell wall synthesis, 2) DNA replication, 3) RNA synthesis, 4) Protein synthesis (Rudi *et al.*, 2012).

Importance of antibiotics:

Since antibiotics discovery in the 20th century, World's demand for antibiotics is progressively growing. As antibiotics have significantly decreased the risk of

infectious diseases. The practice of these "marvel drugs", along with the developments in sanitation, food, housing and the beginning of bulk immunization programs, is leading to an extreme drop in deaths from infections which were once prevalent and frequently incurable. From the years, these antibiotics have protected many lives and relieved the pain of millions. By controlling various severe infections or diseases in the latter 20th century, these antibiotics have increased the chance of survival (Rudi *et al.*, 2012).

Problem with antibiotics:

Antibiotics have also been involved in some problems like changing or altering the microbial flora of the body by abolishing one or other groups of harmless or advantageous organisms, may resulting in infection due to the to over-growth of resistant organisms. Most of these lateral effects are associated with broad spectrum antibiotics. Resistance may arise in these microorganisms against the antibiotics due to incorrect dosage or over-prescription leading to super-infection. Increase in resistance in pathogenic organism's leads to severe infections which are difficult to treat with additional complicated the condition, likewise in case of carbapenem-resistant *Klebsiella pneumoniae*, (Dienstmann *et al.*, 2010) (Tuon *et al.*, 2011) and other microorganisms (Rudi *et al.*, 2012). Resistance may cause extend illness and greater risk of death. As well as increase rates of resistance infected people socializing in the community may cause increase exposure risk of contracting a multidrug-resistant strain (Costelloe *et al.*, 2010).

Bacteria develop resistant to different generation antibiotics (first, second or third generation drugs) may require treatment to be changed which are a lot expensive and sometimes toxic. Examples of such case, those drugs which are required to treat multi-drug resistant *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Mycobacterium tuberculosis* cost 100 times more than that of first generation antibiotics used against non-resistant species. Utmost troublesome thing is that resistance to essentially all antibiotics has been greater than before (Rudi *et al.*, 2012). However the pharmaceutical industry has exaggerated their efforts to replace present drugs with new drugs, the existing trends is leading to propose that within the following ten years there will be no effective therapies for some infections. As antibiotics use is one of the critical factors in the development of resistance (Takesue *et al.*, 2010; Kiffer *et al.*, 2005). In contradiction, underuse due to shortage or no access

and inadequate treatment facilities also play an important role in development of resistance as overuse. Due to these reasons, priority is the proper use to prevent the development and spread of bacterial resistance. Main causes of incorrect practice of antibiotics are greatly related to patient. Example of which is countless patients preferring new and expensive drugs over older drugs. Intense and extended use of antibiotics in exceedingly susceptible patients and patients with severe infections, has caused development of resistant nosocomial infections, which are uncontrolled and expensive eradication of the pathogen (Rudi *et al.*, 2012).

In many developing countries, self-medication and antibiotics are bought in a single doses and practiced till the patient feeling better is another vital factor that adds to develop resistance. As well as physicians are forced or pressured to prescribe antibiotics to meet patient expectations or by manufacturer's influence. In addition of triggering unnecessary expenses, this kind of perception encourages the development of resistance to the new drugs, as well as to the older drugs of these class. Antibiotics becomes ineffective due to its use against viral infections. In some arears oral antibiotics are considered less effective than injected one. Hospitals are the main sources of resistant microbes throughout the world (Borg *et al.*, 2010; Hulscher *et al.*, 2010).

Genome and new antibiotics:

With the accessibility to the genomic sequences of large number of pathogens, hundreds of genes have been estimated as targets for new antibiotics. Essential genes are recognized as the one which are important for survival of bacterium and such genes can become a target particularly if even a small molecule can block its activity (Simmons *et al.*, 2010) but genetic analysis has revealed that a gene may translate a function which is vital in one bacteria but not in another (Payne *et al.*, 2007). About 167 genes have been considered as possible targets for new antibiotics because of its importance in bacterial growth (Freiberg *et al.*, 2005; Falconer *et al.*, 2009) For example, GlaxoSmithKline has managed several studies on an antibiotic such as GKS299423 which act on topoisomerase II, in order to avoid development of resistance by the bacteria (Jones *et al.*, 2010).

Resistance:

Conferring to Nikaido (2009) report about 100,000 tons of antibiotics are manufactured every year, which are used in food, health and agriculture. The use of antibiotics has obstructed the bacterial populations, bringing antibiotics resistance. The development of resistance may be associated to genetic changes such as acquirement of resistance genes through horizontal transfer or mutation, the transfer of genes occurs mostly in organisms of diverse taxonomy (Aminov *et al.*, 2009; Martinez *et al.*, 2009). Mutations which hamper the action of the antibiotic do this by altering at the site of drug action (Andersson *et al.*, 2010). Mostly these resistance genes are found to be on same cluster as of the antibiotic biosynthesis gene (Allen *et al.*, 2010).

Naturally, these antibiotics are produced in response to another competitor to stop their proliferation, while in defense to these antibiotics, the competitor acquire different strategies to overcome these antibiotics by chemically modifying e.g. hydrolysis, and through changing the permeability of membrane or by changing the antibiotics target site (Garza *et al.*, 2009). Several *streptomyces* studied from the urban soil, showed resistance to most of the antibiotics hence showing that these resistance genes are common in that environment (Nikaido, 2009). Plasmids contains various genes for resistance which can express there on these stable plasmids and through conjugation is passed to another compatible strain (Wright *et al.*, 2010). Physiological condition and antibiotic concentration affect the susceptibility of bacteria to a specific antibiotic. The mechanism called persister formation is studied in biofilms where bacterial small population still survive after treatment with higher concentration of antibiotic having no other known mechanisms for resistance although this does not results in advance level resistance (Sheng *et al.*, 2009). Biofilm forming microorganisms are resistant to antibiotics and cause chronic and repeated infections in humans (Hassan *et al.*, 2011). The resistivity in microorganisms is caused greatly by antibiotics uses and also through spreading of resistant strains amongst people due to migration of people to the faraway societies where antibiotics are not used regularly (Allen *et al.*, 2010).

In September 2001, the World Health Organization (WHO) took an initiative by launching a global strategy to battle the severe problems like antimicrobial resistance emergence and its spread. Known to be WHO Global Strategy for the Containment of Antimicrobial Resistance, (WHO, 2011) the strategy is to recognize that antimicrobial

resistance is a global problem and to be addressed in all nations. However it is not effective, no nation can close its boundary to resistant bacteria, hence proper control is vital in all nations. Considerable accountability lies with national governments, with proper strategy and particular care to involvements like introduction of policies and legislation prevailing the licensing, development, distribution and sale of antibiotics (Lerma *et al.*, 2010).

Requirement of novel antibiotics:

With the increase in the incidence of multi-drug resistant pathogenic bacteria an urgent demand in the pharmaceutical industry produced for new antibiotics screening by more sensible methodologies and tactics developing broad spectrum activity antibiotics, which may resist the inactivation by the microbial enzymes (Motta *et al.*, 2004).

Although, effective novel antibiotics discovery against resistant bacteria is difficult and ambitious field of research but it can be possible. Pharmaceutical industries are not so much interested in this field of research due to low profit and high criteria for developing the drug also has role in absence of attention.

Beside these problems there is still need for novel antibiotics to treat the current emerging resistant pathogens and to fulfill requirement of the pharmaceutical industry to face the challenge of new type of infections (Rudi *et al.*, 2012). There is large potential in the nature to isolate different types of antimicrobial agents from different sources i.e. from plants and from microbes. Microbial growth is considered to be most suitable in soil environments (Cavalcanti *et al.*, 2006), for those microorganisms which have been isolated from the soil. Among them *Streptomyces*, a filamentous bacteria have strong potential for producing different types of antimicrobial agents many of which is currently in practical use and many has been banned due to toxicity and some are not in use due to emerged resistance to it.

There is large area of microbial world that is undiscovered. In this undiscovered world there may be much such type of microorganisms which may have the potential for producing new types of antibiotics some with enhanced ability to target resistant pathogens and some with the potential to target new sites in currently resistant pathogens. While there is obstruction in search for new antibiotics, the pharmaceutical industries have few strategies to overcome the resistance in pathogens by making changes in the natural antibiotics forming semi synthetic antibiotics called second

generation, third and fourth generation antibiotics. These antibiotics are further effective and is not easily inactivated by enzymes responsible for resistance (Fernandes *et al.*, 2006; Fischbach *et al.*, 2009). Even with the use of novel approaches to develop new antibiotics such as combinatory chemistry tools have been pursued, still insufficient new antibiotics are manufactured by the pharmaceutical industry these days. Additional strategies to develop new antibiotics include organic synthesis or modification in the drug pharmacokinetics by using nanotechnology or searching of molecules with unexploited mechanisms of action (Arasu *et al.*, 2013).

Marine: A new source of novel antimicrobials

Oceans covers 70% of the earth and it is believed that life on the earth has origin from these oceans. Experts have an estimate that diversity is higher than that of tropical rain forests, in coral reefs and in deep marine floor. The microorganisms found in marine environment are different one from that of terrestrial environment as both environment are extremely different from one another, so the compounds produced by microorganisms of both environment might also be so much different from one another (Vimal *et al.*, 2009). The focus of research has been diverted from terrestrial environment to new sites for search of novel metabolites, as production frequency of novel metabolites from terrestrial environment has decreased greatly. Microorganisms from marine ecosystem has a great potential for production of active metabolites as needed for its adaptation in the sea environment Like terrestrial environment, here in marine environment also reported that the *streptomyces* genus of actinomycetales order is leading in secondary metabolites production (Thenmozhi *et al.*, 2011). Marine environment have about 10% of actinomycetes out of the total bacterial population found there. Marine secondary metabolites have higher potency than that of terrestrial environment and that is due to the fact that sea water have diluting influence. Very few reports are available on the diversity, distribution and occurrence of potential actinomycetes in the marine environment (Usha *et al.*, 2011).

MATERIALS AND METHODS

Marine soil samples collection

Total six soil samples were taken from six different locations on the Karachi sea shore in September, 2014. The sample area have a distance from each other of 100 meters, 3 were taken from dry soil while 3 from mud alongside the water from the Karachi sea coast in sterile polythene bags (Arasu *et al.*, 2013) and were kept at 4^0C (Valli *et al.*, 2012). The samples were brought to the MRL lab Quaid-i-Azam University (QAU) Islamabad for further study. All used chemical and glass ware used were sterile and media were autoclaved at 121^0C for 15 minutes at 15 psi pressure. The chemical and other materials that could not be autoclaved were sterilized in hot air oven and some were sterilized with 70% ethanol if not possible with hot air oven.

Pre-treatment of soil samples

The samples were air dried and kept in hot air oven at 50^0C for 1 hour to decrease the microbial load (Salam *et al.*, 2014) and get only those microorganisms which have the ability to survive in harsh environment.

Sample processing

Serial dilution of the sample were done up to 10^{-9} in the test tubes containing sterile normal saline (Nacl: 9 g/L). For this purpose 1 g from each soil sample were taken and dissolved in 9 ml sterile normal saline and mixed thoroughly by vertex for 15 minutes (Arasu *et al.*, 2013). This serves as stock culture for further processing and diluting. Then 1 ml from this stock culture were transferred to next test tube containing 9 ml of sterile normal saline and mixed thoroughly. This test tubes makes the dilution of 10^{-1} and then 1 ml from this test tube was transferred to next test tube containing 9 ml of sterile normal saline and making dilution of 10^{-2} and hence making dilution by the same way up to 10^{-9}. The separation was carried out on Tryptic Soy Agar (TSA) medium autoclaved at 121^0C for 15 minutes at 15 psi pressure, and before pouring supplemented with nystatin (50 mg/L) and cycloheximide (50 mg/L) for inhibiting the growth of fungi and yeasts (Zereini, 2014). Then 100 µl from 10^{-4} to 10^{-9} dilutions of each sample was taken and spread over the surface of TSA medium by the help of sterile glass spreader. The plates were labelled properly with sample name and its dilution and incubated at 30^0C for 15 days because some of *actinomycetes* are slow grower.

Isolation of the strains

From the 15 days plates, the plates were observed after every 24 hour (hr) for growth up to 15 days continuously and the colonies having different size, color, shape and appearance were picked up and streaked again and again on TSA plates to get the pure colonies. Then the pure colonies were preserved on TSA slants. The plates were also marked with the help of permanent marker on the sites where the colonies were picked up to decrease the chances of selecting the same colony again. The morphology of every isolate was recorded.

Primary screening of isolates for antimicrobial activity

Primary screening of all marine isolates were carried out on Muller Hington Agar plates by preparing lawn of 0.5 McFarland standard from several bacterial and fungus test strains.

Test organisms

Gram positive bacteria: *Staphylococcus aureus* ATCC 6538, *Bacillus spizizenii* ATCC 6633.

Gram negative bacteria: *Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 10145, *Salmonella typhimurium* ATCC 14028, and *Klebsiella pneumoniae* clinical isolates.

Fungi

Unicellular fungi: *Candida albicans* clinical isolate.

Filamentous fungi: *Aspergillus fumigatous* FFBP 66, *Aspergillus niger* FFBP 0198 and *Aspergillus flavus* food isolate.

Afterward the marine isolates were point inoculated in the center of the plates and properly labelled. The bacterial lawn were incubated at 37^0C for 3 days and fungal lawn at 30^0C for 7 days. The zones of inhibition against different bacterial and fungal strains were observed, the diameter of zone was measured and recorded in term of mm.

Secondary screening of isolates

All positive isolates were then screened for its activity against several clinical isolates of bacteria and fungi. The Multi-Drug Resistance (MDR) bacterial strains were *K.pneumoniae, E.coli, P.aeruginosa, S.aureus, E.cocci* and MDR fungal strains were *C.albican, A.flavus, A.fumigatous, and A.niger*. Lawn from these strains were made on MHA plates of 0.5 McFarland standard and then point inoculation were done in the center of the plate from the producer strain. The plates were properly labelled and fungal lawn were incubated at 30^0C and bacterial at 37^0C for 7 days and 3 days respectively. Measured the diameter of zones and noted.

Preservation of isolates

All producer strains against clinical and MDR pathogens were preserved in TSA and Nutrient Agar slants for short time preservation. For long time preservation it was preserved in Luria-Bertani (LB) broth with 17% glycerol. For this 773 µl of Nutrient broth was added into cryo-vials. Culture was added into each vial except for control vial and was labelled properly on top and from sides by codes and the codes were written in note book along with their full details. The cap were closed loosely for aeration and kept in shaker incubator at 35^0C for 16 hours (hrs) to increase the microbial growth. After 16 hrs of incubation 227 µl of 75% glycerol (autoclaved at 121^0C for 15 minutes at 15 psi) was added making total volume of 1ml. The caps were tightly closed and parafilm were wrapped around head then vortexed for 5 minutes, kept in a box and stored at -20^0C. Each sample was preserved in triplicate. After a week the viability test was performed by taking a loopful from a cryo-vial of 3 different isolates and streaking it on LB agar plates. The plates were incubated at 37^0C for 24 hrs and colony morphology were noted.

Morphological characteristics of the isolates

General characteristics of all producer isolates against clinical and MDR pathogens was studied. Colony morphology was studied after refreshing the isolates on Nutrient Agar plates for 24 hrs and then morphological characteristics was noted based on color, size, appearance, consistency, margins, elevation and transparency. Gram staining, endospore staining and microscopy were performed for all producer isolates.

General characteristics of the highly producer isolate KSS 1.4

Out of all producer isolates one isolate named KSS 1.4 was selected for further study. This isolate was highly active against multiple clinical MDR pathogens. The colony morphology was studied by growing on Nutrient agar medium. Gram staining, endospore staining and microscopy was performed for this isolate.

Growth ability on different solid media i.e. Nutrient agar, Luria-Bertani (LB) agar, Tryptic Soy agar, Starch Casein agar was studied. The isolate was inoculated on these solid media and incubated at 37^0C for 24-72 hours.

The isolate was grown on ISP-1 (Tryptone-Yeast extract agar) and ISP-7 (Tyrosine agar) media to check the melanin production ability of the isolate. The selected isolates were observed for melanin production.

The temperature range for growth was find out by inoculating the culture on LB agar media and incubating at different temperature i.e. 4^0C, 15^0C, 25^0C, 30^0C, 37^0C, 45^0C, 50^0C, 60^0C and 70^0C for 24 to 72 hours .

Growth on solid media in presence of different NaCl concentration i.e. 0%, 2%, 4%, 6%, 8%, 10% and 12% was studied to check the NaCl tolerance level of the isolate.

Several biochemical and physiological characteristics i.e. catalase, urease, gelatinase etc. enzyme production and starch hydrolysis of the selected isolate was studied.

Optimization of the culture conditions for antimicrobial production

Several parameter were optimized to find out the optimum conditions for growth and antimicrobial production for selected isolate KSS 1.4. Parameters optimized were temperature, carbon source, nitrogen source, production medium, pH, sea water, optimal NaCl concentration, and best extraction solvent. Once a parameter was optimized, keeping it constant next optimization process was carried out. Each experiment was carried out in triplicate.

Temperature optimization

Temperature optimization is the basic step in optimization processes for antimicrobial production. For this purpose the selected isolate was grown in Nutrient broth at 37^{0}C for overnight in a shaker incubator. Then 2% of the refreshed culture was added to different flasks containing 50 ml of Nutrient broth prepared in distilled water and kept at various temperature i.e. 25^{0}C, 28^{0}C, 30^{0}C, 37^{0}C in shaker incubator at 155 rpm. Then each 24 hrs the samples was taken, up to 5 days, in sterile Eppendorf tubes from each flask and centrifuged for 20 minutes at 12,000 rpm at 4^{0}C. The supernatant was stored in another sterile Eppendorf tubes at 4^{0}C in the refrigerator for further study. The lawn from different bacterial and fungal pathogens were prepared and left for an hour, wells were made with sterile borer then 100 µl of supernatant was added in the wells from each product of different temperatures. The plates were left in the Laminar Flow Hood for 2 hrs until the supernatant dissolved in the media and then incubated, fungal lawn at 30^{0}C and bacterial lawn at 37^{0}C for 16-18 hrs according to Kirby-Bauer technique. Diameter for zone of inhibition was measured in mm with scale, for each day product to find out optimum temperature and optimum incubation time for antimicrobial activity.

Another 1 ml of the cultured broth was taken in the sterile test tubes from each flask of different temperature after every 24 hrs up to 5 days and O.D was taken to find out growth and the optimum temperature for the growth selected strain.

Best carbon source optimization

The carbon source plays very important role in the fermentation process. This decides the cost and production capacity of the product. Using cheap substrate as a carbon source gives us valuable products with less cost. For this purpose the carbon source was optimized after temperature optimization. Then 9 different carbon sources were used keeping peptone as a common nitrogen source for all carbon sources. The different mono saccharides, di saccharides, and complex sugars were used as carbon sources. They were sucrose, starch, glucose, glycerol, sorbitol, maltose, fructose, galactose and xylose. 2% of the carbon source and 1% of the nitrogen source were used in the broth, autoclaved and checked for sterility. The selected isolate was grown in Nutrient broth (prepared in distilled water), at 37^{0}C for overnight in a shaker incubator. Then 2% of the refreshed culture was added to different flasks containing 50 ml of broth (prepared in distilled water), having different carbon sources and kept at optimized

temperature (37^0C) in shaker incubator at 155 rpm. The remaining steps were repeated as in temperature optimization process for 6 days to find out best carbon source and optimum incubation time for antimicrobial activity. Another 1 ml of the cultured broth was taken in the sterile test tubes from each flask of different carbon sources after every 24 hrs up to 6 days and O.D was taken to find out growth and the optimum carbon source for selected strain.

Optimization for best nitrogen source

Nitrogen source is another important factor in the fermentation process of antimicrobial production after carbon source. Keeping the best carbon source common 6 different nitrogen sources were used. These nitrogen sources were tryptone, peptone, casein, L-arginine, beef extract and KNO_3. For antimicrobial activity above same procedure was repeated as used in carbon source optimization process for 4 days.

Screening for best production medium

The generally used media in microbiology laboratory were also used to screen out which is best for strain growth and antimicrobial production. There were 4 different generally used medium were selected i.e. Nutrient broth, Luria-Bertani broth, Tryptic Soy broth and Starch Casein broth. According to the manufacturer instruction medium were dissolved in the $d.H_2O$, autoclaved and checked for sterility by incubating overnight. Same procedure was used as in carbon source optimization for 5 days.

Initial pH optimization

The pH of medium have very important role in the production of antimicrobials. It also provide a stress condition for the microorganisms which then produce its secondary metabolites in response to the stress condition. The best carbon and nitrogen source was used to prepare broth for pH optimization experiment. Initial pH of the medium was adjusted at 10 different pH i.e. 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12. The media was autoclaved and kept overnight to check sterility of the medium. The same process were repeated used in other optimization processes for 5 days.

Optimization of sea water

Antimicrobial production from marine isolates are sea water dependent (Valli *et al.*, 2012). To find out best concentration of the sea water different percentages of

sea water was used with d.H_2O using optimized conditions in different flasks. The concentration of the sea water were 0% (containing total amount of d. H_2O and no sea water), 25%, 50%, 75% and 100% (having total amount of sea water with no d.H_2O.

Effect of NaCl on antimicrobial production

NaCl concentration provide osmotic pressure to the microorganism and also provide stress condition to them so secondary metabolites are produced as a result of stress condition. While in higher amount NaCl blocks the production of antimicrobials and also increase in microbial growth can be ceased in much higher amount. Different concentration of NaCl i.e. 0%, 2%, 4%, 6%, 8% and 10% were used with d.H_2O with previously optimized conditions. The medium were autoclaved with standard protocol and incubated for overnight to check sterility.

Optimization with best extraction solvent

The previously optimized parameters were used for the production of antimicrobial. The antimicrobial produced are to be extracted from the cultured broth with best extraction solvent. Different organic and inorganic, polar and non-polar solvents were used for extraction purpose depending on the nature of secondary metabolite produced. These 4 types of solvents used were ethyl acetate, n-hexane, petroleum ether and chloroform. These solvents are constantly and vigorously used for the extraction of antimicrobial compounds from cultured broth. The isolate KSS 1.4 was refreshed overnight in Nutrient broth at 37^0C at 155 rpm. Then 2% of the refreshed culture was transferred to 100 ml of previously optimized production medium. So starch was used as carbon source, peptone as nitrogen source, pH 5 as initial pH with 50% sea water and kept at 37^0C temperature for 2 days of incubation time in shaker incubator at 155 rpm. Then the medium were harvested and centrifuged for 20 minutes at 12,000 rpm at 4^0C. The supernatant was stored in sterile screw capped bottles at 4^0C in the refrigerator for further study. Then 20 ml of the supernatant was mixed thoroughly with equal amount of the solvent in extraction funnel and left to stand for 4 hours to form two layers which can easily be separated and help in extraction. Then taking aqueous phase and organic phase separately in different flasks. The extraction with organic solvents was carried out twice and organic phase of both was mixed. It was let open in air until the solvent evaporates leaving some mass in the bottom which was harvested. Then it was dissolved in 2 ml of DMSO. Different MDR bacterial and fungal pathogens

lawn were prepared and left for an hour, then well were made with sterile borer. 100 µl of the product extracted with different solvents and dissolved in DMSO was added to the well. The antibiotic discs (Methicillin for MRSA, Vancomycin for VRE, and Cefotaxime for Salmonella) were used as positive control and 100 µl simple production broth without culture was used as negative control. Then 100 µl of production broth (that was not used for extraction) and 100 µl of extracted broth (the broth left after extraction with solvents) were used to find to find the difference in their zone of inhibition. Then about 100 µl of DMSO was also used to subtract any possible antimicrobial activity of this from diameter for zone of inhibition leaving only diameter for zone of inhibition of the extraction solvent. The plates were left in Laminar Flow Hood (L.F.H) until the product dissolves then incubated at desired temperature for 16-18 hrs according to Kirby-Bauer method. The diameter for zone of inhibition was measured in mm and selecting the best extraction solvent for further studies.

Molecular identification:

Genomic DNA extraction

The selected isolate of KSS 1.4 was refreshed on Nutrient agar plate for 24 hrs. A loopful of the refreshed culture was added to an Eppendorf tube containing 570 µl of T.E buffer. Afterward 30 µl of 10X SDS and 4 µl of 20 mg/ml proteinase K were added and mixed it thoroughly. Now this was incubated at 37^{0}C for 1 hour and the solution becomes clear. After that 100µl of 10X Nacl was added to that solution, mixed thoroughly and incubated at 65^{0}C for 2 minutes. Then 80µl of CTAB was added to the solution, mixed and incubated at 65^{0}C for 10 minutes. Chloroform extraction was carried out by adding 800 µl chilled chloroform/isoamyl alcohol (24:1) to the solution, centrifuged for 5 minutes at 10,000 rpm and aqueous phase transferred to another Eppendorf tube. After that 800 µl chilled phenol/chloroform/isoamyl alcohol (25:24:1) was added and centrifuged for 10 minutes at 10,000 rpm and aqueous phase transferred to another tube. Then chloroform extraction was repeated by adding chilled chloroform/isoamyl alcohol (24:1) to that aqueous phase and centrifuged for 10 minutes at 10,000 rpm and aqueous phase was transferred to next tube. Then DNA wool was obtained by adding 700 µl chilled isoamyl propanol to that aqueous phase and centrifuged for 5 minutes at 10,000 rpm to get DNA in the form of pellet. Afterward isoamyl propanol was removed by pouring and pellet was washed with 500 µl chilled

ethanol (70%), centrifuged at 10,000 rpm for 5 minutes. Ethanol was removed and pellet was dried and then re-suspended in 60 µl T.E buffer and stored at 4^0C.

Gel Electrophoresis

Gel electrophoresis was done for the sample DNA to confirm the extraction of genomic DNA. For this purpose 1% agarose gel was prepared in T.B.E buffer by adding 0.8 g of agarose in 80 ml of T.B.E buffer (1X), heated in hot air oven at 100^0C until the solution became clear, then allowed to cool. 8 µl of ethidium bromide solution (10 mg/ml) was added to it, mix thoroughly and poured it in to gel tray having combs. After solidifying combs were removed from the gel and load into gel tank filled with T.B.E buffer. 5 µl of the T.E buffer having DNA sample was mixed with a small drop of loading dye and added to the well in gel carefully. Terminals of the gel tank were connected to power supply, giving 110 volts of voltage at 400 ampere for 30 minutes. Then terminals were disconnected from power supply, gel was taken and observed under UV trans-illuminator for observation of DNA band.

Amplification, 16S rRNA sequencing and phylogenetic analysis

The amplification of 16S rRNA gene was carried out using PCR with Taq DNA polymerase and using the primers, 27F primer (5′ AGAGTTTGATCGTGGCTCAG 3′) and 1492R primer (3′GGTTACCTTGTTACGACTT 5′). The conditions for thermal cycling were as follows: denaturation of the target DNA at 94 ^{0}C for 5 min followed by 30 cycles at 94 ^{0}C for 45 sec, primer annealing at 55°C for 30 sec and primer elongation at 72 ^{0}C for 60 sec followed by final extension for 10 min at 72 °C (15). PCR amplification was detected by agarose gel electrophoresis and was visualized by ultraviolet (UV) fluorescence after ethidium bromide staining. The PCR product obtained was commercially sequenced by Macrogen Inc. South Korea. The obtained sequence was compared for similarity with other micro-organisms 16S rRNA gene sequence in the nucleotide database, using NCBI BLAST at http://www.ncbinlm-nih.gov/.

The sequences showing highest similarity to the blasted sequence were download from NCBI. The Evolutionary analyses were conducted in MEGA6 software and the tree were obtained by Neighbor-Join method also in this software (Tamura *et al.*, 2013).

RESULTS

Screening of isolates for antimicrobial activity

A total of 172 isolates were isolated from these six (6) marine soil samples of Karachi sea shore. The number of isolates were 29, 38, 22, 31, 20, and 32 from sample 1, 2, 3, 4, 5, and 6 respectively. Out of these 172 isolates 84 have antimicrobial activity against several gram positive, or gram negative or both of them and some were also active against fungi in primary screening. A total of 32 isolates out of these 84 were found having antimicrobial activity also against multi-drug resistant pathogens in secondary screening against clinical isolates (Figure 4.1).

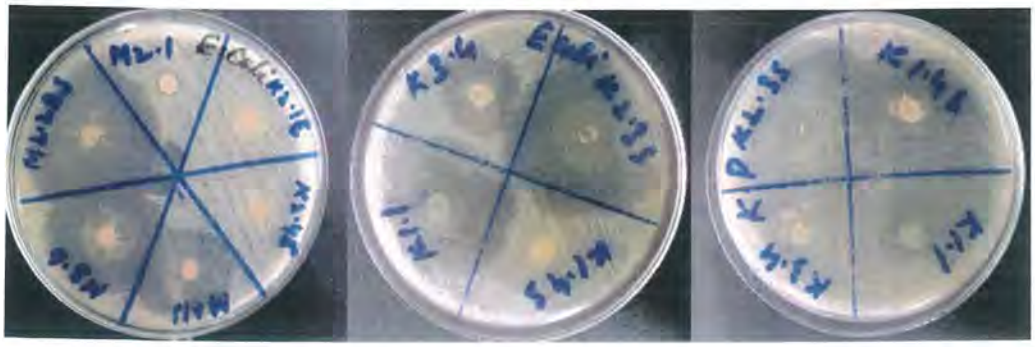

Figure 4.1. Diameter of Inhibition Zone (DIZ) of point inoculation of some isolates against clinical isolates in the secondary screening.

The total number of isolates from each sample and number of isolates having activity in primary screening against ATCC strains and in secondary screening against MDR pathogens are given in table 4.1.

Table 4.1. Number of bacterial isolates from different samples of Karachi sea shore.

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Total CFU	87	73	97	45	65	43
No of isolates	29	38	22	31	20	32
No of isolates with activity against ATCC	12	18	16	11	12	15
No of isolates with activity against MDR bacteria and fungi.	02	3	10	3	5	9

Morphological characteristics of the isolates

Gram staining, endo spore staining, microscopy and colony morphology results of all the 32 isolates having activity against MDR pathogens are given in appendix 1.

Preservation of isolates

The preservation process was successful as the preserved strains showed growth after growing on LB agar for 24 hrs.

General and morphological characteristics of the highly producer isolate KSS 1.4

The selected isolate KSS 1.4 was Gram positive, large rod shaped, some shorts and long Chains (Fig. 4.2) were also observed having terminal and Sub-terminal endo-Spore under 1000x magnification of microscope. The visible colony appearance of this isolate studied by growing on nutrient agar was creamy color, small in size, round, flat, Shiny appearance, having Irregular margins, and translucent (Fig. 4.3).

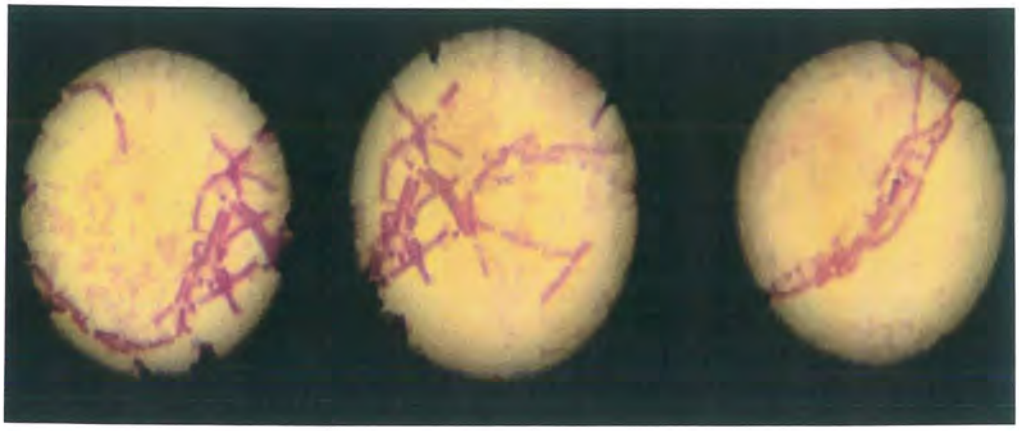

Figure 4.2. Gram staining and Microscopy.

The isolate growth was observed on general media i.e. Nutrient agar, Luria-Bertani (LB), Tryptic soy agar, and starch casein agar on 37^0C after 24 hrs.

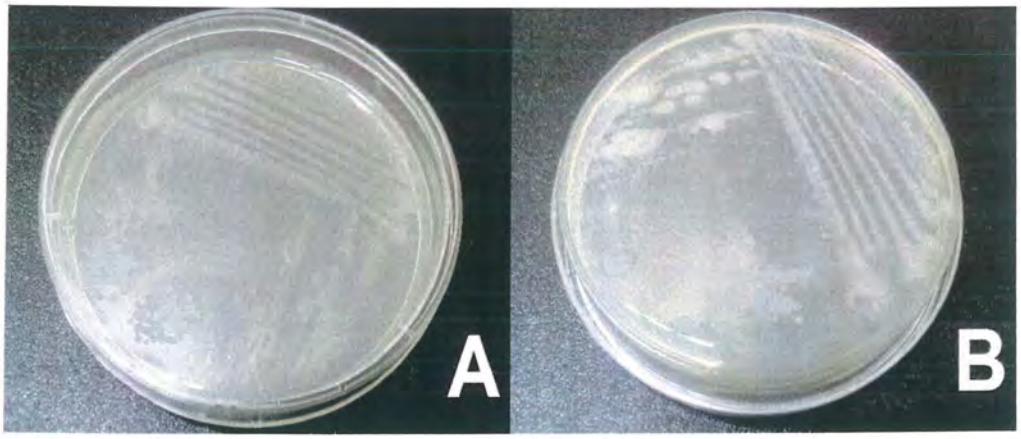

Figure 4.3. Colony visible growth and appearance on nutrient agar medium. (A) Top view. (B) Bottom view.

The isolate showed growth on ISP-1 (Tryptone-Yeast extract agar) and ISP-7 (Tyrosine agar) media but haven't the ability to produce any type of melanin (colored pigment) compound (Fig. 4.4).

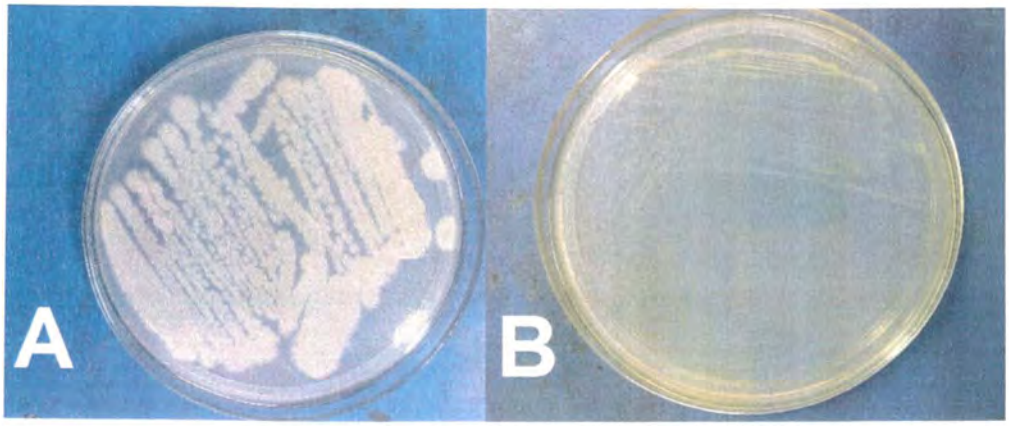

Figure 4.4. Growth of KSS 1.4 on Melanin production media. (A) Growth on ISP-1 solid medium. (B) Growth on ISP-7 solid medium.

The isolate KSS 1.4 showed significant growth up to 8% NaCl presence in the solid media of nutrient agar (Fig. 4.5) and growth level at different concentration are given in Table 4.3.

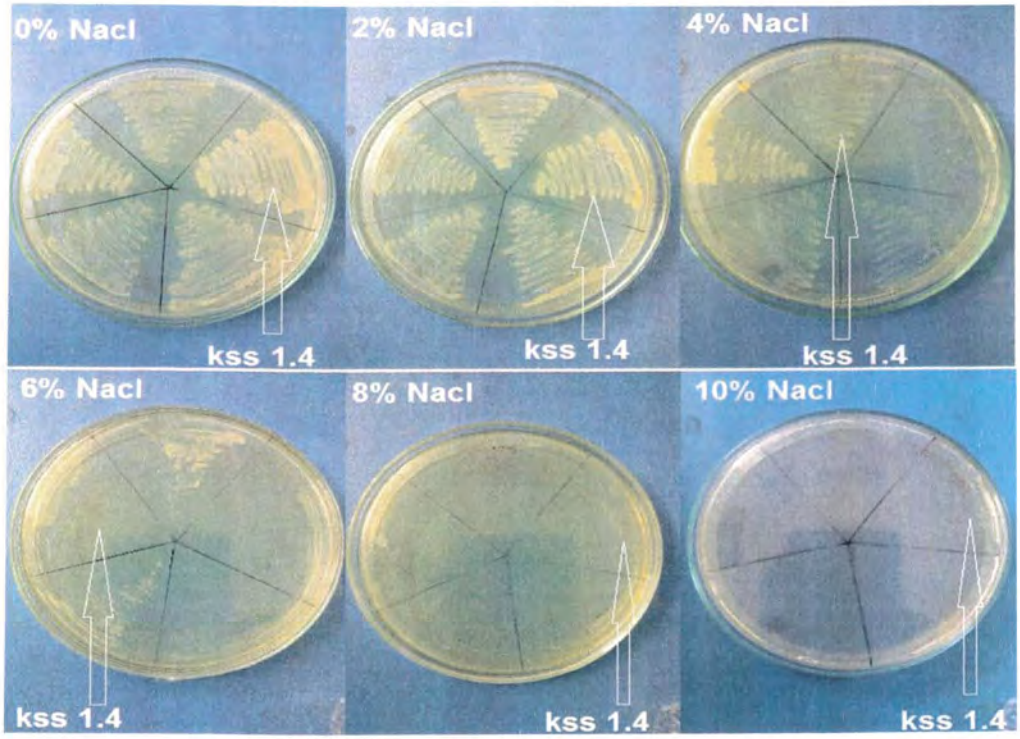

Figure 4.5. NaCl tolerance of KSS 1.4 on nutrient agar media.

Table 4.2. Growth of KSS 1.4 at different concentration of NaCl. (+ low growth, ++ medium growth, +++ high growth, - no growth).

NaCl concentration	Growth
0%	+++
2%	+++
4%	+++
6%	++
8%	+
10%	-

The isolate showed significant growth on different temperature range from 4^0C to 60^0C but was unable to grow on 70^0C. The growth level at each temperature is given below in table 4.4.

Table 4.3. Growth level of KSS 1.4 at different temperatures. (+ low growth, ++ medium growth, +++ high growth, - no growth).

Temperature	Growth
4^0C	++
15^0C	++
25^0C	+++
30^0C	+++
37^0C	+++
45^0C	+++
50^0C	+++
60^0C	++
70^0C	-

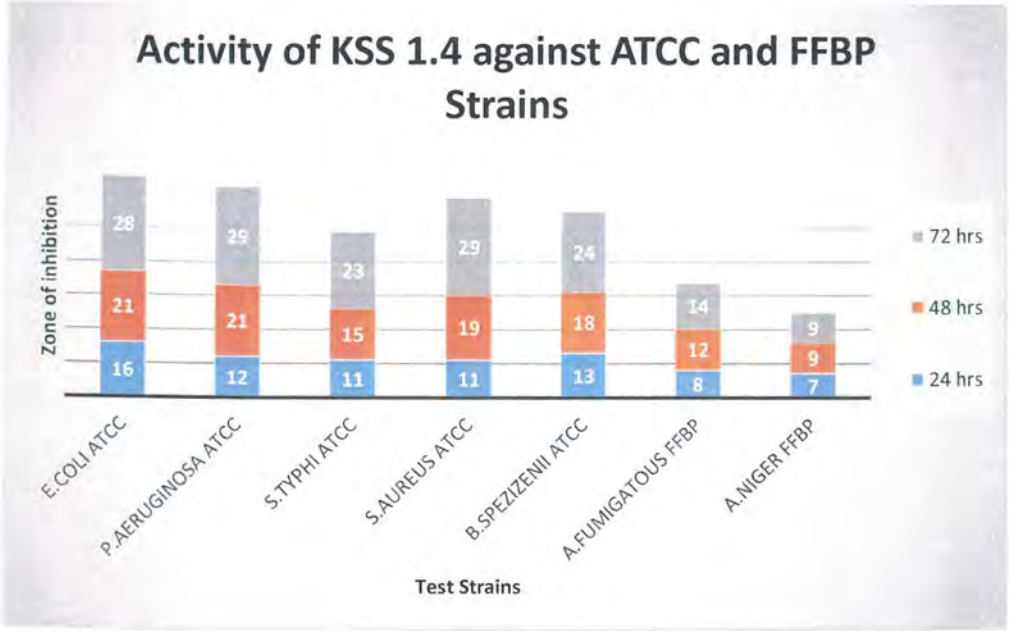

Figure 4.6. Zone of inhibition in mm of isolate KSS 1.4 against different bacterial ATCC and Fungal FFBP strains. The number shows DIZ (Diameter of Inhibition Zone) in mm (Milli Meter) and each color shows different time of incubation in hours.

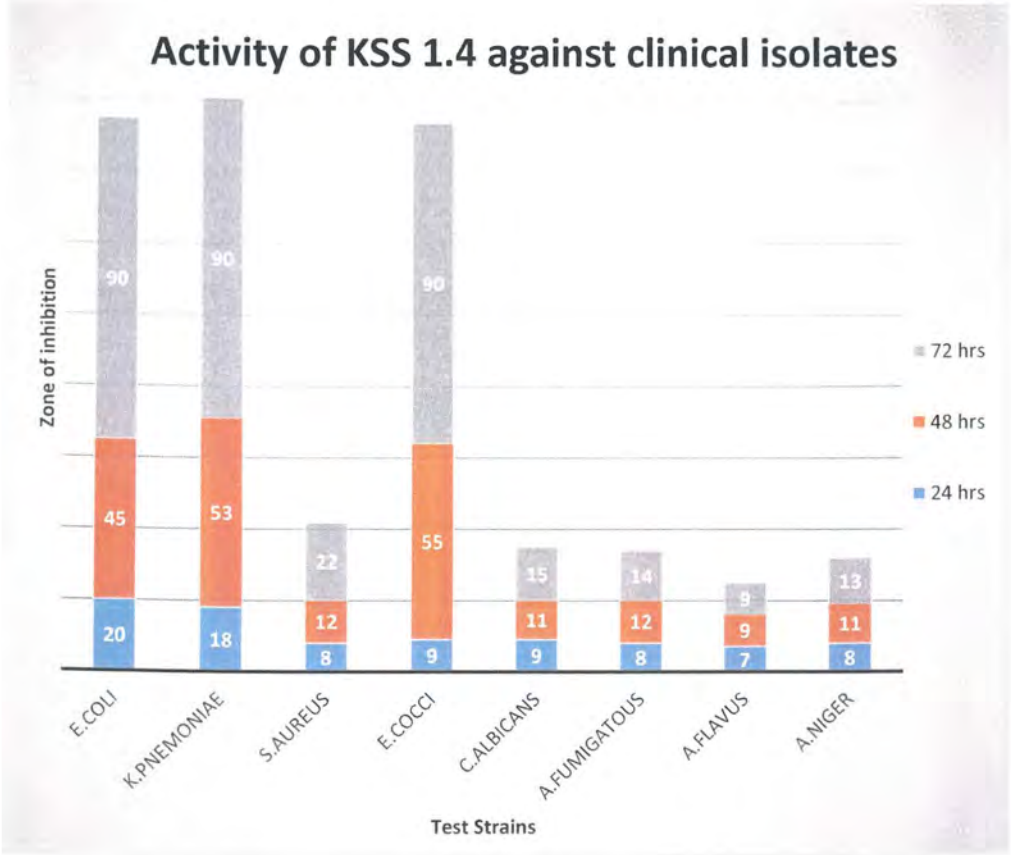

Figure 4.7. Zone of inhibition in mm against different bacterial and fungal clinical strains. The number shows DIZ (Diameter of Inhibition Zone) in mm (Milli Meter) and each color shows different time of incubation in hours.

Biochemical properties of the selected isolate

The isolate KSS 1.4 was catalase positive, amylase positive, urease negative, and gelatinase positive (Fig. 4.8, 4.9).

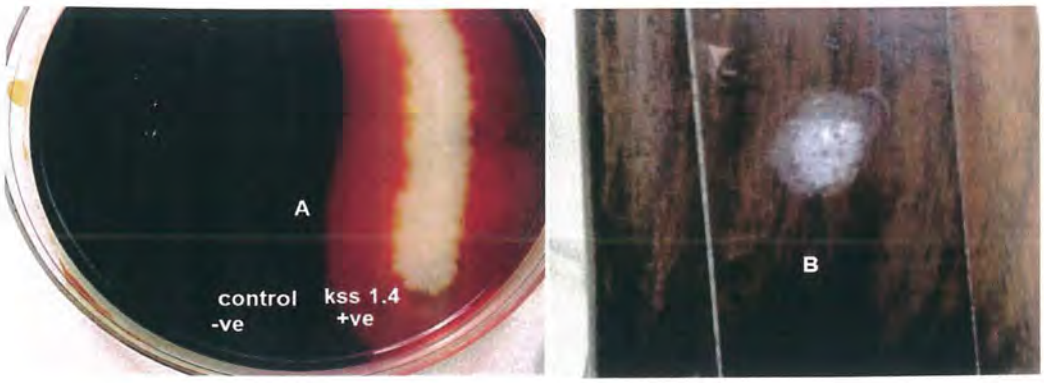

Figure 4.8. (A) Starch hydrolysis test (B) Catalase test. Control: *Escherichia coli*

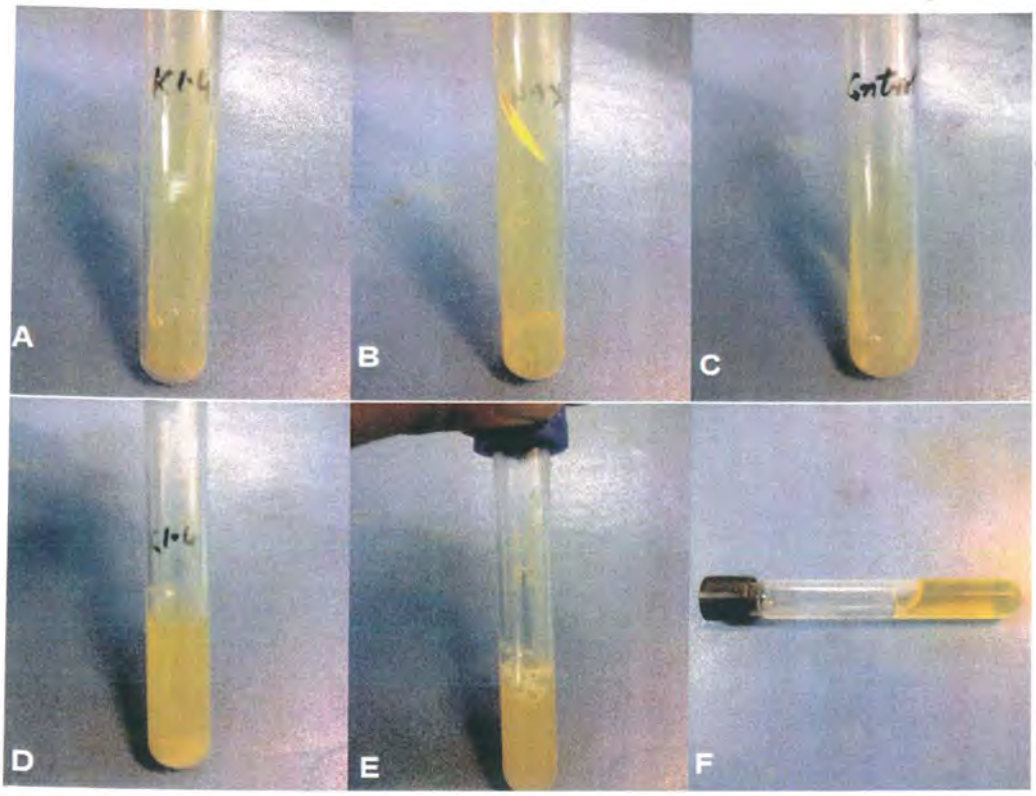

Figure 4.9. Above, Urease production test of KSS 1.4 (A) Front (B) Back (C) Control. Below, Gelatinase production ability test of KSS 1.4 (A, B) KSS 1.4 (C) Control.

Optimization of the culture conditions for antimicrobial production

Different optimization of culture conditions results are given below. These include temperature optimization, best carbon source, best nitrogen source, best media screening, initial pH optimization, optimization with sea water, optimization with NaCl, and optimization with solvents for extraction.

Temperature optimization

In the temperature optimization process the best temperature for the growth of isolate KSS 1.4 was 25^{0}C on day 3rd while for antimicrobial activity, the best temperature was 37^{0}C on day 3rd (Fig. 4.10, 4.11, 4.12, 4.13). So 37^{0}C was used as optimal temperature for optimization of other parameters.

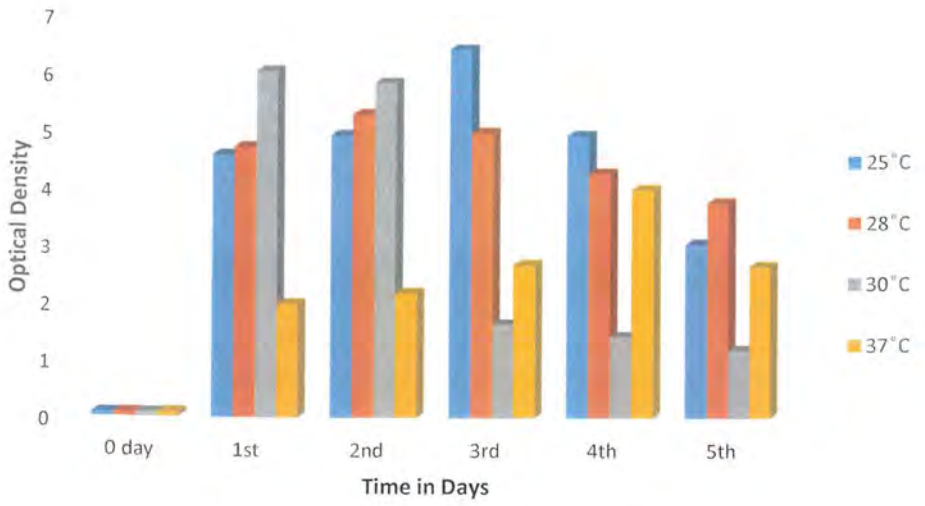

Figure 4.10. Temperature optimization for growth of isolate KSS 1.4.

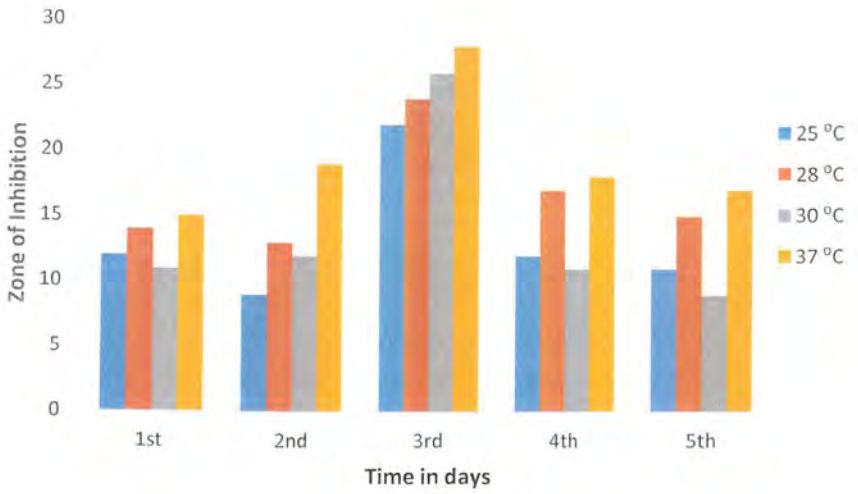

Figure 4.11. Antimicrobial activity of KSS 1.4 on different temperature against *P. aeruginosa*.

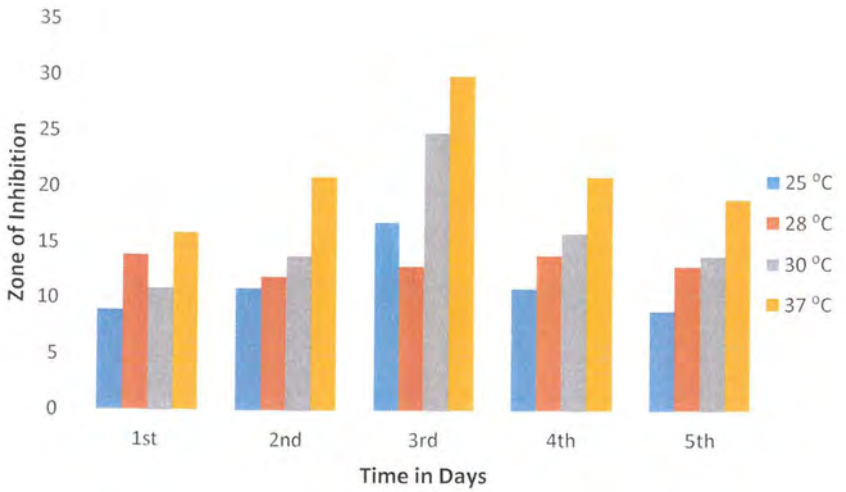

Figure 4.12. Antimicrobial activity of KSS 1.4 on different temperature against *S. aureus*.

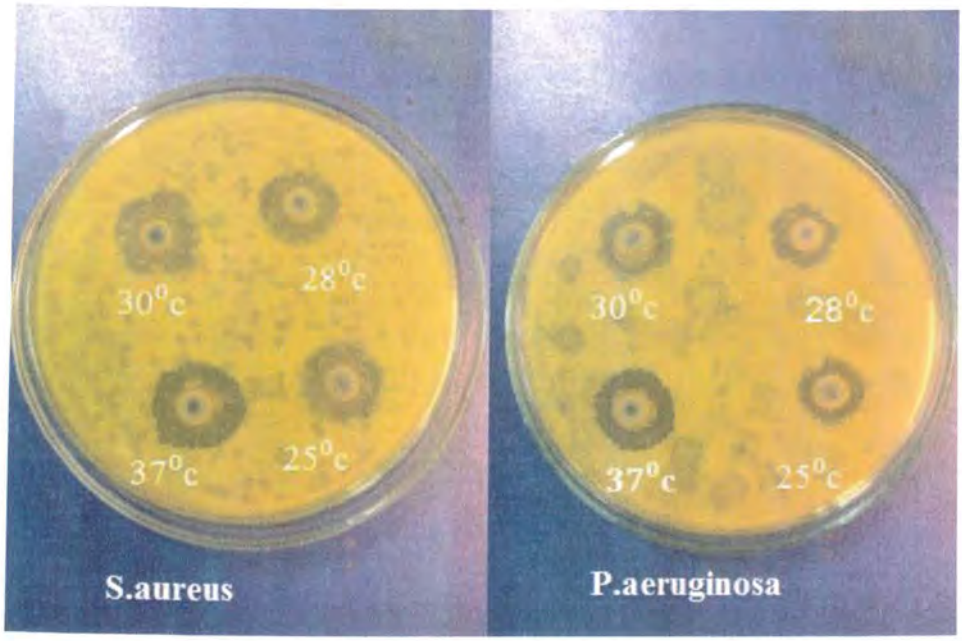

Figure 4.13. Antimicrobial activity of KSS 1.4 on different temperature.

Best carbon source optimization

Out of total 9 carbon sources used, the best carbon source for both the growth of KSS 1.4 and for its antimicrobial activity was starch. The highest growth was on day

3rd while best activity showed was on 2nd day (Fig. 4.14, 4.15, 4.16, and 4.17). So starch was used as carbon source in next optimization processes.

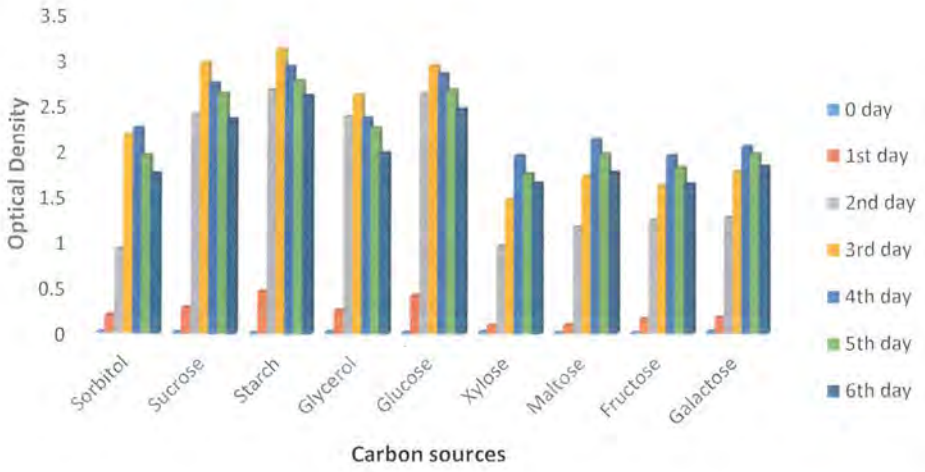

Figure 4.14. Growth of KSS 1.4 in different Carbon source.

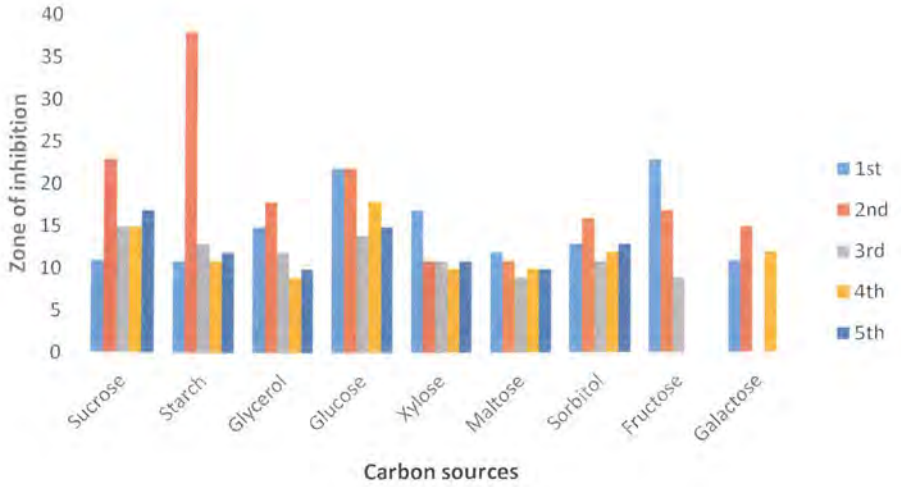

Figure 4.15. Antimicrobial activity of KSS 1.4 against *P. aeruginosa* in different Carbon sources (Zone of inhibition in mm).

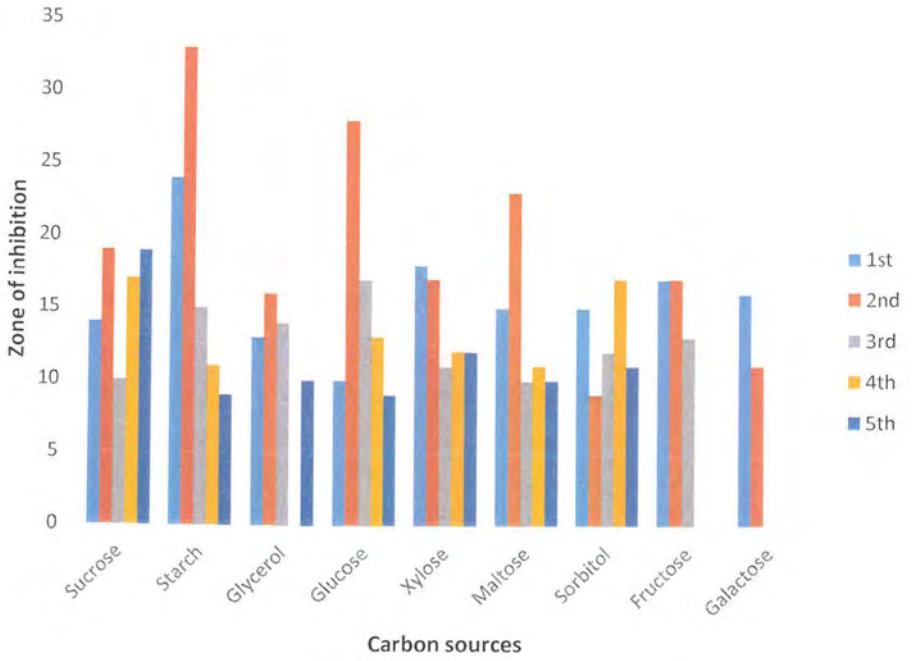

Figure 4.16. Antimicrobial activity of KSS 1.4 against *S. aureus* in different Carbon sources (Zone of inhibition in mm).

Figure 4.17. Antimicrobial activity of KSS 1.4 in different Carbon sources.

Optimization for best nitrogen source

The best nitrogen source out of total 6 used nitrogen sources was peptone. The highest growth was in beef extract on day 2nd while best antimicrobial activity was in peptone on day 2nd (Fig. 4.18, 4.19, 4.20, 4.21). So peptone was as nitrogen source for next optimization processes.

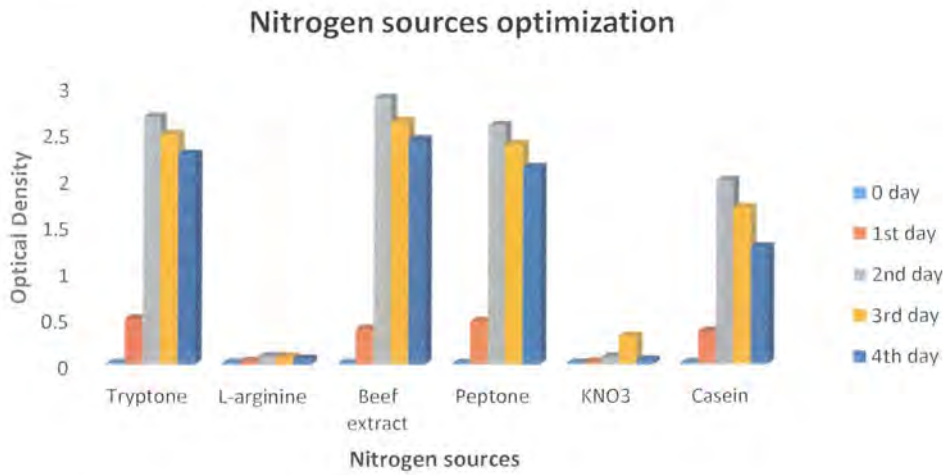

Figure 4.18. Growth of KSS 1.4 in different Nitrogen sources.

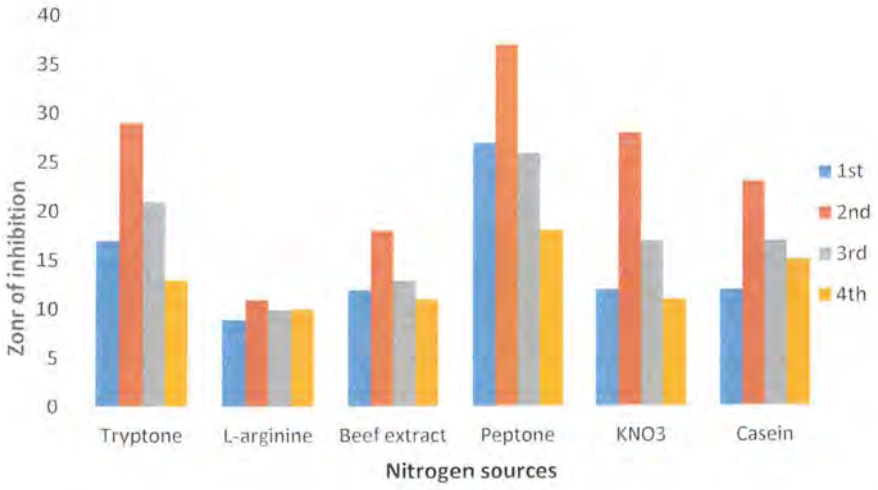

Figure 4.19. Antimicrobial activity of KSS 1.4 against *P. aeruginosa* in different Nitrogen sources (Zone of inhibition in mm).

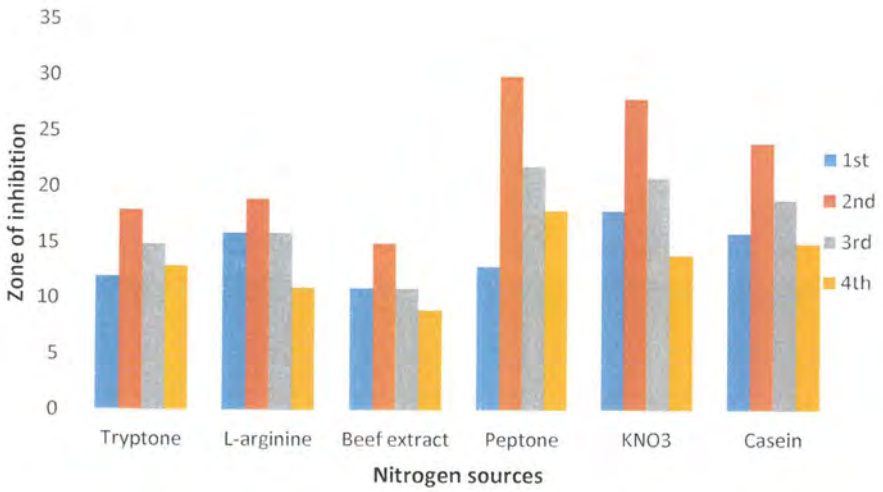

Figure 4.20. Antimicrobial activity of KSS 1.4 against *S. aureus* in different Nitrogen sources (Zone of inhibition in mm).

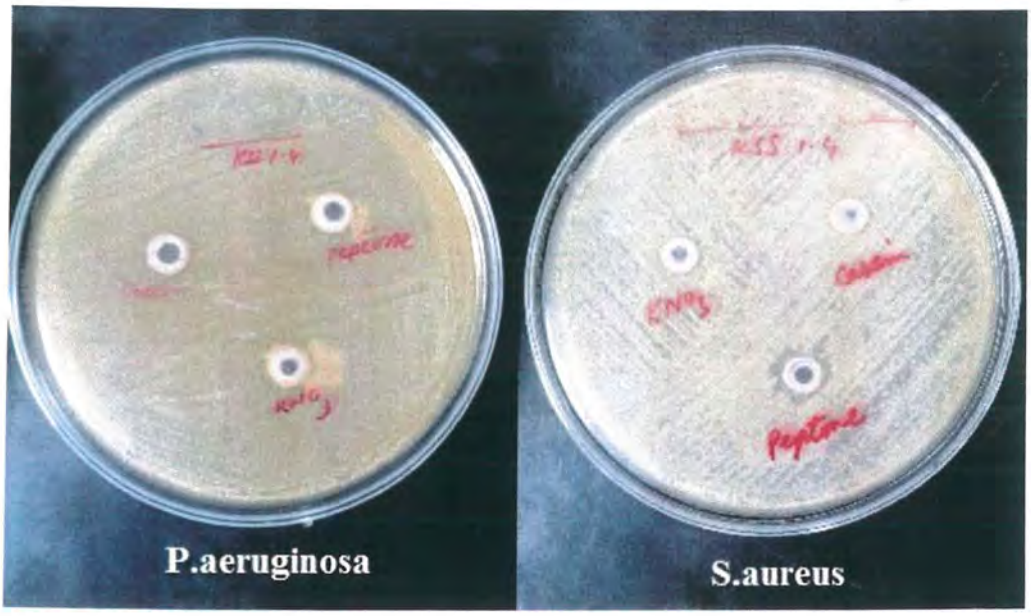

Figure 4.21. Antimicrobial activity of KSS 1.4 in different Nitrogen sources.

Screening for best production medium

Out of 4 generally used prepared medium i.e. Nutrient Broth, Luria Berteni, Tryptic Soy Broth, and Starch Casein Broth, the best one for growth of KSS 1.4 was SCB media showing best growth on day 3rd while this prepared medium was also best

for antimicrobial production showing best activity on day 2nd (Fig. 4.22, 4.23, 4.24, 4.25).

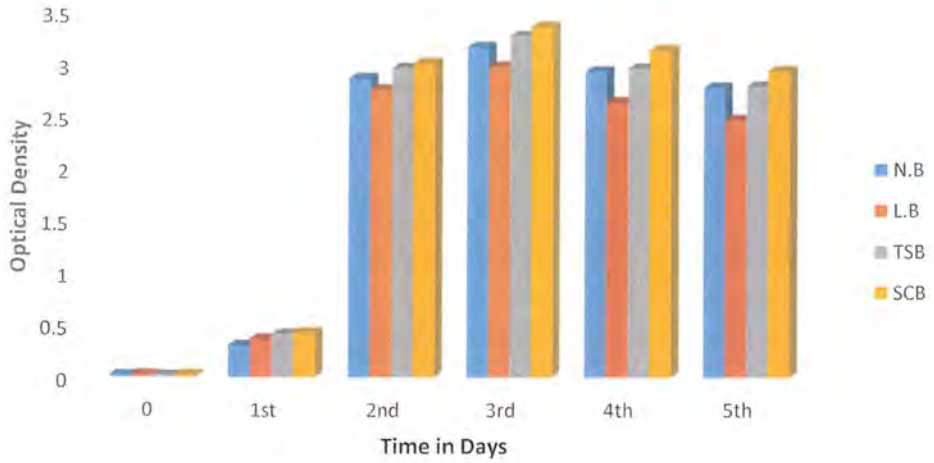

Figure 4.22. Growth of KSS 1.4 in different generally used media.

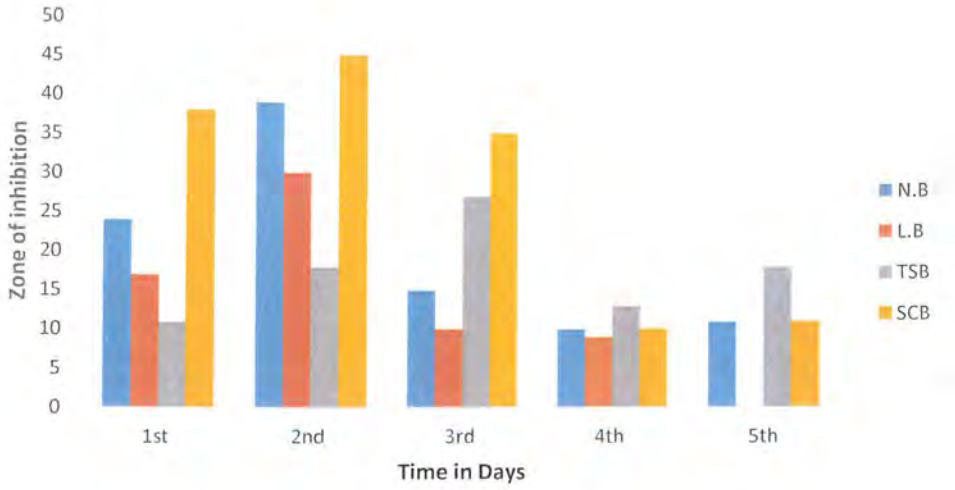

Figure 4.23. Antimicrobial activity of KSS 1.4 against *P. aeruginosa* in different generally used media (Zone of inhibition in mm).

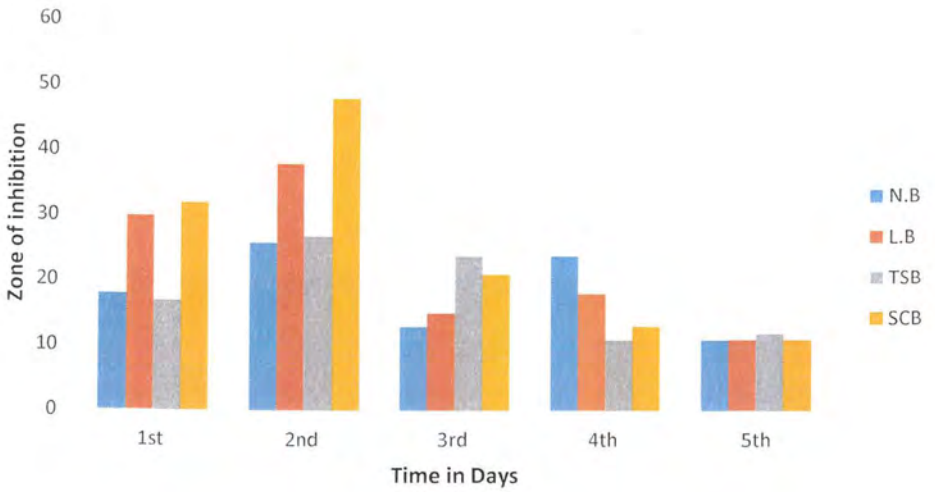

Figure 4.24. Antimicrobial activity of KSS 1.4 against *S. aureus* in different prepared media (Zone of inhibition in mm).

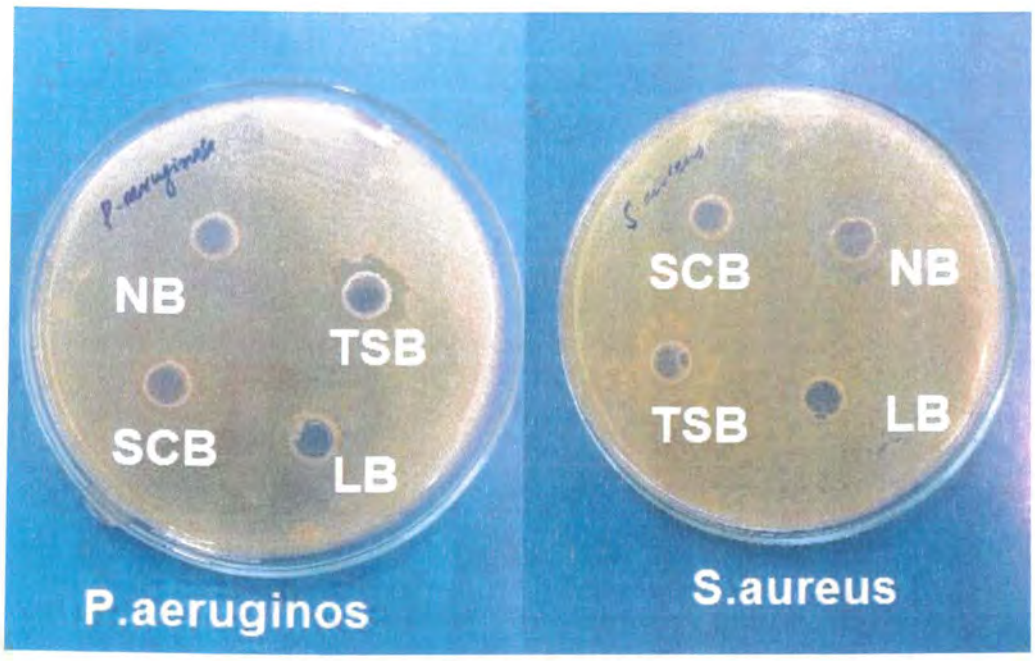

Figure 4.25. Antimicrobial activity of KSS 1.4 in different generally used media.

Initial pH optimization

The best initial pH for the growth of KSS 1.4 was pH 11 showing while best initial pH for activity was pH 5 showing best antimicrobial activity on day 1st (Fig. 4.26, 4.27, 4.28, and 4.29). So the pH 5 was used as optimized initial pH for next experiments.

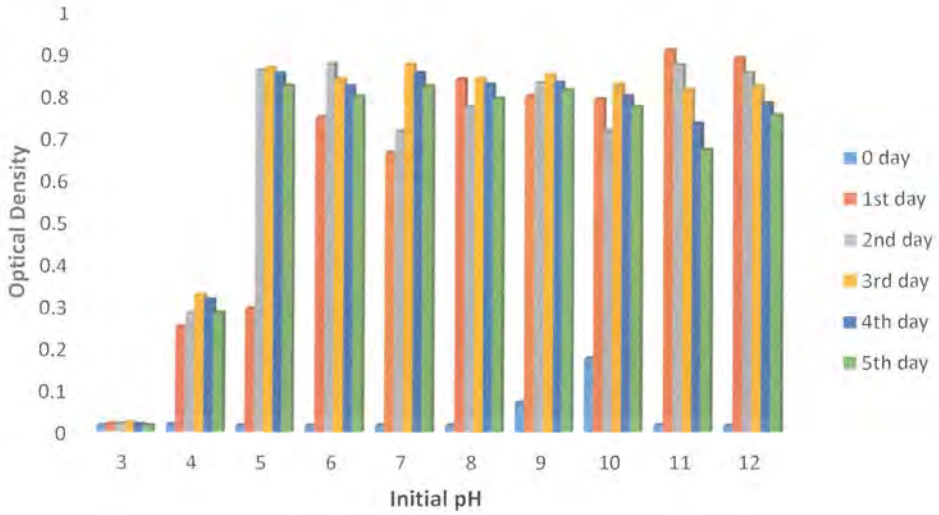

Figure 4.26. Growth of KSS 1.4 in different initial pH.

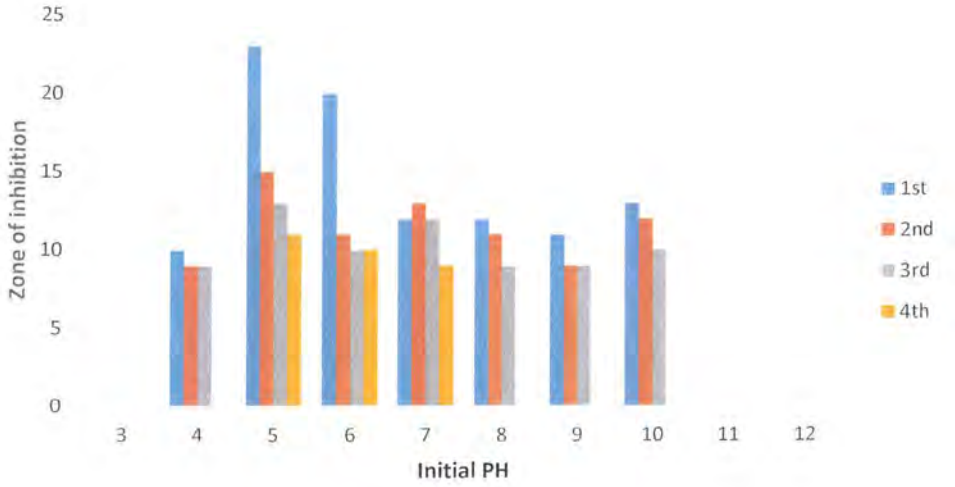

Figure 4.27. Antimicrobial activity of KSS 1.4 against *P. aeruginosa* in different initial pH (Zone of inhibition in mm).

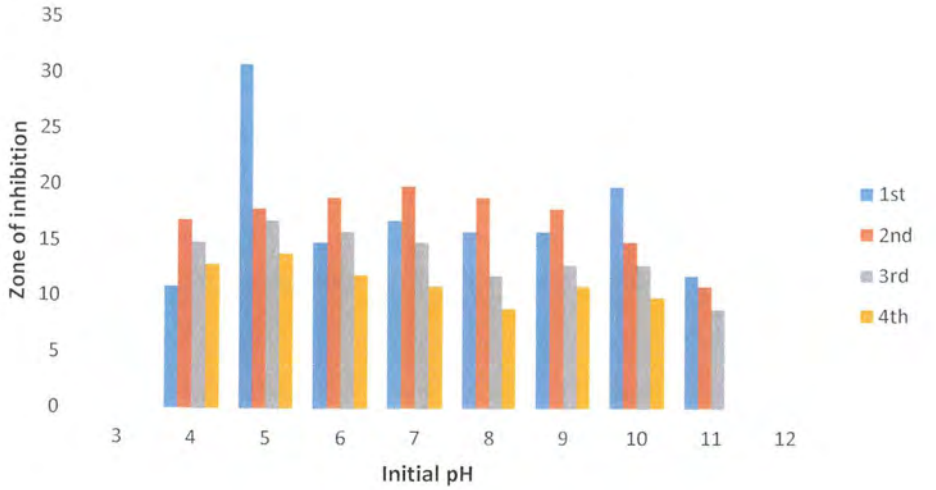

Figure 4.28. Antimicrobial activity of KSS 1.4 against *S. aureus* in different initial pH (Zone of inhibition in mm).

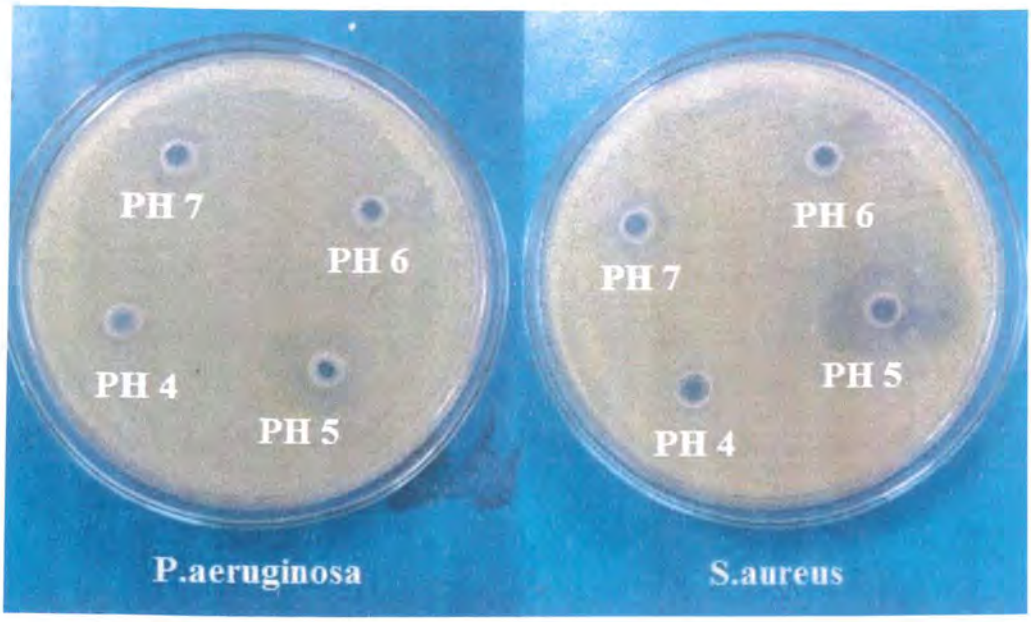

Figure 4.29. Antimicrobial activity of KSS 1.4 in different initial pH.

Optimization with sea water

Out of the different concentrations of sea water with d.H_2O used (0%, 25%, 50%, 75% and 100%) with previously optimized parameters i.e. starch-peptone media at initial pH 5 and temperature 37^{0}C, the best sea water with d.H_2O concentration for

growth was 25:75, showing highest growth on day 1st (Fig. 4.30). For antimicrobial activity this ratio was 50:50, showing best activity on day 2nd (Fig. 4.31, 4.32, 4.33). So this ratio of sea water with distilled water was used for further optimization experiments.

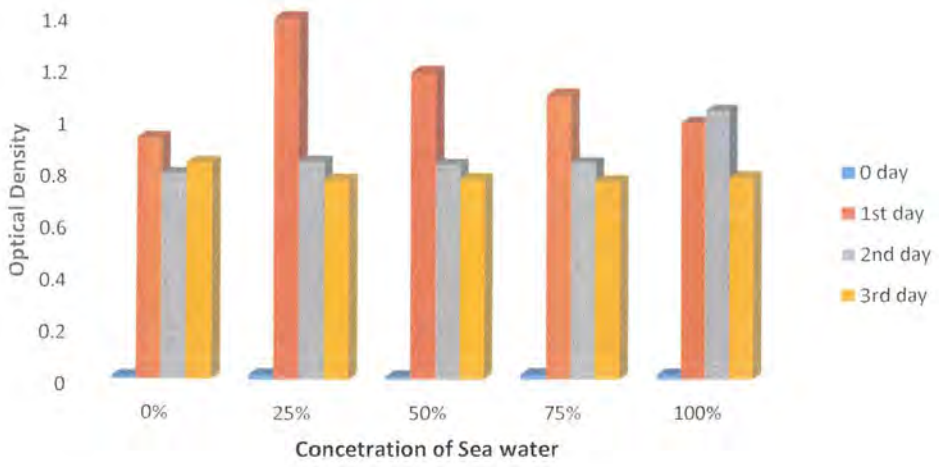

Figure 4.30. Growth of KSS 1.4 in different Sea water concentration.

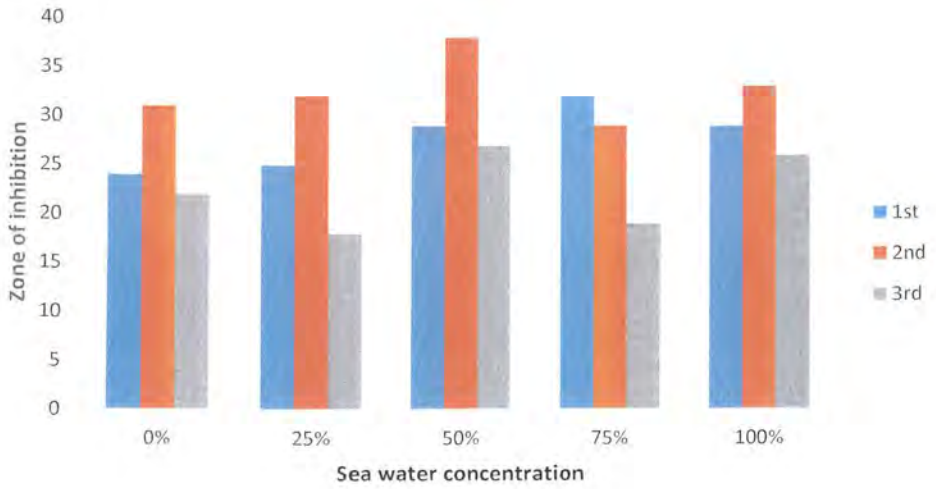

Figure 4.31. Antimicrobial activity of KSS 1.4 against *P. aeruginosa* in different Sea water concentration (Zone of inhibition in mm).

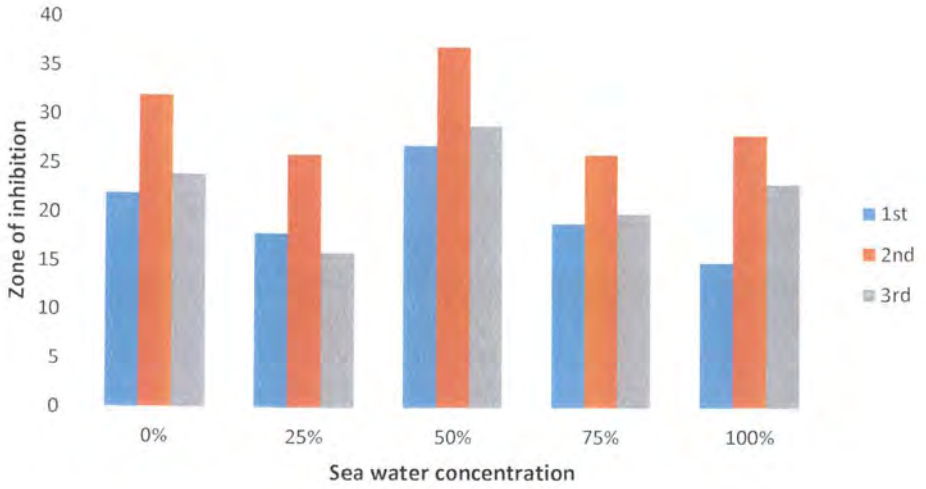

Figure 4.32. Antimicrobial activity of KSS 1.4 against *S. aureus* in different Sea water concentration (Zone of inhibition in mm).

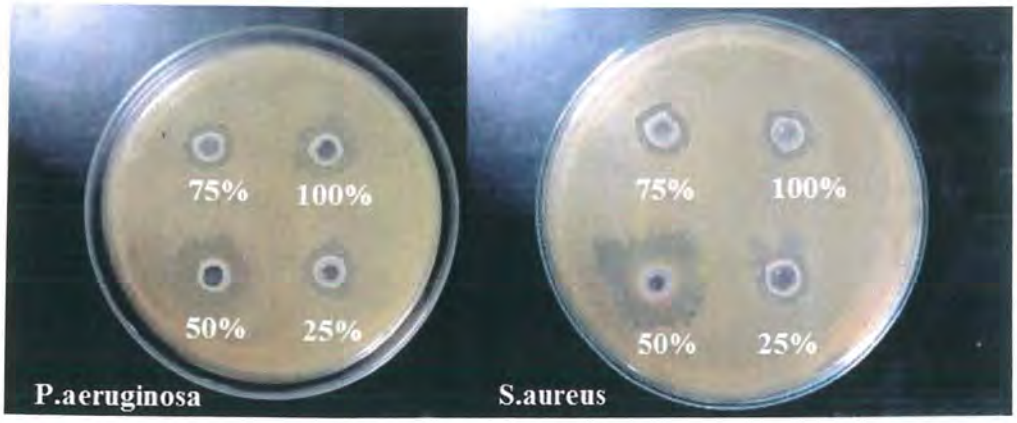

Figure 4.33. Antimicrobial activity of KSS 1.4 in Sea water concentration.

Optimization with NaCl concentration

The best growth of KSS 1.4 was in 0% NaCl presence in the starch-peptone broth at pH 5 prepared in d.H_2O, showed on day 1st (Fig. 4.34) while highest antimicrobial activity was showed on day 2nd (Fig. 4.35, 4.36, 4.37) in the presence of 2% NaCl in the starch-peptone broth at pH 5 prepared in d.H_2O.

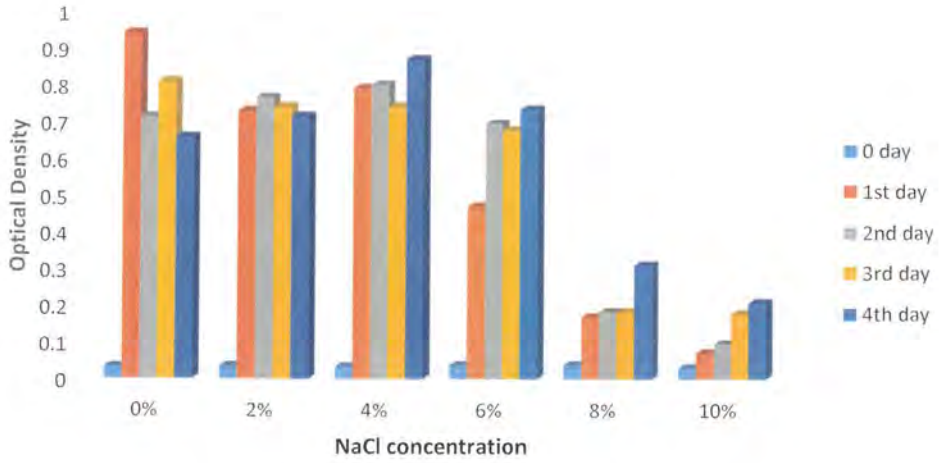

Figure 4.34. Growth of KSS 1.4 in different NaCl concentration.

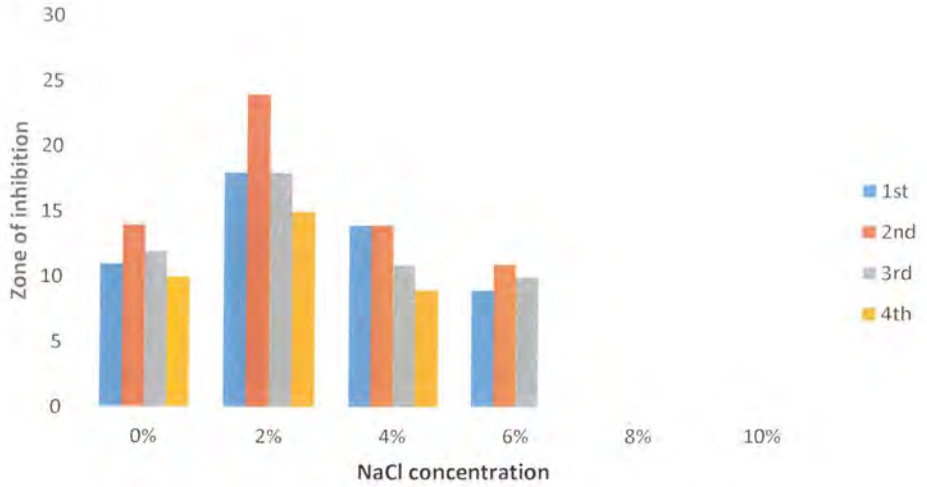

Figure 4.35. Antimicrobial activity of KSS 1.4 against *P. aeruginosa* in different NaCl concentration (Zone of inhibition in mm).

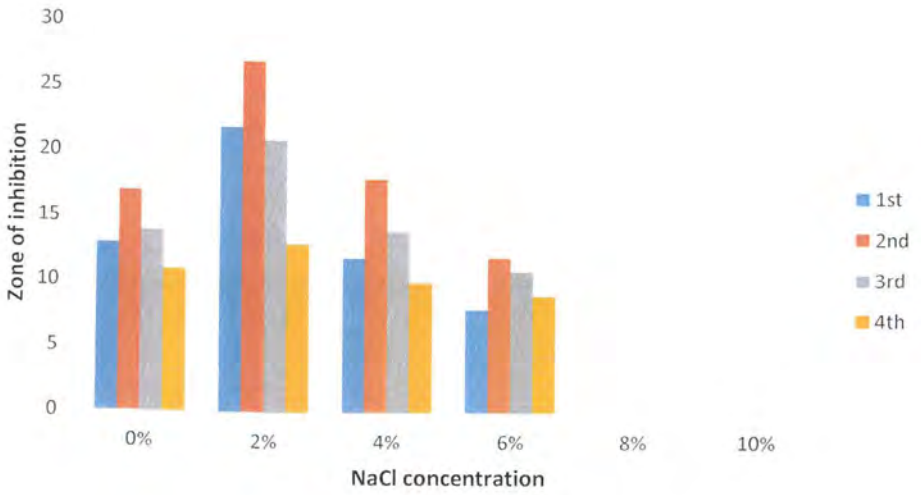

Figure 4.36. Antimicrobial activity of KSS 1.4 against *S. aureus* in different NaCl concentration (Zone of inhibition in mm).

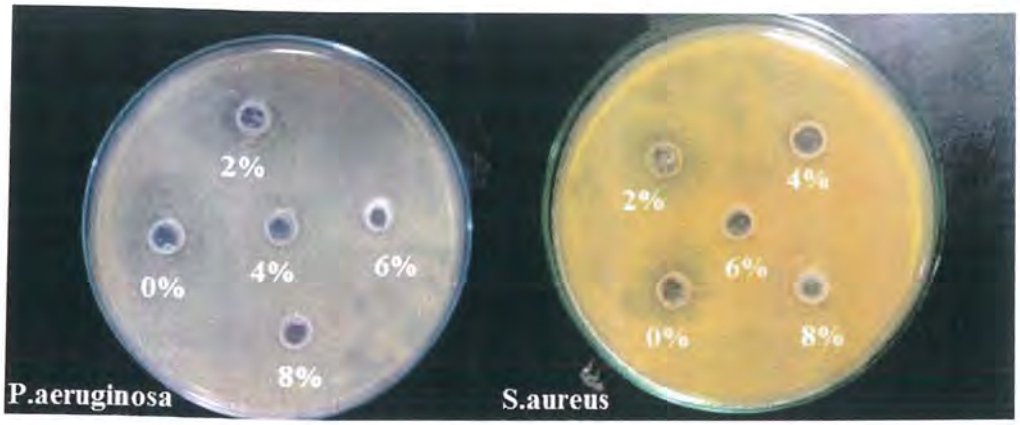

Figure 4.37. Antimicrobial activity of KSS 1.4 in different NaCl concentration.

Optimization with best extraction solvent

The best extraction solvent was n-hexane out of 4 solvents used for extraction. While with petroleum ether and ethyl acetate has lower extraction respectively and lowest extraction was with chloroform (Fig. 4.38, 4.39). So n-hexane is used for extraction of antimicrobial from this isolate.

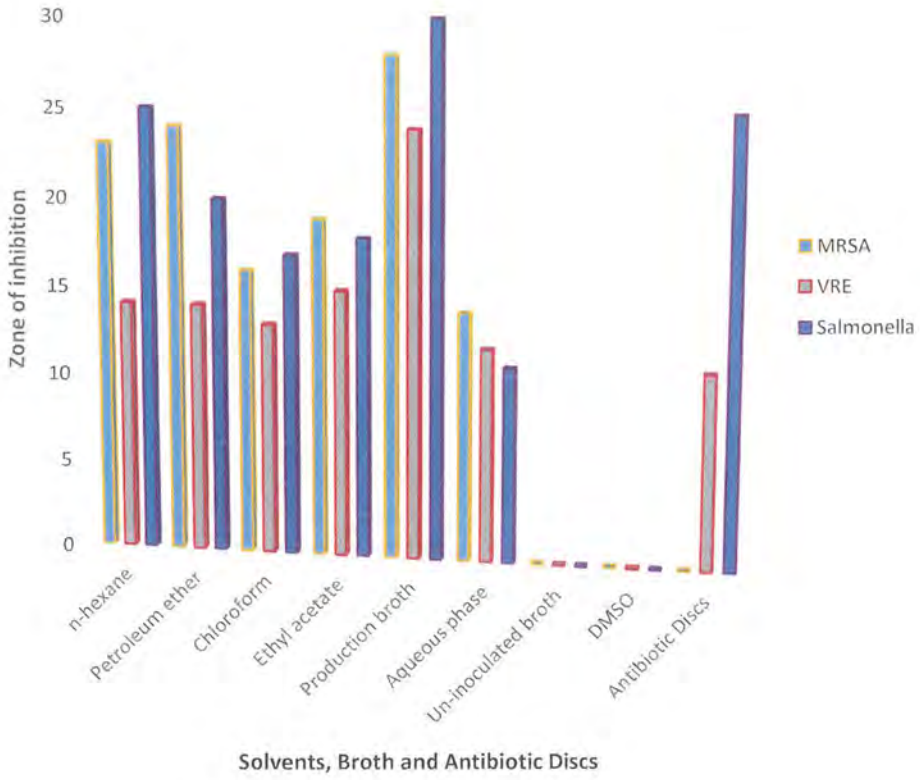

Figure 4.38. Antimicrobial activity of KSS 1.4 against different MDR pathogens after extraction with solvents (Zone of inhibition in mm).

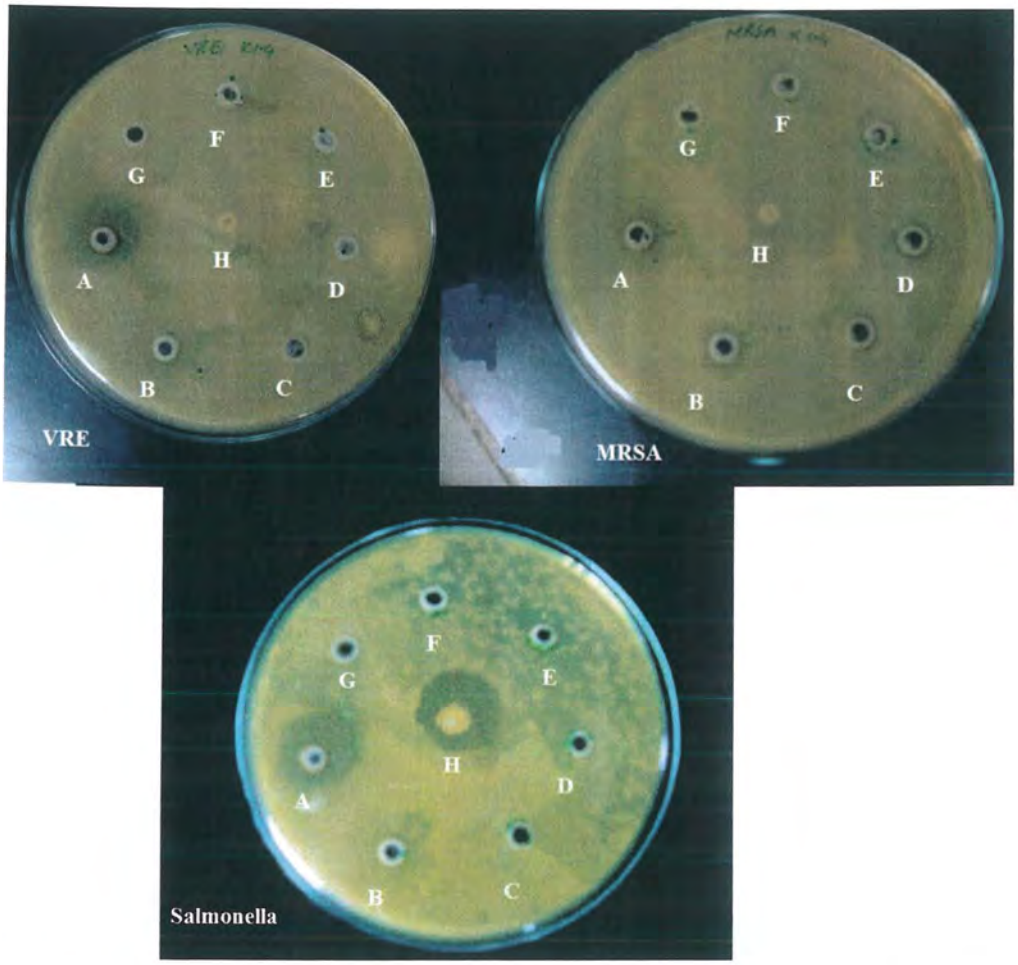

Figure 4.39. Antimicrobial activity of KSS 1.4 in different solvents. A. Production broth, B. n-hexane, C. Chloroform, D. Petroleum ether, E. Ethyl acetate, F. DMSO, G. Broth without inoculation, H. Antibiotic discs (Methicillin for MRSA, Vancomycin for VRE, and Cefotaxime for Salmonella).

Phylogenetic analysis

The obtained sequence from NCBI showed 94% similarity to *Streptomyces sp. LK-2 (JF710609)* on blast search analysis. The phylogenetic tree revealed that the studied isolate KSS-1.4 clustered into *Streptomyces sp.*

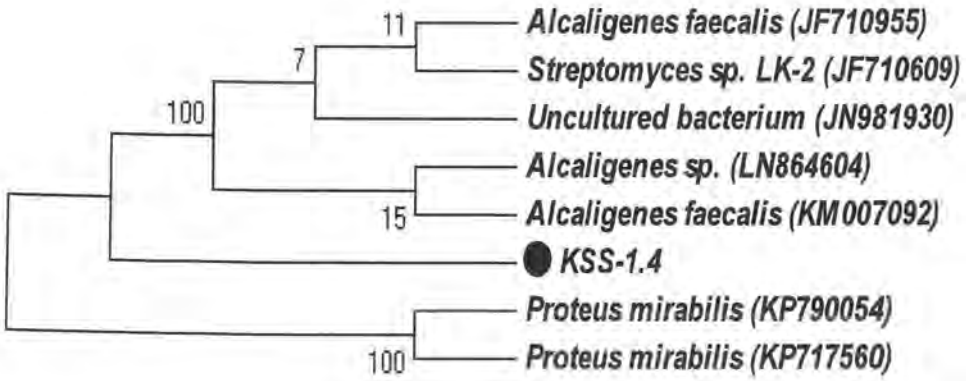

Figure 4.40. Molecular Phylogenetic analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura K. and Nei. 1993). The tree with the highest log likelihood (-1349.6898) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 8 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 619 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013).

DISCUSSION

The main sources in the past for antibiotics isolation or for its precursor molecules were from natural compounds (Butler and Buss, 2006). The β-lactam antibiotics were the first isolated antibiotics from the microbial sources (Demain and Elander, 1999) then aminoglycosides, polyketides, macrolides, quinolones, glycopeptides, and different generations i.e. second, third and fourth generation of β-lactam antibiotics, then daptomycin and oxazolidinones antibiotics were developed (Singh and Barret, 2006). From last two decades the isolation and production of novel antimicrobial compounds has been in declined greatly. Due to the excessive use of antibiotics their efficiency has greatly decreased mainly due to development of resistance to these drugs by pathogenic bacteria (Maestro and Sanz, 2007). New methodologies and techniques have been adopted by the pharmaceutical industries to develop novel antibiotics such as combinatory chemistry tools, organic synthesis and other such techniques, but satisfactory results has not been achieved (Luzhetskyy *et al.*, 2007; Arasu *et al.*, 2013). Although synthetic antimicrobial compounds are of great importance, used for treatment of microbial diseases nowadays but there is still a great potential in the nature to isolate and develop novel antibiotics and other compounds of industrial importance from it (Newman and Cragg, 2007). Due to the excessive increase in the demand for novel antibiotics and to handle the resistant pathogens, the research has been shifted for the isolation of novel antimicrobials to the new habitats having unusual conditions (Arasu *et al.*, 2013).

Environment had a great impact on the metabolism of microorganism and have the ability to alter it, so it is necessary to study the microorganisms from its natural habitats and screen them for its antimicrobial activity. For this purpose the endophytes from terrestrial plants and sea organisms have been screened and studied for its capacity to produce antimicrobial compounds as well as other sources has been also the focus of such studies (Gunatilaka, 2006; Gandhimathi *et al.*, 2008). Marine environment has greater capacity and is the larger pool for biological and chemical variety than terrestrial environments (Arasu *et al.*, 2013). Reports about the antibiotics and enzymes production from marine isolates have been published, but there is still a greater potential in the marine microorganism specifically in the marine actinomycetes to produce new types of antimicrobials and other industrially important compounds (Sharma and Pant,

2001). Many types of resistance pathogens has been emerged and causing serious infection such as MRSA, MDR *P.aeruginosa*, reports are also available of Vancomycin resistant enterococci pathogens which is a drug of choice against MRSA pathogen, so it is the need of time to develop anti MRSA and anti VRE strategies (Chopra, 2003).

The Indian Ocean has not been explored so much for its microbial diversity and important metabolites and it has a greater chance to isolate new types of microorganisms, novel antimicrobials and other industrially important substances from there (Arasu *et al.*, 2013). Soil has been considered important source of antimicrobial producing microorganisms (Ouhdouch *et al.*, 2001; Lee and Hwang, 2002; Prabavathy *et al.*, 2006) and many of it has been isolated from marine samples (Sujatha *et al.*, 2005; Maldonado *et al.*, 2005; Fenical and Jensen, 2006). So Karachi sea coast was selected for this study as this is also the part of the Indian Ocean and not so much attention has been given to it. The purpose of this study was to isolate the potent antimicrobial producing microorganisms from the marine samples of Karachi, optimize the cultural condition for antimicrobial production and to produce antimicrobials from them. This study also involved the identification and characterization of the selected isolate.

A total of 172 isolates from six soil samples of Karachi sea shore were screened for its antimicrobial activity. Out of these 172 screened isolates 84 isolates were found having activity against many standard microorganisms including various bacteria and fungi. When these 84 isolates were screened for its antimicrobial activity against MDR pathogens, 32 isolates were found active against MDR pathogens. This is so much high rate that about 49% of the isolates were active metabolite producer against standard microorganisms and 38% of them were also active against MDR pathogens e.g. MRSA, VRE and clinical *P.aeruginosa*. This confirms the hypothesis of Sharma and Pant (Sharma and Pant, 2001) that marine environment has a great potential of antimicrobial producing microorganisms. There is also other such reports that, as a result of screening, new types of microorganisms have been isolated having strong antimicrobial activity (Donadio *et al.*, 2007). It has the ability to produce novel compounds having antimicrobial activity or these can be used as precursor molecules for developing novel antibiotics (Sofia and Boldi, 2006). Out of the total 32 active metabolites producers against MDR pathogens 23 (%) are gram positive bacteria while 9 (%) are gram negative bacteria, hence it shows that the active metabolite producers in the marine environment are mostly gram positive bacteria, previously reported by M.V. Arasu *et*

al. (2013), H.M Atta (2012) and Valli *et al.* (2012). About 28% (9 isolates) having endospores shows that it have the ability to survive in the harsh environment. The media were supplemented with nystatin (50 mg/L) and cycloheximide (50 mg/L) for inhibiting the growth of fungi and yeasts (Zereini, 2014). The selected isolate KSS 1.4 is gram positive, large rods and long chains, while it also have the ability to produce terminal and sub-terminal endospore which is the main characteristic of antibiotic producing microorganisms for the survival in the harsh environments. It has the antimicrobial activity against almost all of the tested standard and MDR pathogens. These findings are almost similar to that of Valli *et al.* (2012) and Arasu *et al.* (2013). The isolate KSS 1.4 can grow on Nutrient Agar, Luria-Bertani Agar, Tryptic Soy Agar and highest on Starch Casin Agar which is same as studied by Usha *et al.* (2013) and Salam *et al.* (2014). The islolate KSS 1.4 showed highest growth on 0% NaCl and 2% NaCl, medium growth on 4% NaCl, poor growth on 6% NaCl and very poor growth in 8% NaCl presence in the medium which is much higher than the previous study of Esmaeil *et al.* (2012) and Salam *et al.* (2012), in their study the isolate PG-01 and A5 showed growth up to 5% NaCl presence in the medium while somehow similar to study of Arasu *et al.* (2013). The isolate showed positive results for the amylase, gelatinase, protease, and catalase enzymes production reported earlier by Arasu *et al.* (2013) and Salam *et al.* (2014) while it is negative for melanin pigment and urease enzyme production which is contrary to the study of Usha *et al.* (2011), H.M Atta (2012) and Usha *et al.* (2013). The isolate showed medium growth from 4^0C to 15^0C while highest growth from temperature 25^0C to 50^0C and medium growth on 60^0C while no growth on 70^0C this results are almost different from the previous studies of Usha *et al.* (2011), H.M Atta (2012), Usha *et al.* (2013), Salam *et al.* (2014), in which the selected isolate showed growth from 20^0C to 50^0C or less. It suggest that isolate KSS 1.4 has a diverse temperature and have the ability to survive in different temperatures from psychrophilic to thermophilic nature i.e. from 4^0C to 60^0C so we can suggest that it is of mesophilic nature as it have optimum temperature in the mesophilic range from 25^0C to 45^0C while growth on 50^0C-60^0C suggest that it is a kind of thermo-tolerant. This suggestion is also supported by the temperature range of the Karachi sea surface temperature which ranges from 20^0C to 47^0C throughout the year (Pakistan Meteorological Department, 2015). The isolate KSS 1.4 have antimicrobial activity against large number of clinical isolates. In secondary screening where spot inoculation was used as the incubation time

was increased from 24 hrs to 72 hrs the zone of inhibition against these clinical isolates also increased in some case clearing the whole 90 mm petri plate from the clinical isolates. This may be due to the increasing competition for space and nutrition in the petri plates as the incubation time increased and it may be due to the increase in production of antimicrobials with the time. No such study has been reported until now of isolating so much high producer microorganism from marine samples. This also suggest that the isolate KSS 1.4 is the potent antimicrobial producer against clinical isolates as showed in the results.

The temperature optimization has the basic role in the optimization of parameters for fermentation. Temperature provide optimal condition for growth increase as well as it can be a stimulus to the bioactive metabolite production from the microorganisms. The isolate KSS 1.4 showed its highest growth on temperature 25^{0}C while the antimicrobial production was highest on 37^{0}C. These results is same to the climatic condition of the Karachi sea surface temperature which has the average low temperature 20.3^{0}C, daily mean 26^{0}C and high temperature 31.7^{0}C throughout the year (Pakistan Meteorological Department, 2015). Our findings for the isolate KSS 1.4 is approximately same to the daily mean for its optimum growth which is 25^{0}C while temperature 37^{0}C which is best temperature for antimicrobial production, is higher than that of average high temperature throughout the year which is 31.7^{0}C so the temperature 37^{0}C provide stress condition to the isolate so why it produces antimicrobial substances in higher rate, the same results were reported by Esmaeil *et al.* (2012). Other thing which strengthen our findings is that as the temperature increases the production of antimicrobials by isolate KSS 1.4 also increases as mentioned in results.

Among the optimization processes the other most important parameter for fermentation is carbon and nitrogen source optimization. The strain KSS 1.4 has the ability to utilize a wide range of carbon sources and different sugars. It showed satisfactory growth in all 9 types of carbon sources. The best carbon source for the growth of this isolate was starch which is also best for the antimicrobial activity. Our results is assisted by the finding of Usha *et al.* (2013) and Salam *et al.* (2014) where their isolates showed highest growth in the medium containing Starch. The isolate KSS 1.4 showed highest growth in the presence of starch as a carbon source already reported as best carbon source by Ababutain *et al.* (2013) and as temperature 37^{0}C provided

stress condition to it so the antimicrobial production was also higher than other carbon sources due to the presence of higher number of cells. In the nitrogen sources used the best nitrogen sources was peptone already reported by Balagurunathan *et al.* (2011), Ababutain *et al.* (2013), Saleh and Paragati (2015).

In this study the starch casein broth was the best prepared medium for the growth as well as for the antimicrobial activity of the isolate KSS 1.4 while this medium has also been reported as best medium for the growth of marine *Streptomyces* (Valan *et al.*, 2008) and Arasu *et al.* (2013). The initial pH of the medium has also the critical role in the antibiotic production. The highest antimicrobial production was shown on initial pH 5 by the isolate KSS 1.4 in this study which is approximately near to the finding of Balagurunathan *et al.* (2011) and Ababutain *et al.* (2013) and similar to the findings of Tomprefa *et al.* (2011). The highest growth showed on pH 11 was approximately similar to the findings of H.J. Son *et al.* (2006). So the results from this study suggest that isolate KSS 1.4 is alkaliphile (Horikoshi and Koki, 1999) as alkaliphile has survival range from pH 8.5 to 11 while the isolate KSS 1.4 also showed second highest growth on pH 12 after pH 11 suggesting that it has the enzyme system to play role in pH homeostasis for survival on so much high alkaline pH by promoting capturing and retention of proton, metabolic changes are made which lead to increase acid production and proton retention in the cytoplasm is increase by changing layers on cell surface (Padan *et al.*, 2005). There is no antimicrobial activity on pH 11 and pH 12, the possible explanation for is survival of the isolate in that high alkaline pH. In place of antimicrobial production the metabolism of the isolate may be shifted to the survival so why no antimicrobials are produced.

As antimicrobial production from marine isolates are sea water dependent so sea water optimization was carried out. The isolate KSS 1.4 has optimum sea water concentration with that of distilled water is 50%:50%. The Indian Ocean sea surface salinity (SSS) level is from 3.2% to 3.7% (Wikipedia.org) Karachi sea is part of the India Ocean having salinity level is approximately 4% so when we dilute the sea water with equal amount of distilled water the level of salinity decreases to 2% so these findings are similar to that of Esmaeil *et al.* (2012) where the optimum NaCl concentration for antibiotic production in marine broth was 2%. These results were also confirmed by the results of NaCl concentration optimization for antibiotic production which showed highest antimicrobial activity in 2% NaCl presence which is similar to

the findings of Esmaeil *et al.* (2012). While highest growth was observed in the 4% NaCl presence in the broth but the antimicrobial activity was much less than that of 2%, their growth pattern is approximately similar but activity in 4% NaCl is less than 2% NaCl, it may be less due to the survival mechanism of the isolate in which metabolism of the isolate divert toward survival in higher NaCl concentration so the antimicrobial production decreases.

In the extraction process with solvents the best solvent was n-hexane (Illic *et al.*, 2007) showing highest activity to MRSA and *salmonella* while activity to the VRE was lower than that of ethyl acetate extract. So n-hexane is the solvent of choice as reported earlier by Christen and Veuthey (2001).

The selected isolate showed 94% similarity to the genus *Streptomyces* LK-2 as this genus is known for production of maximum number of antibiotics and other industrially important metabolites (Rudi *et al.*, 2012).

Finally it is necessary to produce the antimicrobial from the isolate KSS 1.4 in larger amount and purify it. As this isolate have the antimicrobial activity against MDR pathogens and the superbug like MRSA and VRE, so it can be very helpful in treating the diseases caused by these pathogens.

CONCLUSION

It was found in this study that marine environment of Karachi, Pakistan has a great potential of new types of microorganisms and majorly *Streptomyces sp.* The isolates from this area showed higher antimicrobial metabolites production against several microbial pathogens. The isolates KSS 1.4 which was identified as a novel *Streptomyces sp.* and showed highest antimicrobial metabolite production against all types of standard and clinical pathogens. This isolate was also actively producer of metabolites against MDR pathogen such as MRSA and VRE, both of them are causing life threatening diseases in the present world. This study shows that different culture parameter optimization are very important for the production of antimicrobials from marine isolates. The optimized culture conditions for the isolate KSS 1.4 studied were temperature 37^0C, carbon source starch, nitrogen source peptone and starch casein broth was best medium. While other parameters optimized were pH 5, 2% NaCl, or 50% sea water concentration in the medium for production of antimicrobials from this isolate. The best extraction solvent for antimicrobial extraction was n-hexan. Further study on this isolate will lead to the isolation of some new types of metabolites. This study also shows that marine environment has some naturally occurring new types of actinomycetes which have a greater potential to produce potent antimicrobial metabolites and having the chances to lead to the discovery of new type of antibiotics.

FUTURE PERSPECTIVES

- The antimicrobials from the marine isolate KSS 1.4 can be produced in large scale using the optimized culture parameters.
- The antimicrobial compound can be checked for its purity and determination of active ingredients.
- The physical and biochemical properties of the compound can be find out which will be helpful in the therapeutic studies with the antimicrobial produced as well as in the characterization of the compound.
- The minimum inhibitory concentration of the compound can be found which will be used in other in-vitro and in-vivo experiments.
- The elemental analysis and structure elucidation of the compound can be done using FTIR, GC-MS, and NMR etc, that will help in the identification of the antimicrobial produced.
- Mechanism of action for the active metabolite can be studied.
- The structural properties of the isolate KSS 1.4 can be studied using electron microscopy technique which will be helpful in identification of the isolate.

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Appendix

Appendix 1: Gram staining, endo spore staining, microscopy and colony morphology results of all the 32 isolates having activity against MDR pathogens.

(Endospore: $+^{T}$, Terminal endospore. $+^{ST}$, Sub-Terminal endospore. $+^{C}$, Central endospore)

(Gram staining: G +, Gram Positive. G -, Gram Negative)

(Colony Morphology: C, Color. S, Size. CL, Colony)

Sr.No	Sample I.D	Endospore	Gram Staining/ Microscopy	Visible Colony Morphology
1	KSS 1.1	-	G +, Single and diplo-Cocci	C: Off-white S: Large CL: Irregular, Flat, Shiny, Transparent.
2	KSS 1.4	$+^{T, ST}$	G +, Large Rods and short + long Chains.	C: Creamy S: Small CL: Round, Irregular margins, Flat, Shiny, Translucent.
3	KSS 2.1	-	G +, Single + diplo-Cocci	C: off-white S: Small /Medium CL: Round, Regular margins, Flat, Shiny, Transparent.
4	KSS 2.3	-	G +, Single + diplo-Cocci, some Short Chains	C: Creamy S: Small/Medium CL: Round, Raised, Irregular margins, Shiny, Transparent.
5	KSS 2.4	-	G +, Single + diplo-Cocci	C: Creamy S: Small CL: Round, Irregular margins, Raised, Dry Translucent.
6	KSS 3.2	$+^{C}$	G +,	C: off-white

			Single + Double Rods,	S: Medium CL: Flat, Irregular, dry, Opaque.
7	KSS 3.4	-	G -, Cocci + diplo-Cocci, abundant thin filament.	C: Creamy S: Medium CL: Round, Irregular margins, Flat, Shiny, Opaque.
8	KSS 3.6	-	G +, Single + diplo-Cocci and Short Chains.	C: Creamy S: Small CL: Round, Shiny, Raised, Regular margins, Transparent.
9	KSS 3.7		G -, Single + double Small size Cocci.	C: Creamy S: Medium CL: Raised, Round, Shiny, Irregular margins, Opaque.
10	KSS 3.8	$+^{C}$	G +, Single + double Medium size Rods.	C: Creamy S: Small CL: Flat, Irregular, Shiny, Translucent.
11	KSS 3.9	-	G +, Single + Double Rods.	C: Creamy S: Large CL: Flat, Shiny, Irregular, and Translucent.
12	KSS 3.10	-	G +, Single + double Cocci.	C: Creamy S: Large CL: Flat, Round, Shiny, Regular margins, Translucent.
13	KSS 3.11	-	G -, Single + double Cocci, a few	C: Creamy S: Small/Medium

			short chains and some long thin Filaments.	CL: Round, Regular margins, Flat, Shiny, Translucent.
14	KSS 3.12	-	G +, Single Rods.	C: Creamy S: Medium CL: Shiny, Flat, Irregular margins, Opaque.
15	KSS 3.13	-	G +, Single + double Medium size Rods.	C: Creamy S: Medium CL: Flat, Irregular margins, Shiny Translucent.
16	KSS 4.2	-	G -, Single + double Rods.	C: Creamy S: Small CL: Round, Raised, Regular margins, Shiny, Translucent.
17	KSS 4.3	-	G -, Single Rods.	C: Creamy S: Small CL: Round, Flat, Regular margins, Shiny, Transparent.
18	KSS 4.6	-	G +, Single + double Medium Size Rods.	C: Creamy S: Medium CL: Round, Raised, Regular margins, Shiny, Translucent.
19	KSS 5.1	$+^{ST}$	G +, Thick Rods With Some Filaments.	C: off-white S: Medium CL: Round, Shiny, Flat, dry, Irregular margins, Opaque.
20	KSS 5.5	$+^{C}$	G +, Single and Pair Rods.	C: off-white S: Large

				CL: Flat, dry, Irregular margins, Translucent.
21	KSS 5.8	$+^{C}$	G +, Single + double Rods and some long Chains.	C: off-white S: Large CL: dry, raised like fungus, irregular margins, and opaque. Black color in mix culture initially. Reverse: yellowish in center with off-white and smooth margins.
22	KSS 5.17	-	G -, Single + double Rods.	C: Creamy S: Small CL: Round, Flat, Irregular margins, Shiny, Transparent.
23	KSS 5.18	-	G +, Single + double Rods, Short Chains.	C: off-white S: Large CL: Flat, dry, Irregular margins, Translucent. Black color in mix culture initially. Reverse: C: Creamy with White Irregular margins.
24	KSS6.1	-	G +, Single + double medium size Rods.	C: Creamy S: Small CL: Raised, Round, Shiny, With Irregular dry Margins, Translucent.
25	KSS 6.7	$+^{ST}$	G +,	C: off-white S: Small/Medium