Isolation and Characterization of Antibiotic Producing Bacterial and Fungal Strains from Red Soil of Himalayan Region of Pakistan

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Antimicrobials

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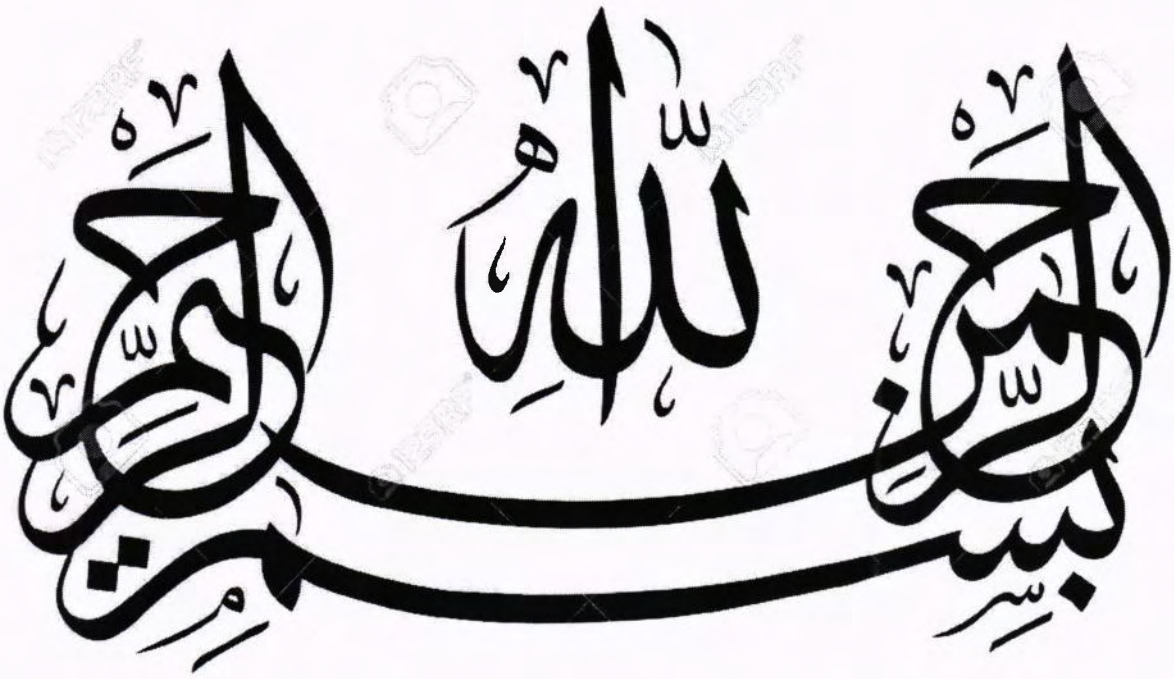
بسم الله الرحمن الرحيم

Dedicated to

My Beloved Parents


بسم الله الرحمن الرحيم

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Dated: 20.9.17

FINAL APPROVAL

It is certificated that we have read and evaluated the thesis 'Isolation and Characterization of Antibiotic Producing Bacterial and Fungal Strains from Red Soil of Himalayan Region of Pakistan' submitted by Ms. Neelam Firdous and it is our judgment that this project is of sufficient standard to warrant its acceptance by International Islamic University, Islamabad for the M.S Degree in Biotechnology.

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Dedicated to

My Beloved Parents

DECLARATION

I hereby declare that the work present in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Date 12.10.17

Neelam Firdous

Neelam

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Acknowledgments

All praises to **Almight ALLAH**, the Omnipotent and the most merciful and Beneficent, Who is the creator of universe Who bestowed the man with intelligence, knowledge, sight to observe, and mind to think and judge. Peace and blessing of ALLAH be upon the **Holy Prophet, Muhammad** (Peace Be Upon Him), who is everlasting torch of guidance and knowledge for humanity and who exhorted his followers to seek for knowledge from cradle to grave.

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Neelam Firdous

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LIST OF ABBREVIATIONS

µl	Micro litre
AIDS	Acquired Immunodefeciency syndrome
BHIB	Brain Heart Infusion Broth
Ca	Calcium
CAP	Community acquired pneumonia
Cm	Centi meter
$CuSo_4$	Copper sulphate
ddH_2O	Double distilled water
DMSO	Dimethyl sulfoxide
gm	Gram
HGT	Horizontal gene transfer
hrs	Hours
ICU	Intensive care units
K	Potassium
MA	MacConkey Agar
MDR	Multidrug resistant
Mg	Magnesium
mins	Minutes
ml	Milli litre
mm	Milli meter
mM	Milli molar
MRSA	Methicillin resistant *Staphylococcus aureus*
NA	Nutriet Agar
Na_2So_4	Sodium sulphate
NaCl	Sodium chloride

NB	Nutrient Broth
P	Phosphorous
PDA	Potato Dextrose Agar
psi	Pounds per square inch
SDA	Sabouraud Dextrose Agar
sec	Seconds
TB	Tuberculosis
TSA	Tryptone Soya Agar
TSB	Tryptone soya Broth
UTI	Urinary tract infections
UV	Ultra violet
VRE	Vancomycin Resistant Enterococci
WHO	World Health Organization

ABSTRACT

In this day and age due to the emergence of multi drug resistant bacterial pathogens, crucial challenges for efficient treatment of diseases have appeared. As a consequence, there is an increased burden of resistant pathogens on the globe which has resulted in dire requirement of searching new effective antimicrobials. Soil is an ultimate reservoir of biologically active microflora, which provides nearly all of the clinical antimicrobial drugs used today.. Hence aim of the present study was an attempt to isolate and identify the antibiotic producing microbial strains from the red soil of Pakistan. From 10 different soil samples only one bacterial strain was isolated which was capable of antimicrobial activity. Strain was identified by biochemical characteristics and final identification was done by API 20 NE kit which showed 99% homology with *Pseudomonas aeruginosa.* Antibacterial and antifungal activity of the *P. aeruginosa* showed that *Staphylococcus aureus* was extremely sensitive to it with a zone of inhibiton of 42mm. *Staphylococcus epidermidis, Enterobacter aerogenes,Aspergillus fumigatus* and *Candida albicans* were also inhibited by the isolated strain. Crude extract was also prepared by solid state fermentation and solvent extraction method and was evaluated for the antibacterial activity against different clinical test strains. Results showed that crude extract exhibits antibacterial property certains bacterial strains pathogenic to humans. The unexplored Kashmir Himalayas are of great significance because of its richness in biodiversity and need to be explored for isolation and characterization of native microbes for biologically active secondary metabolites.

CHAPTER 1
INTRODUCTION

1.1 Antibiotics

Antibiotics are also known as "antimicrobial compounds", they are the low molecular-weight and non-protein molecules that are produced by the living microorganisms as secondary metabolites (Sethi *et al.*, 2013; Eman and Noor, 2014). Technically defined, antibiotics incorporate a chemically heterogeneous group of small organic molecules of microbial origin that at lower concentrations are lethal to the growth or metabolic activities of other microorganisms (Ahmed *et al.*, 2013).

In one form or another, they have been in use for centuries, for instance antimicrobial compounds were used therapeutically for the control of infectious diseases (Eman and Noor, 2014).

By providing roadmaps for the biosynthesis of numerous synthetic and semi-synthetic drugs, secondary metabolites carry a significant role in human health. Microbial bioactive products are present as mycotoxins or bacteriosins and are derived from filamentous and non-filamentous bacteria and fungi (Woappi *et al.*, 2013).

In the nature, antibiotics are widely distributed where they play a fundamental role in regulating the microbial population of soil, water, sewage, and compost. At the present several hundred naturally produced antibiotics have been purified, however only a few have been adequately non-toxic to be of use in medical practice (Sethi *et al.*, 2013).

From the past seven decades various infectious diseases have been treated by the antimicrobial agents (Demain and Sanchez, 2009). Antibiotics have been primarily useful in lowering the duration of illness and death caused by the contagious diseases (Girish and Lakshmi, 2017).

1.2 History of antibiotics

In 1928 with the discovery of penicillin by Sir Alexander Fleming, the modern era of antibiotics was started (Piddock, 2012; Sengupta *et al.*, 2013). From that time antibiotics have transformed modern medicine and saved millions of lives (Gould and Bal, 2013) (http://www.cdc.gov/drugresistance/threat-report-2013).

Antibiotics or antimicrobials were the first to be prescribed in the 1940s for the treatment of serious infections (http://www.cdc.gov/drugresistance/threat-report-2013).

Bacterial infections amongst World War II soldiers were successfully controlled by Penicillin (Sengupta *et al.*, 2013). However, once the antibiotic was used commonly, penicillin resistance became a considerable clinical problem, resulting in threatening of many of the advances of the prior decade (Spellberg and Gilbert, 2014).

Later on new beta-lactam antibiotics were discovered, developed, and installed in response to re-establish the confidence (Sengupta *et al.*, 2013; Spellberg and Gilbert, 2014). However, during that same decade, in the United Kingdom and in United States in 1962 and in 1968 respectively, the first case of methicillin resistant *Staphylococcus aureus* (MRSA) was identified (Sengupta *et al.*, 2013)(http://www.cdc.gov/drugresistance/threat-report-2013).

Unluckily, resistance has sooner or later been seen to almost all antibiotics that have been developed (http://www.cdc.gov/drugresistance/threat-report-2013).

Afterwards in 1972 for the treatment of methicillin resistant *S. aureus* coagulase negative staphylococci Vancomycin was introduced, and it was believed that it would be very difficult to induce vancomycin resistance (Sengupta *et al.*, 2013)(http://www.cdc.gov/drugresistance/threat-report-2013).

But unfortunately in 1979 and 1983 the cases were reported in coagulase negative staphylococci of vancomycin resistance (Sengupta *et al.*, 2013).

Later on to resolve the resistance crisis many new antibiotics were introduced by the pharmaceutical industry but after that the antibiotic production began to shrink and as a result fewer new antibiotics were introduced (Spellberg and Gilbert, 2014).

While antibiotic resistance crises continued to extend we no longer had new antibiotics weapons to deal with the threat of resistant microbes, so as result in 2015 the time decades past when initially the antibiotics were introduced to treat the and patients, yet again the bacterial infections became a threat to people lives and wellbeing (Spellberg and Gilbert, 2014).

1.3 Benefits of antibiotics

Human development was revolutionized by the antibiotic drugs in such a way that only some of the other scientific discoveries have. Antibiotics have not only enabled human beings to save lives of patients but they have also played a key role in achieving major advances not just in infections but also in medication and surgical treatment (Gould and Bal, 2013).

Antibiotics have successfully treated and prevented the infections that may occur in:

- Chemotherapy receiving cancer patients
- Rheumatoid arithritis patients
- Diabetic patients
- End stage renal disease patients
- Patients who have had major complicated surgical procedures such as cardiac surgery, joint replacement or organ transpalantation.

(Gould and Bal, 2013; Rossolini *et al.*, 2014; Wright, 2014)

(http://www.cdc.gov/drugresistance/threat-report-2013)

By changing the outcome of bacterial infections the anticipated life spans could be expected to extend through the help of antibiotics (Piddock, 2012; Rossolini *et al.*, 2014).

A Congressional Research Service Report Life expectancy in the U.S. states that the average life span of people in the U.S. is increased from 56.4 years that was in 1920s to almost 80 years now (http://www.cnie.org/nle/crsreports/05mar/RL32792.pdf).

Globally these antimicrobial drugs have had same beneficial effects. In those countries of world which are underdeveloped and have poor sanitation systems, the disease and death rates caused by the food borne pathogens and various other infectious diseases associated to poverty are decreased by the antibiotics (Rossolini *et al.*, 2014).

1.4 Antibiotic resistance crisis

One of the most important public health concerns of the 21st century is microbial resistance to antibiotics (Woolhouse and Farrar, 2014; Laxminarayan *et al.*, 2016).

It is stated by (Roca *et al.*, 2015) that infections caused by the multidrug resistant (MDR) bacteria are considered as a major public health concern and an emergent disease, happening worldwide, by the organizations of the European Centre for Disease Prevention and Control (ECDC), the US Centers for Disease Control and Prevention (CDC), and the World Health Organization (WHO).

No matter what microorganisms adapts themselves to become resistant when we apply any type of selective pressure on them. For instance a national data from U.S. indicates that strains of *Klebsiella* spp., *Pseudomonas aeruginosa,* and *Acinetobacter baumannii* were isolated from the intensive care units (ICU), 10%,20% and 50% of these strains were resistant to carbapenems respectively (Shlaes *et al.*, 2013).

1.5 Causes of antibiotic resistance

1.5.1 Antibiotic overuse

In 2014 it was anticipated that the global consumption of antibiotics was more than 70 billion doses per annum (Van *et al.*, 2014). The progression of resistant bacteria is

undoubtedly driven by the antibiotic overuse (Read and Woods, 2014)(http://www.cdc.gov/drugresistance/threat-report-2013).

Antibiotic resistance is transferred among different bacterial species by the horizontal gene transfer (HGT) and it can also arise through spontaneous mutations (Read and Woods, 2014).As a result of natural selection, resistant strains of bacterial species are left behind to reproduce, whereas the drug sensitive strains or the competitors are removed by the antibiotics(Read and Woods, 2014).

Antibiotics are still overprescribed globally regardless of the warnings concerning antibiotic overuse. In several countries the antibiotic drugs are accessible as over the counter drugs, without any medical recommendation (Michael *et al.*, 2014).

Hence the overuse of antibiotics is promoted by this nonregulation which makes them easily reachable, economical and ample (Michael *et al.*, 2014).

Online purchasing options in the countries where antibiotics are regulated also make them easily accessible (Michael *et al.*, 2014).

1.5.2 Inappropriate prescription of antibiotics

The promotion of resistance in bacteria is associated with the inaccurate prescription of antibiotics (http://www.cdc.gov/drugresistance/threat-report-2013). It is estimated by the studies that 30% to 50% cases of the suggested treatments, choice of antibiotics and the duration of antibiotic dosage is inaccurate, which collectively results in inappropriate prescription (Luyt *et al.*, 2014)(http://www.cdc.gov/drugresistance/threat-report-2013).

There is a reported U.S. study which says that, 17,435 patients were hospitalized due to community acquired pneumonia (CAP), and only 7.65% of those patients were identified with pathogen (Bartlett *et al.*, 2013). Moreover 30% to 60% of the prescribed antibiotics in intensive care units ICUs are inaccurate, redundant or less than optimal (Luyt *et al.*, 2014).

Patients are exposed to the impending complications of antibiotic therapy due to the inaccurate prescription of antibiotics (Lushniak, 2014).

Changes in the genetic expression, mutations and the horizontal gene transfer results in alteration of the genetic makeup of microorganisms to make them resistant, and it is promoted by the concentration of antibiotics which are subtherapeutic and subinhibitory (Viswanathan, 2014). Because of the altered gene expression which is induced by antibiotics, virulence is increased, whereas the antibiotic resistance and its spread are caused by the increase in horizontal gene transfer and mutagenesis (Viswanathan, 2014).

In some strains such as *Pseudomonas aeruginosa* diversification of strains can be found which is attributed to the low levels of antibiotics (Viswanathan, 2014). In *Bacteroides fragilis* extensive proteomic alterations are induced by the tazobactam and piperacillin's subinhibitory concentrations (Viswanathan, 2014).

1.5.3 Extensive use of antibiotics in agriculture

Globally, antibiotics are widely used as growth supplements for farm animals (Bartlett *et al.*, 2013) (http://www.cdc.gov/drugresistance/threat-report-2013). According to an assessment 80% of the antimicrobials sold are used to avoid infections in livestock and to promote their growth (Bartlett *et al.*, 2013; Gross, 2013; Spellberg and Gilbert, 2014). It is said that generally health of the animals could be enhanced; better yields and high quality products can be obtained by the use of antibiotics (Michael *et al.*, 2014).

Antimicrobials are transferred to the human body when they consume food from antibiotic treated livestock (Golkar *et al.*, 2014). 35 years ago this transmission of resistant bacteria was noticed when antimicrobial resistance was found in the intestinal flora of famers and their livestock (Bartlett *et al.*, 2013).

Recently it has been verified through the molecular detection methods that meat products (of the antibiotic treated animals) are also one the sources through which consumers get resistant bacteria (Bartlett *et al.*, 2013). It occurs through the following procedure:

1. Treating farm animals with antimicrobials kills the sensitive bacteria while allows the resistant strains to replicate.
2. Through the food products, these resistant strains are transferred to human beings.
3. Infections are caused in humans by these resistant bacteria.

(http://www.cdc.gov/drugresistance/threat-report-2013).

Microorganisms present in an environment are also affected by the extensive use of antimicrobial compounds in agriculture (http://www.cdc.gov/drugresistance/threat-report-2013) (Bartlett *et al.,* 2013). Livestock excretes 90% of the antibiotics given to them, which is then spreaded through the fertilizers, overland flow and the ground water, in the environment (Bartlett *et al.,* 2013) (http://www.cdc.gov/drugresistance/threat-report-2013). Through this practice, environmental microbiome is exposed to the antimicrobial agents, resulting in the disturbance of ecology of the environment by increasing the percentage of resistant microorganisms from the sensitive strains which are suppressed (Golkar *et al.,* 2014).

1.5.4 Availability of Few New Antibiotics

In the past an approach was developed for the production of new antibiotics, it was effective at combating the resistant microorganisms at that time but it had been slowed down due to certain economic and regulatory issues (Bartlett *et al.,* 2013).

In view of pharmaceutical industries the development of antibiotics is no longer considered as an economically prudent investment (Bartlett *et al.,* 2013).

Antibiotics are not as money making drugs as other ones used for the treatment of chronic conditions for example diabetes, asthma or psychiatric disorders, because antimicrobials are often remedial and are used for comparatively short periods (Piddock, 2012; Gould and Bal, 2013; Bartlett *et al.,* 2013; Gould and Bal, 2013; Golkar *et al.,* 2014). So the medicines that are for chronic conditions have a preference by the pharmaceutical companies for investment as they are more profitable (Gould and Bal, 2013).

Low cost of antibiotics is an additional factor which causes the development of antimicrobials to lack economic appeal (Piddock, 2012; Gould and Bal, 2013; Wright, 2014). Antibiotic's low cost, ease of access and ease of use has resulted in the perception of less valuable among the public (Piddock, 2012).

It is advised by the Microbiologists and the specialists dealing with infectious diseases to restrain the antibiotic use (Piddock, 2012).

To combat serious infections antibiotics are used as the last line drugs, leading to the decrease in new antibiotic use and lessened return from the investment on their development (Piddock, 2012; Gould and Bal, 2013; Golkar *et al.*, 2014).

1.6 Antibiotic resistant bacterial infections

Globally antibiotic resistant infections are prevalent (Golkar *et al.*, 2014). One of the most significant causes of death in humans is infectious diseases (Kraus, 2008). The second most important killer in the world is infectious diseases. Each year 17 million people die from bacterial infections worldwide (Jaroslav *et al.*, 2010).

The rapid emergence of bacteria is described as a calamity or dreadful situation, by various public health organizations, which may perhaps have disastrous consequences (Viswanathan, 2014). In 2013 it was stated that, human beings are in the post antibiotic era, by the CDC, and WHO warned in 2014 that the crisis of antibiotic resistance is becoming terrible (Michael *et al.*, 2014).

Antibiotic resistant bacterial strains were at first detected in hospitals; although, now they are not limited to hospitals only, their occurrence is now more extensively distributed (Jaroslav *et al.*, 2010). In hospitals, e.g., in intensive care units (ICU) resistant bacterial strains can stay alive for a prolonged time and may become a reason of causing epidemics (Jaroslav *et al.*, 2010).

Numerous bacterial strains are causing severe health issues which include: *Enterococcus faecium, Staphylococcus aureus, Acinetobacter baumanni, Streptococcus pneumoniae, Mycobacterium tuberculosis, Salmonella typhimurium, Escherichia coli, Legionella pneumophila, Helicobacter pylori,* etc (Jaroslav *et al.*, 2010).

Gram positive resistant bacterial pathogens such as *S. aureus* and Enterococcus species are the major for human health as they causing deadly diseases globally (Rossolini *et al.*, 2014) (http://www.cdc.gov/drugresistance/threat-report-2013).

Predominantly the gram negative pathogens are more troublesome as they are developing resistance in themselves to almost all of the antimicrobial drugs available nowadays (Golkar *et al.,* 2014; Rossolini *et al.,* 2014) (http://www.cdc.gov/drugresistance/threat-report-2013).

Every field of medicine has been affected by the occurrence of multidrug resistance in gram negative bacilli (Golkar *et al.,* 2014). In communities multidrug resistant gram negative pathogens such as *Escherichia coli* are ever more widespread (Rossolini *et al.,* 2014).

1.6.1 Methicillin resistant *Staphylococcus aureus* (MRSA)

Five decades back MRSA was first identified, and from that time infections caused by MRSA have spread worldwide (Rossolini *et al.,* 2014; Spellberg and Gilbert, 2014). These infections are one of the most frequently occurring and can create very serious antibiotic resistance threats (http://www.cdc.gov/drugresistance/threat-report-2013). MRSA alone is responsible for 11,285 deaths per year in the U.S. (Gross, 2013).

Penicillin-like beta-lactamases are the antimicrobial drugs against which Methicillin-resistant *Staphylococcus aureus* (MRSA) shows resistance, but on the other hand several drugs such as linezolid, daptomycin, ceftobiprole etc still shows antimicrobial activity against MRSA (Sengupta *et al.*, 2013; Rossolini *et al.,* 2014).

1.6.2 Drug Resistant *Streptococcus pneumonia*

This bacterial specie is the cause of meningitis, bacterial pneumonia and sometimes several other acute infections such as ear and sinus infections (Gross, 2013) (http://www.cdc.gov/drugresistance/threat-report-2013). Medical treatment has become even more problematical due to the infections caused by resistant *S. Pneumonia* (http://www.cdc.gov/drugresistance/threat-report-2013). It has affected 1.2 million people, and is a cause of death of 7000 people per year (http://www.cdc.gov/drugresistance/threat-report-2013).

Streptococcus pneumonia has developed resistance against some drugs that are infrequently used, and also against erythromycins and penicillin class drugs (http://www.cdc.gov/drugresistance/threat-report-2013). In most of the cases e.g. 30% of the estimated cases *S. pneumoniae* pathogens are completely resistant to one or more of the antimicrobial compounds that are clinically related (http://www.cdc.gov/drugresistance/threat-report-2013).

1.6.3 Vancomycin Resistant Enterococci

There is a major curative challenge faced by people due to the VRE infections (Sengupta *et al.*, 2013). Hospitalized patients are prone to surgical site, urinary tract infections (UTI) caused by Enterococci (Sengupta *et al.*, 2013) (http://www.cdc.gov/drugresistance/threat-report-2013).

Epidemiological consequences and frequency of VRE is lower than MRSA worldwide (Rossolini *et al.*, 2014). Percentage of vancomycin resistant infections is associated with the type of specie causing it (http://www.cdc.gov/drugresistance/threat-report-2013).

For the treatment of VRE infections only few antibiotics are accessible such as dalfopristin and linezolid etc (Rossolini *et al.*, 2014). That's why these infections are still a major threat, but novel drugs, like oritavancin, could be developed that may have antimicrobial activity against these resistant pathogens (Rossolini *et al.*, 2014).

1.6.4 Multidrug Resistant *Acinetobacter*

A gram negative multidrug resistant (MDR) bacterium, *Acinetobacter,* is the main cause of infections in blood stream and pneumonia (http://www.cdc.gov/drugresistance/threat-report-2013). They are even resistant to "carbapenems", drugs which are considered as last treatment option (http://www.cdc.gov/drugresistance/threat-report-2013).

1.6.5 Drug-Resistant *Mycobacterium Tuberculosis*

Globally, drug resistant Tuberculosis (TB) infections are posing a serious and urgent threat, as it was reported in 2012 by WHO that 170,000 people died from these lethal infections

(Gross, 2013; Sengupta *et al.*, 2013). These infections are usually spreaded through air and frequently appear in lungs but can arise anywhere in the body (http://www.cdc.gov/drugresistance/threat-report-2013).

Lack of new drugs or deficient, inaccurate, and unavailable treatment are the main factors causing drug resistance in Tuberculosis (http://www.cdc.gov/drugresistance/threat-report-2013).

1.7 Antibiotic resistant fungal strains

Antifungal resistance is a broad concept which describes the failure of an infection, caused by fungal pathogens, to act in response to antifungal therapy (Alexander and Perfect, 1997). In pathogenic fungi resistance to antibiotics results in a problem in the control of these fungal infections (Kailash and W. Scott, 2007).

Patients with impaired immunity are susceptible to infections caused by invasive fungal species (Pagano *et al.*, 2006; Sanz *et al.*, 2006). In these types of hosts the spectrum of infections caused by fungal pathogens is raising day by day (Maschmeyer, 2006).

Globally, fungal infections represent a challenging dilemma for clinicians.

Candida specie is the major cause of higher morbidity and mortality rates in the patients with impaired immunity (Wright *et al.*, 1997; Pfaller *et al.*, 1997; Ruhnke, 2007). Candida and Aspergillus species, mutually accounts for 90% of the all nosocomial infections caused by pathogenic fungi (Fridkin and Jarvis, 1996).

Immunocompromised patients and those receiving treatment for cancer and other severe diseases, whose immune system is susceptible, *Cryptococcus neoformans* has became a major fungal pathogen in them (Mitchell and Perfect, 1995). Among several other less common fungi such as *Fusarium* species, *Acremonium* species, *Absidia corymbifera*, and dematiaceous fungi etc are resistant to the antifungal drugs available (Fridkin and Jarvis, 1996).

1.7.1 Candida infections

Candidmia represents the fourth most widespread nosocomial fungal infection in bloodstream, and its rates are increasing globally (Pfaller *et al.*, 2000). In bone marrow transplant recipients that are being treated with flucnazole prophylaxis, emergence of antifungal resistant fungemia has been reported (Marr *et al.*, 1997).

Candida albicans has an ability of forming biofilms on wide range of polymers that are used to develop medical devices e.g. pacemakers, catheters and dental materials etc. (Kojic *et al.*, 2004).

There is a distinctive gene expression pattern of biofilms of this specie (García *et al.*, 2004; Murillo *et al.*, 2005; Yeater *et al.*, 2007) and these biofilms are more resistant to antifungal drugs (Ramage *et al.*, 2002; Kojic *et al.*, 2004).

1.7.2 *Cryptococcus Neoformans* infections

During the past two decades, in general the frequency of cryptococcal infections has increased, due to the higher rates of AIDS, chemotherapy treatments for cancer patients and suppression of immune system in recipients of organ transplants (George *et al.*, 2002). In AIDS patients hardly ever these infections are completely cured and rapid death or failure of treatment can often be seen in immunocompromised patients with neoplatic disease (George *et al.*, 2002). Death rates could be higher such as 25% to 30%, in high risk patients that has been treated for cryptococcal meningitis (George *et al.*, 2002).

At first, for treating cryptococcal meningitis, when flucytosine was used as a solitary agent the resistance was rare (George *et al.*, 2002). But it frequently had developed resistance up to 57% of the cases, which had prohibited its use as a sole agent (Whelan, 1987).

1.7.3 Aspergillus infections

During recent years the incidence of persistent *Aspergillus* infections has increased, by exceeding the frequency of candidiasis, it has achieved higher mortality rates of more than 85% (George *et al.*, 2002). The most common specie for causing aspergillosis globally is *Aspergillus fumigatus*, and it accounts for approximately 90% cases of the *Aspergillus* infections (George *et al.*, 2002).

In bone marrow transplant or solid-organ transplant recipients, and leukemia patients aspergillosis is seen frequently, whereas patients with late or end stage AIDS are subjected to *Aspergillus* infections to a lesser extent (Denning, 1998; Patterson *et al.*, 2000).

Aspergillus species shows resistance to fluconazole (George *et al.*, 2002). In-vitro resistance against itraconazole was also reported by few isolates of *Aspergillus fumigatus* (Chryssanthou, 1997; Dannaoui *et al.*, 1999; Dannaoui *et al.*, 2000; Dannaoui *et al.*, 2001).

1.8 Requirement of novel antimicrobial sources

To surmount the serious crisis of evolving pathogens, resistance bacterial and fungal species and multidrug resistance (MDR) amongst common bacterial strains, there is a continuous requirement of novel antimicrobial compounds (Handelsman, 2005; Alanis, 2005; Sharma *et al.*, 2011).

One of the most important sources of antibiotics is natural products. In the recent past, by using scaffold of natural products, novel antibiotics were developed by adapting some semi-synthetic techniques (Clardy *et al.*, 2006). Natural sources are for the most part unexplored and could serve as a possible source for novel antimicrobial drug discovery (Shah *et al.*, 2017). From microorganisms, natural products up to approximately 50,000 have been discovered (Shah *et al.*, 2017).

More than 10,000 of these are products are reported to have biological activity; moreover roughly 100 microbial products are in today's use as antitumor agents, agrochemicals and antibiotics (Singh and Pelaez, 2008).

Soil is a natural reservoir for microorganisms and an intensively exploited ecological niche, whose inhabitants are capable of producing many useful natural products which

are biologically active, such as clinically important antibiotics (Kumar *et al.,* 2014). Despite that, due to the emerging drug and multidrug resistant in pathogens (Talbot *et al.,* 2006), a continuing search for new antibiotics with effective antipathogenic activity is required (Kumar *et al.,* 2014).

For obtaining novel biologically active antimicrobial compounds, exploring earlier unknown microorganisms is an efficient approach (Fenical *et al.,* 1999).

Development of antibiotics or new drugs is urgently required to control the increase in antibiotic resistant pathogens (Payne *et al.,* 2007; Spellberg *et al.,* 2008; Fischbach and Walsh, 2009) and for the treatment of acute diseases such as cancer (Olano *et al.,* 2009).

Secondary metabolites of microorganisms are rich sources for developing novel therapeutic drugs (Sanglier *et al.,* 1996). From Gram-positive, Gram-negative and filamentous fungal cultures, more than 5000 antibiotics have been identified, however only 100 antibiotics have been commercially used to treat diseases (Bulock and Kristiansen, 1997).

Effective and less toxic antibiotics which can surmount the resistance of MDR pathogens are strongly needed (Kumar *et al.,* 2014). Actinomycetes have provided many important biologically active compounds of high marketable value. Therefore still they are screened continuously for the sake of new antimicrobial bioactive compounds (Kumar *et al.,* 2014).

In soil, bacteria and actinomycetes are the most abundant organisms, but they comprise merely one-third of the soil biomass because of their small size (Lyda, 1981). Many types of novel secondary metabolites including antibiotics can also be obtained from an industrially significant microbial source which is filamentous soil bacteria belonging to genus Streptomyces (Williams *et al.,* 1983a; Crandall and Hamil, 1986; Williams *et al.,* 1989; Korn-Wendisch and Kutzner, 1992).

Actinomycetes bacteria are the source from which 70% of known drugs have been isolated, from these drugs 75% are used in medicine and 60% in agriculture (Miyadoh, 1993; Tanaka and Mura, 1993). 75% of commercially and medically useful antibiotics

are obtained from different Streptomyces species. Streptomyces have provided more than half of the naturally occurring antibiotics discovered to date and still are being explored to be screened for useful compounds (Miyadoh, 1993).

From the past few years, frequency of novel antibiotic compounds is declining, and the findings indicate that the soil microbes had been exhausted or diminished so there is an imperative need to search unexplored resources for microorganisms capable of producing novel antimicrobial compounds (Shah *et al.*, 2017).

One of the most promising ways to isolate new strains of microorganisms endowed with antimicrobial activity is exploring new habitats (Shah *et al.*, 2017). Using folklore or historical records to guide the collection of samples or a good research work on the soil of that area is one of the best approaches to discover novel antibiotics from natural sources (Cordell *et al.*, 1994).

Unexplored and less explored soil habitats have been found to be a rich source of microorganisms that produce novel biologically active metabolites (Baltz, 2008; Meklat *et al.*, 2011; Nachtigall *et al.*, 2011). Acidic soils typically red soils are one such habitat (Xiaoxuan *et al.*, 2015).

1.8.1 Red soil

Red soils are generated by plenteous rainfall and elevated temperature (Xu *et al.*, 2003). These soils are acidic in nature. Red soils been exposed to intensive weathering, are rich in mineral nutrients including calcium (Ca), magnesium (Mg), potassium (K) and phosphorous (P) and have low organic carbon and higher contents of iron oxides (Zhang and He, 2004).

For exploring antimicrobial compound producing microorganisms that are acidophilic and acidotolerant in nature, red soils hence provides a potential source. Even though ammonia oxidizing and sulfate-reducing microbes are reported to be present in such soils (He *et al.*, 2007; Liu *et al.*, 2009), the variety and potential to produce novel bioactive compounds of microorganisms in red soils has hardly been explored.

It was revealed by the anecdotes that red soil has some antibiotic like properties, so this may be a reason that, historically red soil have been used by people to treat skin infections and diaper rash, and still it is used as an easy on the pocket alternative to pharmaceutical products in some communities (Falkinham *et al.*, 2009).

Based on the above considerations, plan of this study is to explore red soil for its antimicrobial activity and to isolate different antibacterial and antifungal compound producing strains and their characterization, for the search of new antibiotics to combat the antibiotic resistance crisis faced by the world.

1.9 Aims and objectives

- Isolation and identification of antibacterial and antifungal compound producing strain from soil sample.
- Characterization of biologically active compounds.

CHAPTER 2
MATERIAL AND METHODS

2 Material and methods

The present research was conducted in Biotechnology research laboratory at Department of Biotechnology and Bioinformatics International Islamic University Islamabad.

Details of materials used and analytical methods employed during the study are given below.

2.1 Sterilization

Glassware used in the whole study such as reagent bottles, conical flasks, beakers, test tubes, micropipette tips, forceps, swabs, filter paper and Petri dishes etc were sterilized every time before use, to prevent contamination. Sterilization was done by autoclaving the wrapped glassware at 121 ° C and 15 psi pressure for 15 to 20 minutes. Media (agar and broth) used in different experiments were also sterilized after preparation. Inoculating loop was sterilized by flame until it become red hot. Working area prior to the experiment was cleaned every time with 70 % ethanol and spirit. Before working in laminar flow hood UV light was switched on for 15 minutes for sterilization of interior. Hands were also sterilized with 70% ethanol or any disinfectant to avoid contamination.

2.2 Chemicals and media

Different types of media were used for the bacterial and fungal growth in the whole experiment. For bacterial growth, Nutriet Agar (NA), MacConkey Agar (MA) and Tryptone Soya Agar (TSA) were used while Sabouraud Dextrose Agar (SDA) and Potato Dextrose Agar were used. Different liquid media such as Nutrient Broth (NB), Tryptone soya Broth (TSB) and Brain Heart Infusion Broth (BHIB) were used for bacterial growth.

Distilled water was always used for the preparation of solid and liquid media. And sample dilutions were prepared by using double distilled water (ddH_2O).

Others chemicals included: 80% acetone, ethyl acetate, Sodium chloride (NaCl), glycerol, copper sulphate, sodium sulphate, hydrogen peroxide, 70% ethanol etc.

2.3 Methodolgy

2.3.1 Collection of soil sample

Red soil samples were collected from selected locations of Kashmir region of Northwestern Himalaya. After removal of approx 3 cm of the soil surface which is mostly debris, soil samples up to the depth of 15 cm to 20 cm were collected in sterile polythene bags and transferred to the laboratory for further procedures. Red soil samples were air dried for 3 to 4 hours, crushed, and sieved prior to use for isolation of microbial strains (Saadoun *et al.*, 1999).

2.3.2 Isolation of bacterial and fungal stains

Microbial strains were isolated by the serial dilution method. One gram of dried soil was weighed and added to 9ml of double distilled water (dd H_2O) in a sterile test tube and shaken well using vortex mixer, this stock solution was then diluted serially up to the dilution of 10^{-5}

At first three types of different media were used for the isolation of bacterial strains from the soil sample named as Nutrient agar (NA), Tryptone soya agar (TSA), and MacConkey agar (MA). And two different media including Sabouraud dextrose agar (SDA) and Potato dextrose agar (PDA) were used for the fungal strains. For the preparation of 500 ml nutrient agar, 14 gm of nutrient agar was added into a reagent bottle having 500 ml of distilled water in it. Agar was dissolved using magnetic stirrer and then sterilized by autoclaving for 15 min at 121° C temperature and 15 psi pressure. Likewise for preparing 500 ml of TSA and MacConkey agar, 20 gm and 26 gm of agar were added into 500 ml of distilled water respectively and same process

was repeated. Same procedure was used for the fungal media preparation only the quantity of the agar was different. After sterilization, media was poured into labeled and sterile Petri dishes.

Spread plate technique was used for the inoculation of sample and strain isolation. From each dilution, 100µl of the aliquot was taken with the help of micropipette and spreaded over the surface of different media, with the help of a glass spreader which was sterilized by dipping in ethanol and then by flaming over the spirit lamp. Plates were incubated at 37°C for 24 to 48 hrs for bacteria and for 48 to 72hrs for fungus.

2.3.3 Colony counting

After incubation the number of colonies present in each Petri dish was calculated by using colony counter. Number of colonies present, determines number of cells present in the dilution.

2.3.4 Purification of isolates

After incubation the morphologically distinct colonies were selected and sub-cultured onto sterile nutrient agar plates, and purified by repeated streaking. Slants were prepared of the isolated strains and stored at 4° C for further use.

2.3.5 Preliminary screening of isolated strains for antibiotic production

Isolated strains expected to be the antibiotic producers were screened for their antimicrobial spectrum. Test organisms used for preliminary screening were isolated from the environment and were suspected to be sensitive strains.

Bacterial strains isolated from the soil samples were inoculated in nutrient broth (NB), tryptone soya broth (TSB) and brain heart infusion broth (BHIB) while the fungal strains were inoculated in Sabouraud dextrose broth (STB) and then incubated at 37° C for 48hrs and 72 hrs for bacteria and fungus respectively. Agar well diffusion method was used to assess their capability of producing antibiotic metabolites. For antibacterial activity a lawn of test bacterial strain was spreaded

over the surface of nutrient agar plates by using sterile swabs, and a hole was punched aseptically, with the help of sterile tip, in the center of agar plate. In the case of fungus, sensitive fungal strains were swabbed on the sabouraud dextrose agar plates and same process was repeated. A volume of 100µl of the bacterial and fungal inoculums, grown in broth, was taken with the help of micropipette and introduced in the well of agar plates. Inoculated agar plates were then incubated at 37° C for 24 & 48 hrs, for bacterial and fungal plates respectively. After incubation zone of inhibition was observed.

Secondary screening of the isolates which showed positive results was done against known bacterial and fungal test organisms.

2.3.6 Identification and characterization of bacterial isolate

After preliminary screening, the suspected antibiotic producer isolate was identified and characterized by morphological, cultural and biochemical tests.

For preliminary identification of bacterial strain gram staining was done. Morphological identification of the selected isolate was done by observing color, shape, size and appearance of the colonies of the bacterial isolate grown on agar plate. Odor of the colonies grown on the agar plate and pigment produced (if any) were also observed. While certain biochemical tests were also performed including catalase test, oxidase test.

Isolated strain was also grown on MacConkey agar which is a selective media helpful in identification of gram negative rods. On the basis of appearance of the strains growing on it, this agar also differentiates lactose fermenting and lactose non fermenting strain. Lactose fermentor colonies appear pink on the agar, while non lactose fermentor colonies are colorless.

2.3.6.1 Gram's staining

The first step in gram's staining is slide preparation and then smear fixation by quickly passing the slide three times in the flame. After that crystal violet was applied on the smear for 1 min and then slide was washed with distilled water by keeping it tilted at an angle of 45°. Then gram's iodine was applied and washed after 1 min. Decolourizer i.e. 95% ethyl alcohol was applied for 10 sec, and slide was again washed. At the end slide was counterstained with safranine for 30 sec and washed. Finally slide was blot dried and examined under light microscope.

2.3.6.2 Catalase test

This test is usually performed to differentiate the bacterial isolates on the basis of catalase production. On a clean slide a single colony of the bacterial isolate was transferred with the help of a sterile loop and then 3% hydrogen peroxide solution was prepared that was poured over the colony. Gas production was observed i.e. bubbling. If bubbling occurs it means the strain is catalase positive (Collins and Lyne, 1980).

2.3.6.3 Oxidase test

This test is used to differentiate the bacteria that produce an enzyme "cytochrome c oxidase". A filter paper drenched with reagent tetramethyl-p-phenylenediamine dihydrochloride was taken, and the bacterial colony to be tested was smeared on it. For 10 to 30 seconds change in color was observed. If the enzyme would be produced by the isolate, purple color appears on the paper where colony was inoculated that indicates oxidase positive result. On the other hand filter paper color remains unchanged if the enzyme is absent.

2.3.7.4 API 20 NE

Standardized identification of the selected strain was performed by using API 20 NE kit. API 20 NE is a standardized system for the identification of non-fastidious, non-enteric Gram-negative rods, combining 8 conventional tests and 12 assimilation

tests. The API 20 NE strip consists of 20 microtubes containing dehydrated substrates. The conventional tests are inoculated with a saline bacterial suspension which reconstitutes the media. During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents. The assimilation tests are inoculated with a minimal medium and the bacteria grow if they are capable of utilizing the corresponding substrate.

Suspension of the test organism to be identified was made in 2ml sterile saline. First eight cupules from NO_3 to PNPG were inoculated from the saline suspension. An API AUX ampoule which is provided with the kit was opened and 4 drops (200µl) of the saline suspension was added to it. Remainder of the cupules was inoculated with this suspension, and then the strip was incubated for 24hrs. Results were recorded on the basis of colour change after incubation.

2.3.7 Test microorganisms

Different clinical strains of bacteria and fungi were used as test strains for the secondary screening of selected isolate for evaluation of antibiotic production. Test bacterial organisms were collected from the microbiology laboratories of Pakistan Institute of Medical Sciences (PIMS), Armed Forces Institute of Pathology (AFIP), Rawalpindi Institute of Cardiology (RIC) and Bilal Hospital Rawalpindi, while fungal strains were collected from National Agriculture and Research Center (NARC).

Test bacterial strains included: *Escherichia coli, Staphylococcus aureus, Shigella flexneri, Klebsiella pneumonia, Enterobacter aerogenes, Lactobacillus acidophilus, Staphylococcus epidermidis, Salmonella typhi, Listeria monocytogenes* and *Bacillus subtilis.*

Test fungal strains included: *Aspergillus niger, Aspergillus fumigatus, Candida albicans* and *Trichophyton tonsurans.*

2.3.8 Secondary screening for antimicrobial activity

Bacterial isolates from soil sample which showed positive results in preliminary screening for antibiotic production were then screened for antibacterial and antifungal activities against test strains mention above. Through agar well diffusion method, antibacterial and antifungal activities were assessed and zone of inhibitions were measured in millimeters (mm). Standard was set according to which Sensitivity patterns of the isolate were evaluated.

For antibacterial activity:

Non sensitive (-): For total diameter smaller than 12 mm

Sensitive (+): For total diameter between 12-25 mm

Very sensitive (++): For total diameter between 26-38 mm

Extremely sensitive (+++): For total diameter larger than 38mm

For antifungal activity:

Non sensitive (-): For total diameter smaller than 10 mm

Sensitive (+): For total diameter between 11-20 mm

Very sensitive (++): For total diameter between 21-30 mm

Extremely sensitive (+++): For total diameter larger than 31mm

2.3.9 Culture media optimization

Before extraction of the bioactive compounds from the bacterial strains, selected through secondary screening, the optimization of culture media was done by using different chemicals such as Copper sulphate ($CuSo_4$), Sodium sulphate (Na_2So_4) and Glycerol.

25 ml nutrient agar was prepared in 4 volumes in 100ml conical flasks, 2mM of the $CuSo_4$, Na_2So_4 and Glycerol were added separately in each flask respectively and one flask was taken as control which contained nutrient agar only. These differently supplemented nutrient agar media were poured into labeled petri dishes, media was allowed to set and then bacterial isolates were inoculated into the culture media and

plates were incubated at 37°C for 24hrs. After incubation the difference in growth of the isolates was observed as compared to the control. And the media which supported the maximum growth was selected for further process.

2.3.10 Solid state fermentation for the preparation of crude extract

Extraction of the bioactive compound produced by the bacterial isolate which showed antimicrobial activity was achieved through solid state fermentation. Seed media used for the bulk growth of bacterial isolate in solid state fermentation was selected after testing various media and the best one was chosen to achieve maximum yield of desired antimicrobial product. 800ml of supplemented nutrient agar was prepared and poured into petri plates, promising bacterial strains were inoculated and plates were incubated at 37°C for 5 to 7 days for maximum growth. 600ml of brain heat infusion broth was also prepared in conical flasks and after inoculation with the bacterial strains; broth was also incubated at same conditions.

After 7 days incubation when maximum growth of the isolates was achieved, the culture media from all the petri dishes was transferred into 1000ml beakers and was chopped in to very small pieces with the help of a spatula. Later on the crushed media was moved into the conical flasks and 10 grams of sodium chloride (NaCl) was added in each conical flask, 80% acetone was prepared, and added in a volume such that the crumbled media were submerged in it. Brain heart infusion broth after incubation was also added in it. Conical flasks were placed on rotary shaker for 24hrs and were checked in intervals to avoid the chances of spillage.

The active metabolite was separated from the solid residues by filtration of the acetone and fermented culture mixture by using Whatman No.1 filter paper. Antibiotics are produced extracellularly so the supernatant filtrate was collected in a conical flask, an equal volume of ethyl acetate was added in it, and was vigorously shaken. To isolate the active substances from the fermented culture supernatant solvent extraction technique was used. The Sample was transferred into the separating funnels in three batches, and was allowed to stand for 15 to 30 minutes, until two layers were clearly visible. One of the layers was aqueous and other was

organic layer. Both of the layers were collected separately in beakers. The organic layer or the solvent phase was evaporated at room temperature and finally the dried residues were dissolved in Dimethyl Sulfoxide (DMSO). The crude extracts obtained from the isolates were stored at 4°C temperature, and then were used for further studies (Lakshmipatipathy and Krishnan, 2010; Raja and Prabakaran, 2011).

2.3.11 Determination of antibacterial activity of the crude extract

Antibacterial activity of the crude extracts of the antimicrobial compound was determined by the Kirby Bauer disc diffusion method. DMSO was used as a control and test organisms against which this activity was performed are described earlier. Nutrient agar media was used and lawn of bacterial test strains were inoculated into the agar plates by using sterile swabs. Sterile filter paper disc of about 6mm diameter which contained 10-20µL of crude extract was placed on the agar plate, with one disc containing DMSO as a control and plates were incubated at 37°C for 24hrs. After incubation the zone of clearance were measured in mm and recorded. Standard was set according to which Sensitivity patterns of the crude extract were evaluated.

Non sensitive (-): For total diameter smaller than 10 mm

Sensitive (+): For total diameter between 11-20 mm

Very sensitive (++): For total diameter between 22-30 mm

Extremely sensitive (+++): For total diameter larger than 31mm

2.3.12 Statistical analysis

The data obtained was statistically analyzed by using student's t test.

CHAPTER 3
RESULTS

3 Results

In the present study area selected for sampling was Northwestern Himalayas of the Kashmir region (Fig.3.1). This area is unexplored but acquires a great biodiversity. From different localities ten different red soil samples (Table 3.1) were collected and evaluated for the isolation of bacterial and fungal strains with antibiotic producing potential.

3.1 Isolation of bacterial and fungal strains

All of the collected samples (Fig. 3.2) were analyzed by serial dilution method; Fig. 3.3 is showing the dilutions of the sample prepared from stock solution. After incubation of the Petri plates by following the spread plate technique, numerous isolated microbial colonies were observed distributed evenly on the surface of agar plate (Fig 3.4). Morphologically distinct colonies were selected and subjected for the evaluation of antibacterial and antifungal activity. A total of 21 morphologically different bacterial strains, and 2 fungal strains were isolated from the soil samples mentioned above. The bacterial isolates were named as S1, S2, S3, S4... S20 and S21, while the fungal isolates were named as T1 and T2.

3.2 Colony counting

Colonies on all the plates were calculated with the help of colony counter. Colony forming unit (CFU) is the measure of the viable bacterial cells. The formula used to determine CFU/ml is:

$$\text{CFU/ml} = (\text{number of colonies} \times \text{dilution factor}) / \text{volume of the culture plate}$$

CFU was calculated with this formula and total bacterial counts were determined which are shown in Table 3.1

Fig. 3.1 Sampling area. Red soil of Kashmir region.

Fig.3.2. Red soil sample

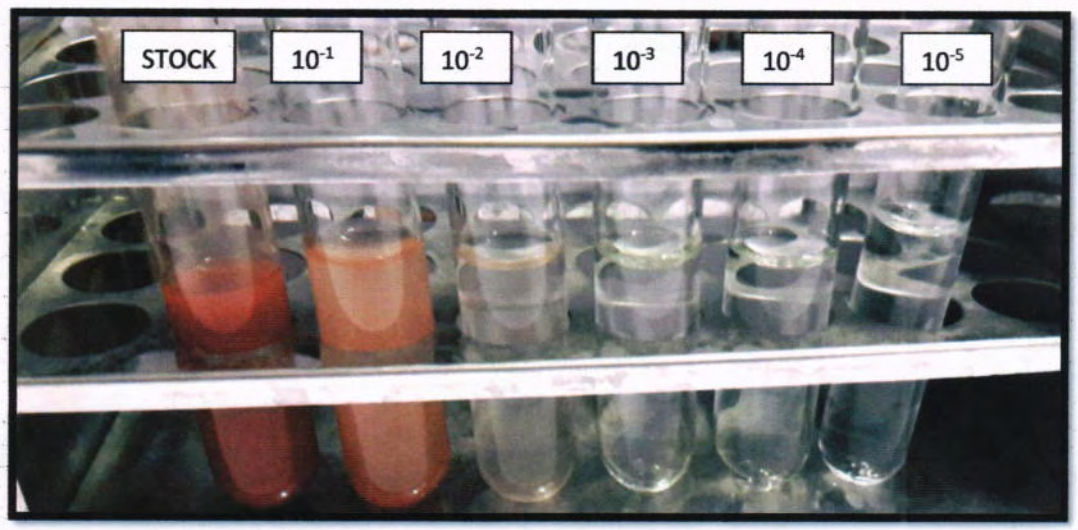

Fig. 3.3: Serial dilution of soil sample.

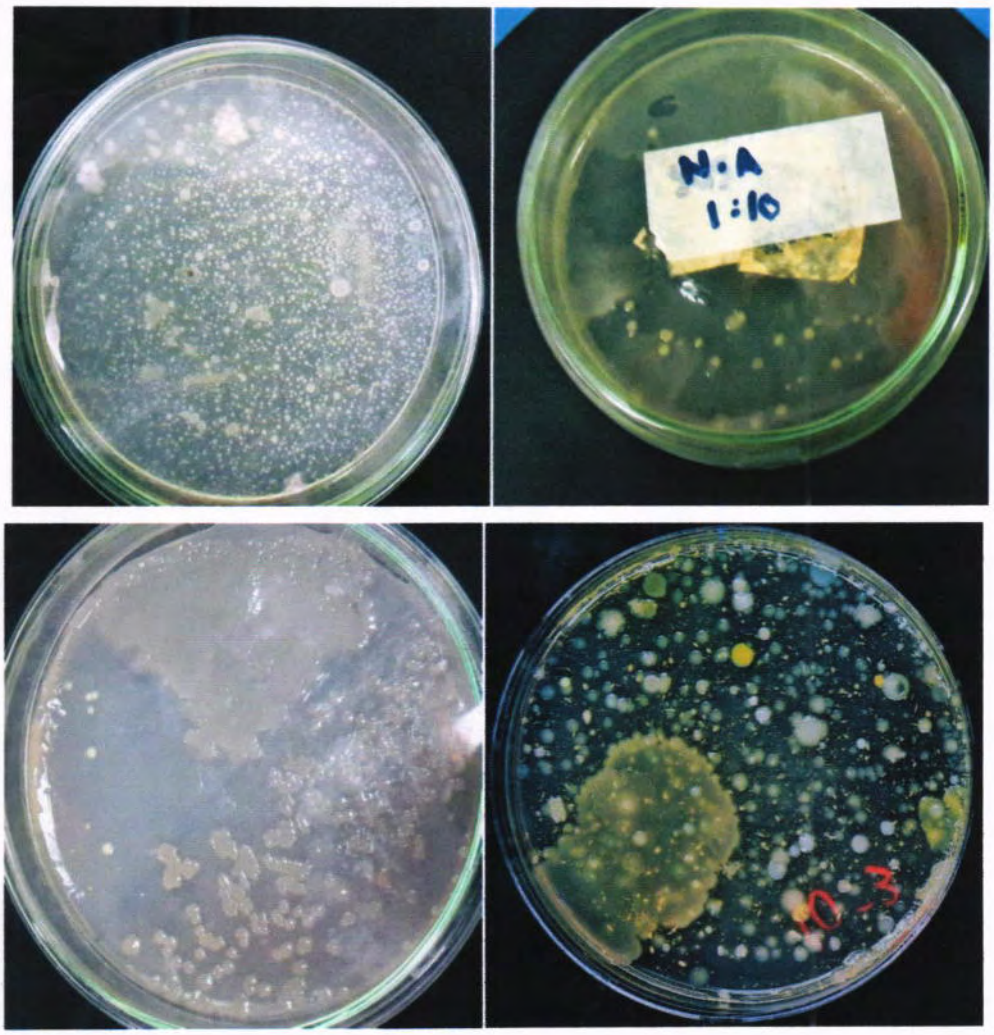

Fig. 3.4. Colonial morphology of the bacterial strains isolated from collected soil sample showing morphologically distinct colonies.

Table 3.1: Samples collected from Kashmir region of the Himalayas and total bacterial count.

Sample codes	Sampling area	Bacterial counts (CFU/ml)
H1	Kotli	1.5×10^5
H2	Hajira	8.1×10^4
H3	Muzafarabad	2.1×10^5
H4	Narh	4.9×10^5
H5	Abbotabad	3.8×10^2
H6	Kohala	2.5×10^5
H7	Thorar	5×10^2
H8	Hattian bala	3.8×10^3
H9	Sudhanoti	7.4×10^5
H10	Khuiratta	6.2×10^4

3.3 Preliminary screening of isolated strains for antibiotic production

After incubation 3 morphologically different bacterial strains and 3 fungal strains, suspected to be sensitive strains, were picked from the agar plates that were exposed to environment. The bacterial strains were named as B1, B2 and B3 while the fungal strains were named as F1, F2 and F3.

Bacterial and fungal strains that were isolated from the soil samples were then subjected to the preliminary screening for antimicrobial activity against the different isolated environmental bacterial and fungal strains. Out of these 21 bacterial isolates only two strains named as S2 and S17 produced zones of inhibition against isolated bacterial and fungal strains (Fig. 3.5(a), 3.5(b), 3.5(c), 3.5(d), 3.6(a), 3.6(b) and 3.6(c)). S2 inhibited the activity of only one bacterial strain while S17 was active against all of the test strains including the fungal and bacterial strains. On the other hand none of the fungal test strain was inhibited by S2.Diameter of inhibition against these strains is shown in Table 3.2. On the basis of high spectrum of activity, against both fungal and bacterial test strains, and larger zone of inhibition S17 isolate was selected for identification and secondary screening against known test strains. Graphical representation of the data is shown in Fig. 3.7.

While both fungal strains (T1 and T2) isolated from the soil sample did not inhibit any of the test strain.

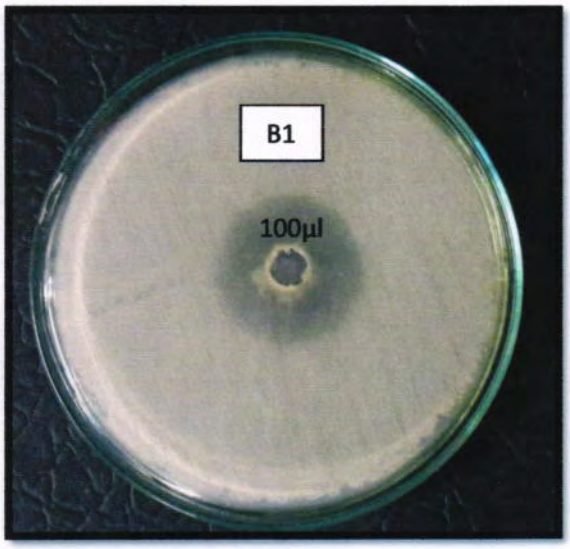

Fig. 3.5(a) Antibacterial activity of S2 isolate against B1

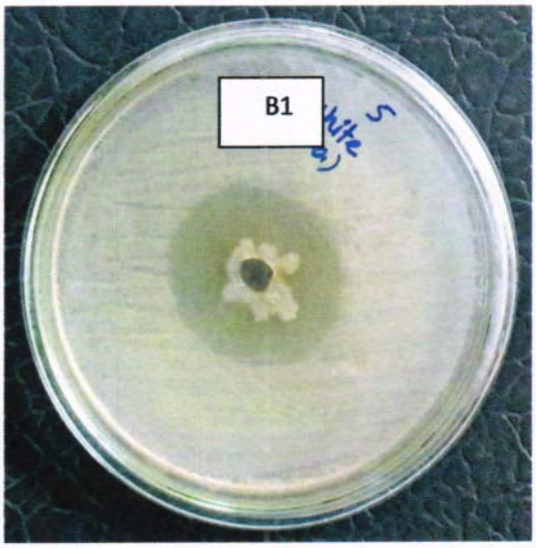

Fig. 3.5(b) Antibacterial activity of S17 isolate against B1

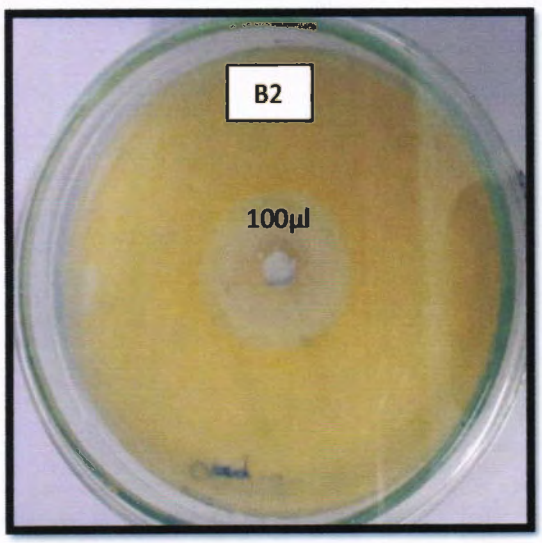

Fig. 3.5(c) Antibacterial activity of S17 isolate against B2

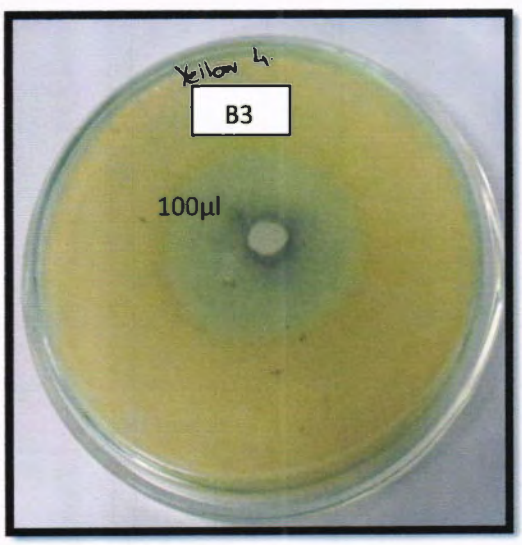

Fig. 3.5(d) Antibacterial activity of S17 isolate against B3

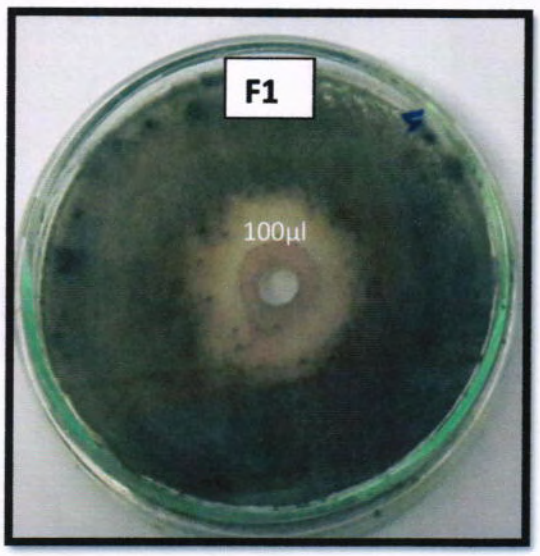

Fig. 3.6(a): Antifungal activity of S17 against F1

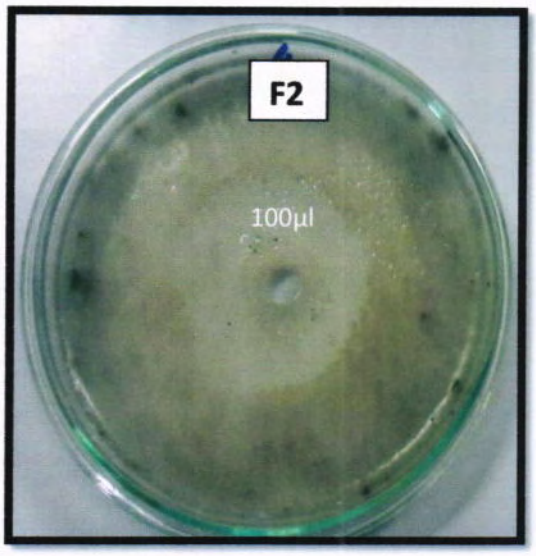

Fig. 3.6(b): Antifungal activity of S17against F2

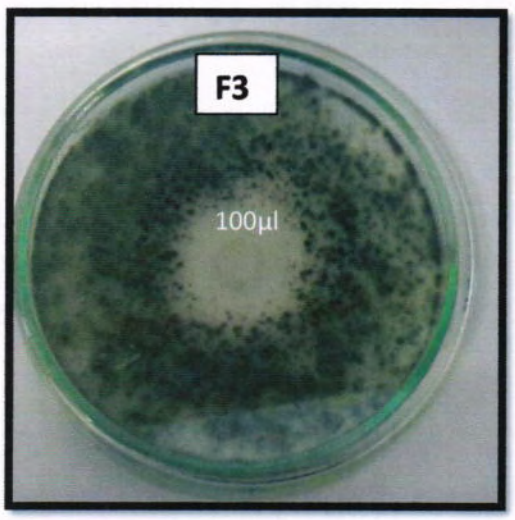

Fig. 3.6(c) Antifungal activity of S17 isolate against F3

Table 3.2: Inhibition diameter of isolates S2 and S17 against isolated bacterial strains

Test strain	Gram stain	Shape	Isolate S2	Isolate S17
B1	Gram positive	Cocci	21mm	29mm
B2	Gram positive	Rod	-	26mm
B3	Gram positive	Cocci	-	39.5mm

mm = milli meter
- = no inhibition

Table 3.3: Antifungal activity of isolates S2 and S17 against isolated fungal strains

Test strain	Morphology	Isolate S2	Isolate S17
F1	Aspergillus spp	-	25mm
F2	Penicillium spp	-	32mm
F3	Aspergillus spp	-	22mm

mm = milli meter
- = no inhibition

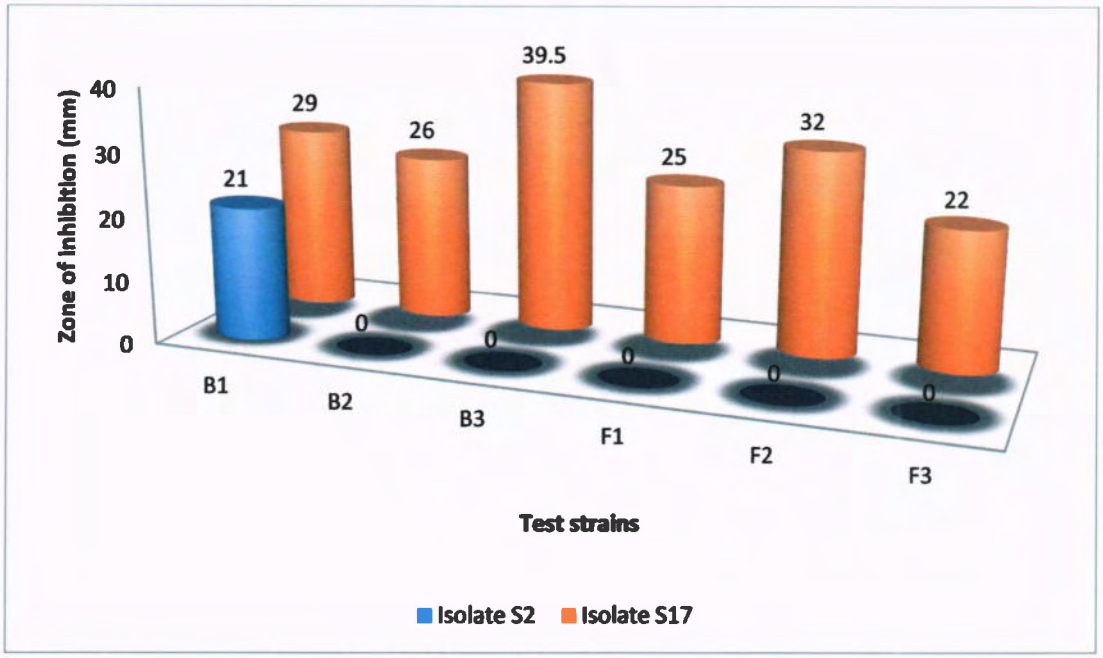

Fig. 3.7: Preliminary screening of selected isolates (S2 and S17) for antibacterial and antifungal activity against isolated test strains

3.4 Identification and characterization of bacterial isolate

For the identification of the isolate, after incubation and appearance of growth on petri plates, careful examinations of colony morphology was done to note colony shape, color, size, texture, elevation and margins. Colonies grown on the agar plate were smooth, spherical and medium in size with wavy margins. Texture of the colonies was mucoid with a fruity odor. Colonies of the isolate also produced a blue-green pigment which was diffusible in the agar (Fig. 3.8). Colonies of the isolated strain grown on the differential media, MacConkey agar, were colorless and no change in the media around colonies was observed, indicating that the strain was non lactose fermentor (Fig. 3.9).

Morphological characteristics of the isolate S17 are shown in the Table. 3.4

3.4.1 Gram's stain

This technique differentiates two large groups of bacteria by the differences in their cell wall composition. Gram positive bacteria are stained purple due to the thick layer of peptidoglycan in their cell wall, while gram negative bacteria doesn't retain crystal violet dye, due to thinner peptidoglycan layer in their cell walls, and are stained pink.

Bacterial isolate S17 was when observed under 100X lens of microscope after gram's staining, it was stained gram negative and pink rods were observed, shown in Fig. 3.10(a) and Fig. 3.10(b)

3.4.2 Catalase test

Isolated strain S17 showed a positive behavior during catalase test i.e. bubble formation when treated with 3% of H_2O_2 (Fig. 3.11)

3.4.3 Oxidase test

When tetramethyl-p-phenylenediamine dihydrochloride soaked filter paper was smeared with the isolated strain, change in color was observed. Purple color on filter paper indicated that the isolated strain was oxidase positive (Fig. 3.12).

3.4.4 API 20 NE

Standardized identification of the strain S17 was performed by using API 20 NE test kits. Results were recorded on the basis of color change after 24hrs of incubation shown in Fig.3.13. According to the results obtained, tests for gelatin hydrolysis, glucose assimilation, mannitol assimilation, N acetyl glucose amine assimilation, gluconate assimilation etc were positive while tests for glucose fermentation, arginine hydrolysis, urea hydrolysis, maltose assimilation etc were negative as shown in Table. 3.15.

Test results showed 99.9% homology with *Pseudomonas aeruginosa.*

Table 3.4: Colonial morphology of the S17 strain

Colony shape	Spherical
Colony size	Small to medium
Texture	Mucoid
Elevations	Umbonate
Margins	Wavy
Odor	Fruity
Pigment	Blue- green pigment production
Growth on MacConkey agar	Colorless colonies -Non lactose fermentor
Gram stain reaction	Gram positive
Gram stain arrangement	Rods
Catalase	Positive
Oxidase	Positive

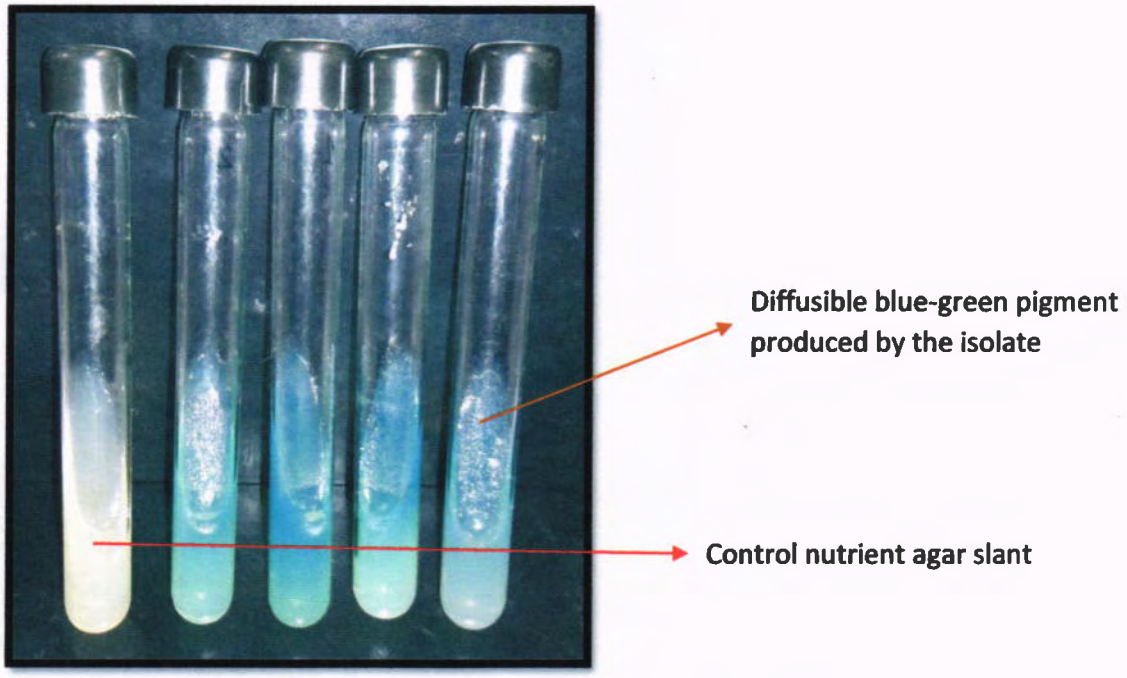

Fig. 3.8: Nutrient agar slants sowing blue-green pigment produced by the S17 strain

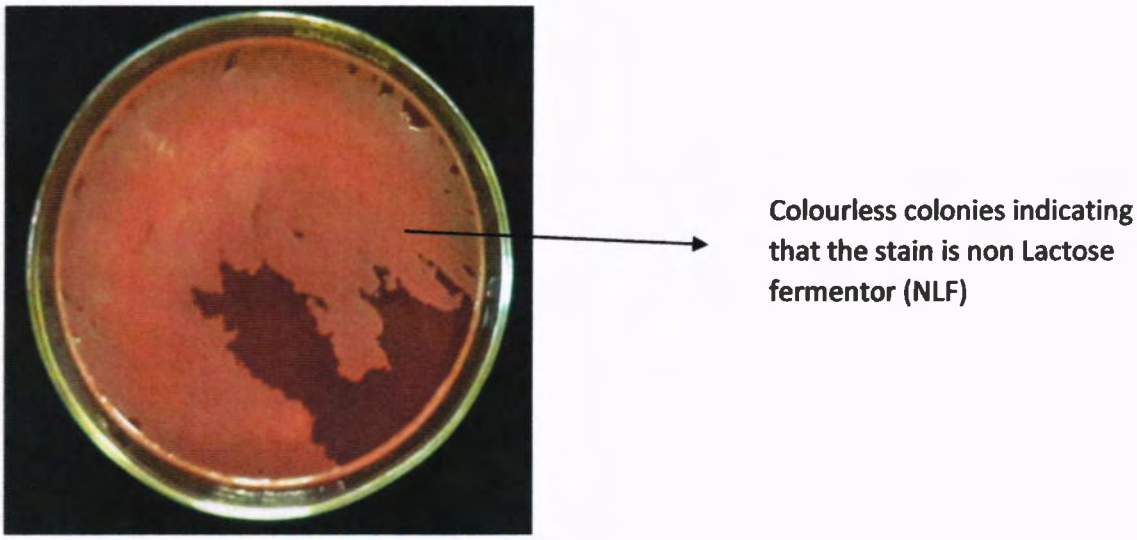

Fig. 3.9: S17 Isolate grown on MacConkey Agar

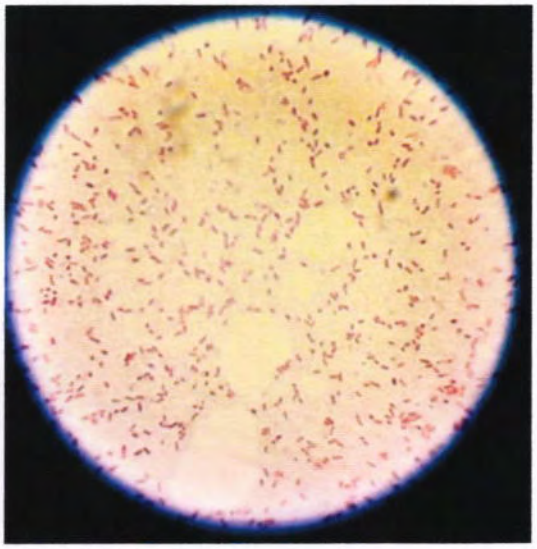

Fig. 3.10(a): Gram stain of isolate showing gram negative rods

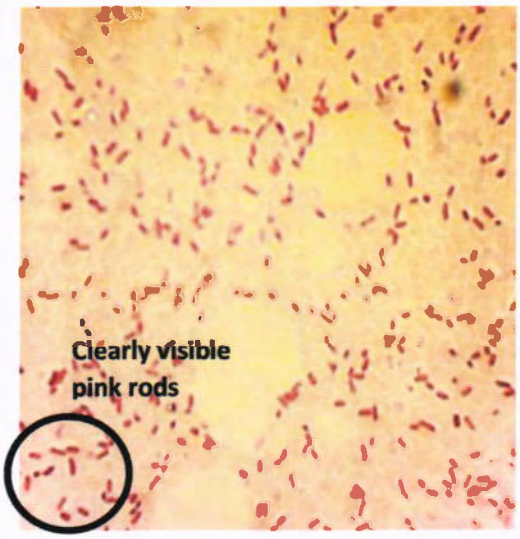

Fig. 3.10(b): Magnified picture of gram stain

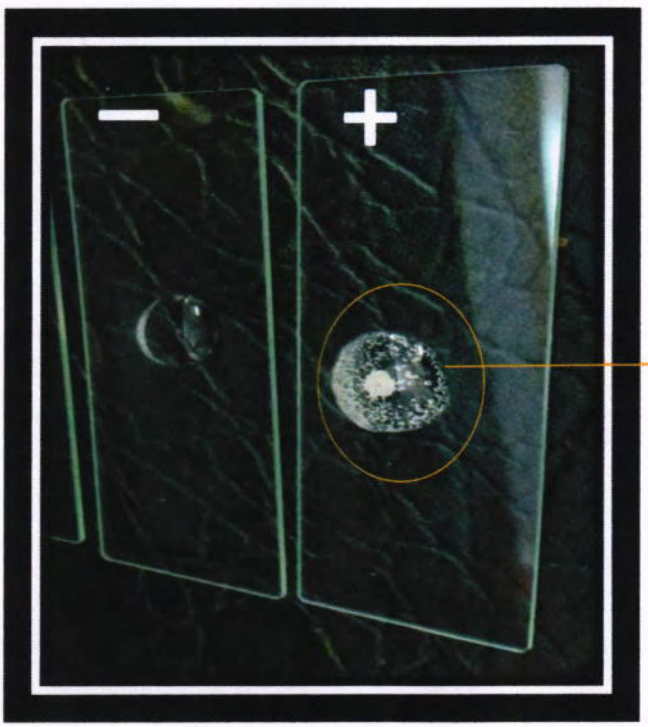

Fig. 3.11: Isolate S17 showing positive behavior during catalase test

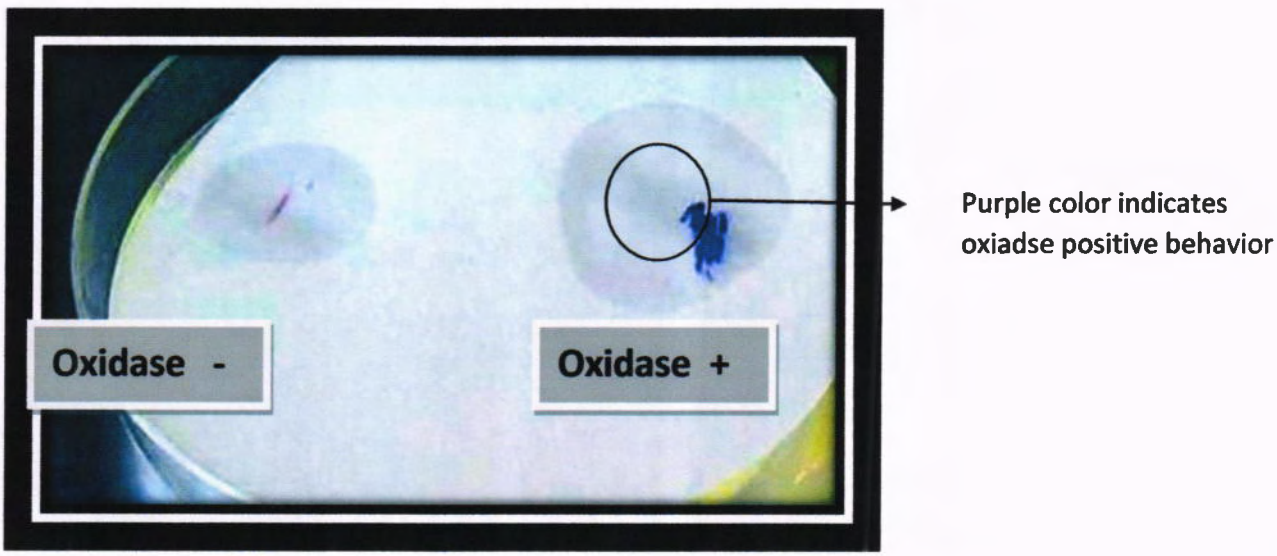

Fig. 3.12: Change in color by S17 strain indicating oxidase positive result

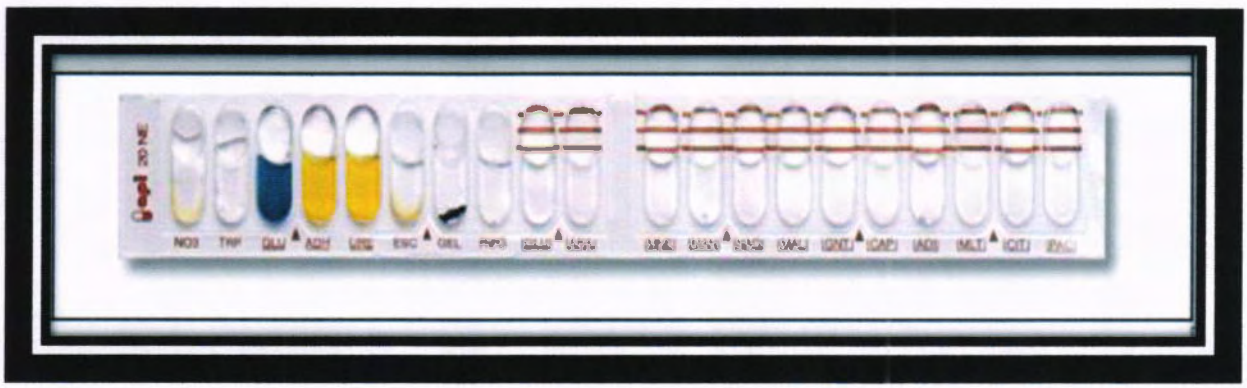

Fig. 3.13(a): API 20NE kit before adding reagents

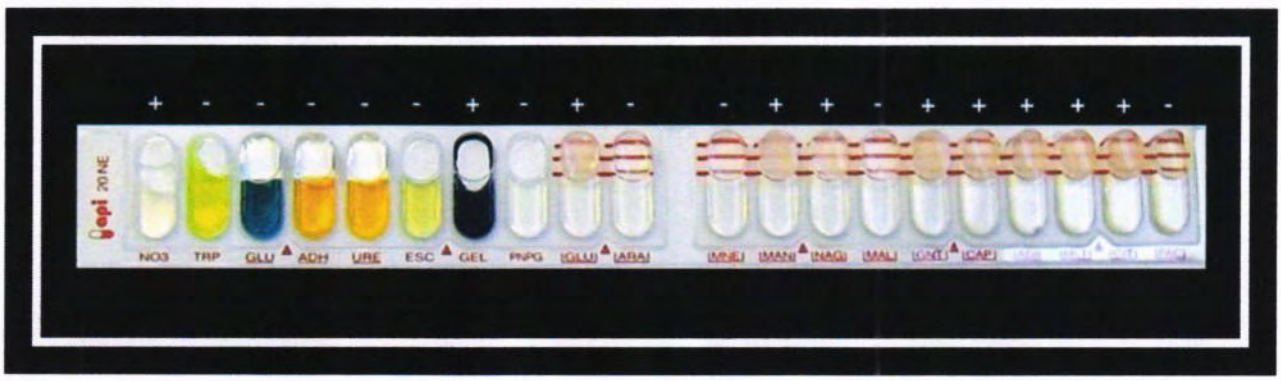

Fig. 3.13(b): API kit results after incubation of 24hrs

Table 3.5: Results of API 20NE

S. No	Test	Reaction	Result
1	$NO_3 \rightarrow NO_2$ $NO_2 \rightarrow N_2$	Reduction of potassium nitrate	+
2	TRP	Indole production from tryptophan	-
3	GLU	Glucose fermentation	-
4	ADH	Arginine hydrolysis	-
5	URE	Urea hydrolysis	-
6	ESC	Aesculin hydrolysis	-
7	GEL	Gelatin hydrolysis	+
8	PNPG	p-nitrophenyl-βD-galactopyranoside hydrolysis	–
9	GLU	Glucose assimilation	+
10	ARA	Arabinose assimilation	-
11	MNE	Mannose assimilation	-
12	MAN	Mannitol assimilation	+
13	NAG	N-acetyl-glucosamine assimilation	+
14	MAL	Maltose assimilation	-
15	GNT	Gluconate assimilation	+
16	CAP	Caprate assimilation	+

17	ADI	Adipate assimilation	+
18	MLT	Malate assimilation	+
19	CIT	Citrate assimilation	+
20	PAC	Phenyl-acetate assimilation	-
21	Oxidase	Cytochrome oxidase	+

+ = positive

- = negative

3.5 Secondary screening for antimicrobial activity

The isolate selected by preliminary screening identified as *Pseudomonas aeruginosa* was evaluated for antimicrobial activity against known clinical bacterial and fungal strains. The antibiotic producing ability of *P. aeruginosa* was tested against 5 gram positive and 5 gram negative bacterial strains via agar well diffusion method. Gram negative strains including *Shigella flexneri, Salmonella typhi, Escherichia coli, Klebsiella pneumonia* and gram positive strains of *Bacillus subtilis, Lactobacillus acidophilus* and *Listeria monocytogenes* showed very weak zone of inhibition, so according to the sensitivity patteren they were non-sensitive to the bacterial strain S17. *Staphylococcus epidermidis* showed sensitivity to the isolated strain, with a clearance zone of 22mm in diameter (Fig. 3.14(a)). Intermediate sensitivity was observed in case of gram negative *Enterobacter aerogenes,* which was inhibited by the antibiotic producer strain, with a zone of diameter 26mm (Fig. 3.14(b)). While extreme sensitivity was shown by Gram positive *Stapylcoocus aureus* and its zone of clearance was calculated to be 42mm (Fig. 3.14(c)). Table 3.6 demonstrates the sensitivity patterns and zone of exhibition of the strains. Fig. 3.15 shows the graphical representation of the antibacterial activity of *P. aeruginosa.*

Antifungal activity of *P. aeruginosa* was assessed against four different fungal strains among which *Aspergillus fumigatus* was extremely sensitive to the isolate with 32mm zone of clearance (Fig. 3.16(a)), while *Candida albicans* was also sensitive to it and its zone of diameter was 29mm (Fig. 3.16(b)). While *Trichophyton tonsurans* and *Aspergillus niger* were resistant to the isolate with no zone of clearance. Table 3.7 indicates the inhibition zones by the antifungal activity of isolated strain. Graphical representation of the data is shown in Fig. 3.17.

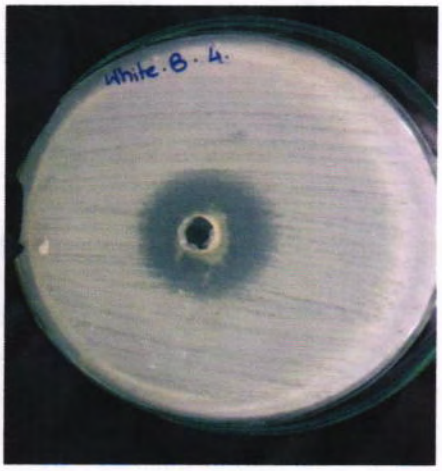

Fig. 3.14(a): Zone of inhibiton against *Staphylococcus epidemidis*

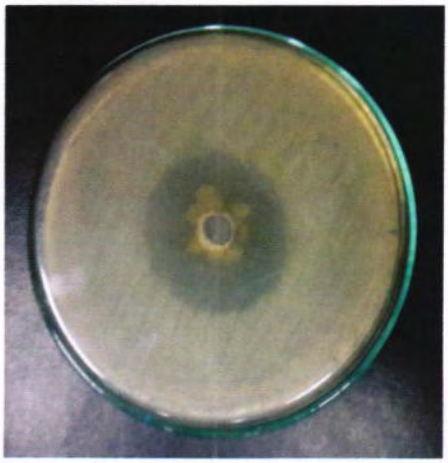

Fig. 3.14(b): Zone of inhibiton against *Enterobacter aerogenes*

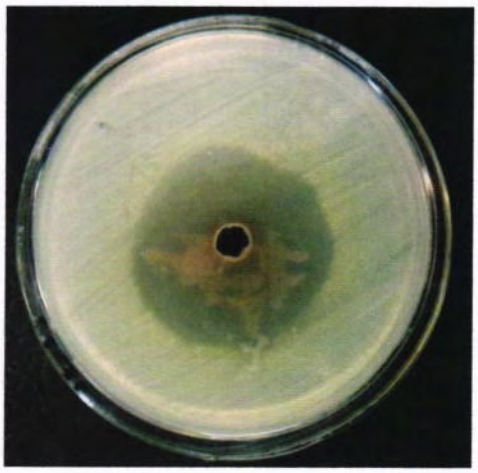

Fig. 3.14(c): Zone of inhibiton against *Staphylococcus aureus*

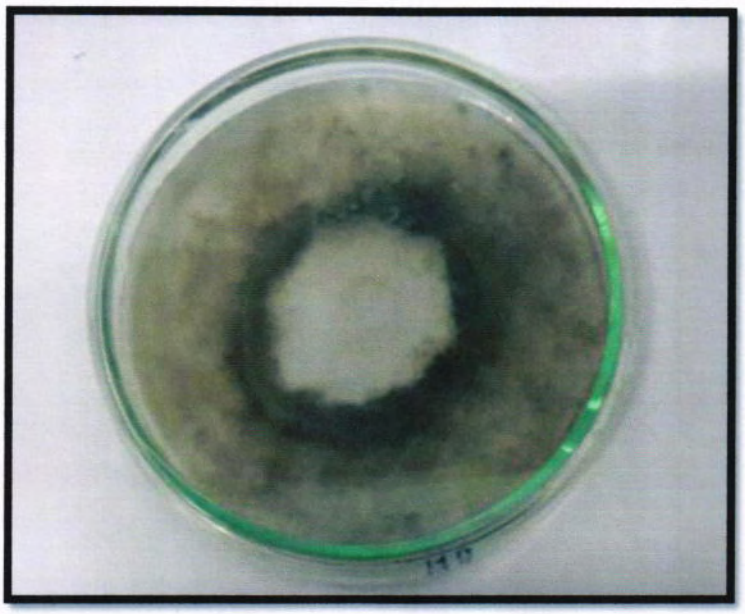

Fig. 3.16(a): Antifungal activity of *P. aeruginosa* against *Aspergillus fumigatus*

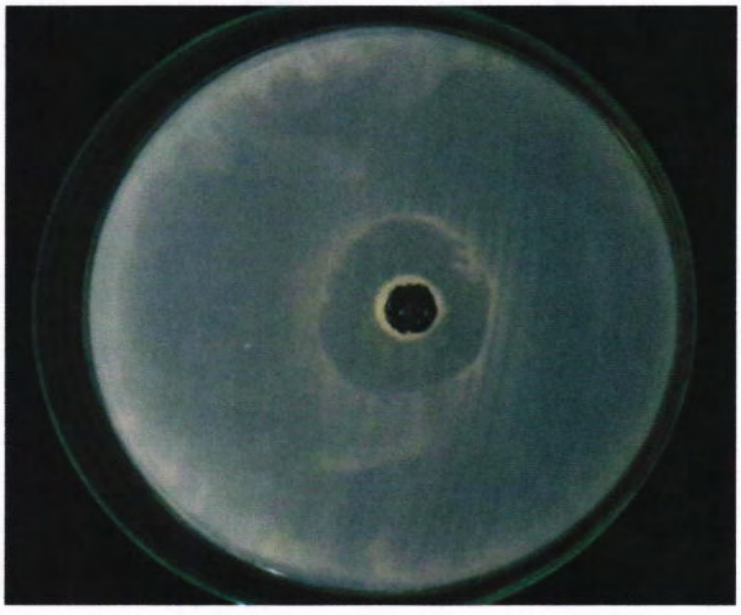

Fig. 3.16(b): Antifungal activity of *P. aeruginosa* against *Candida albicans*

Table 3.6: Antibacterial Activity of *P. aeruginosa* against 10 different known test strains

Test strain	Zone of inhibition	Sensitivity pattern
Escherichia coli	5mm	–
Staphylococcus aureus	42 mm	+++
Shigella flexneri	2 mm	–
Klebsiella pneumoniae	4 mm	–
Enterobacter aerogenes	26 mm	++
Lactobacillus acidophilus	7 mm	–
Staphylococcus epidermidis	22 mm	++
Salmonella typhi	3mm	–
Listeria monocytogenes	5.5mm	–
Bacillus subtilis	1.5mm	–

P > 0.0296

Non sensitive (-): For total diameter smaller than 12 mm

Sensitive (+): For total diameter between 12-25 mm

Very sensitive (++): For total diameter between 26-38 mm

Extremely sensitive (+++): For total diameter larger than 38mm

Table 3.7: Antifungal activity of *P. aeruginosa*

Test strain	Zone of inhibition	Sensitivity pattern
Aspergillus Niger	1mm	–
Aspergillus fumigatus	32 mm	+++
Candida albicans	29 mm	++
Trichophyton tonsurans	0	–

P > 0.1793

Non sensitive (-): For total diameter smaller than 10 mm

Sensitive (+): For total diameter between 11-20 mm

Very sensitive (++): For total diameter between 21-30 mm

Extremely sensitive (+++): For total diameter larger than 31mm

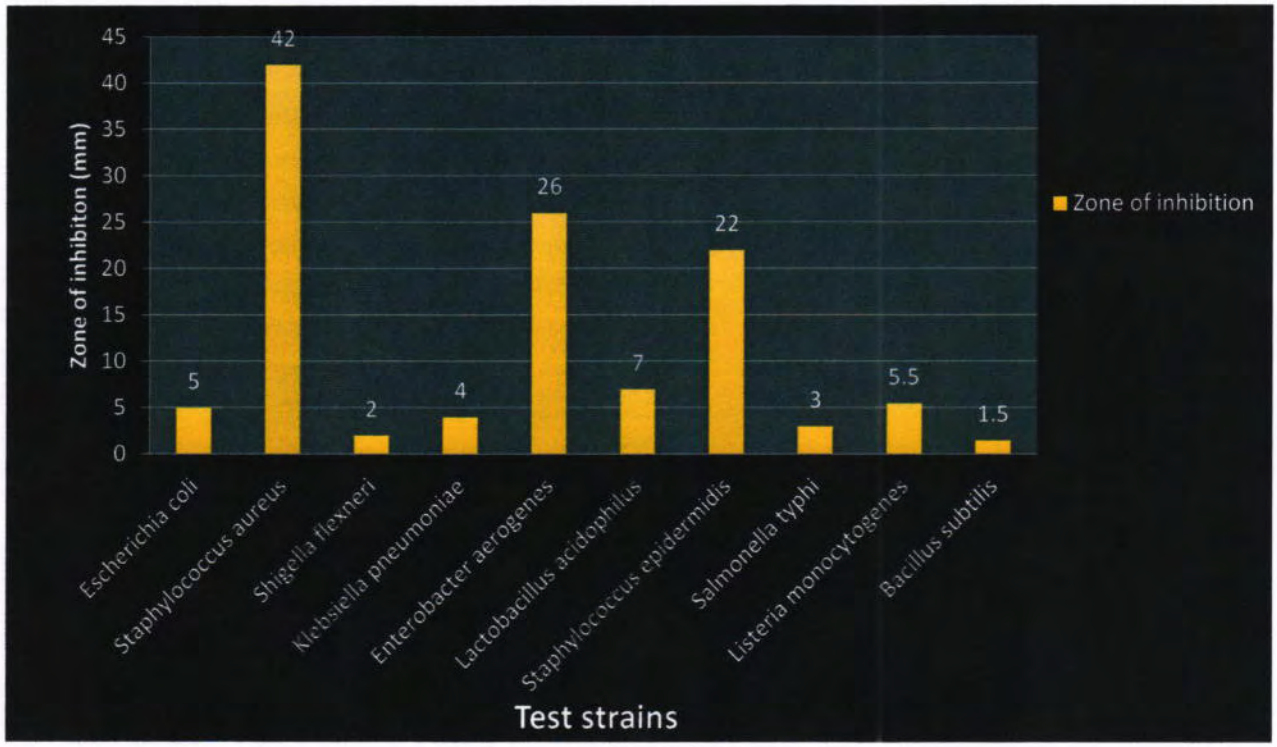

Fig. 3.15: Antibacterial activity of the *Pseudomonas aeruginosa* against 10 different gram positive and gram negative bacterial strains

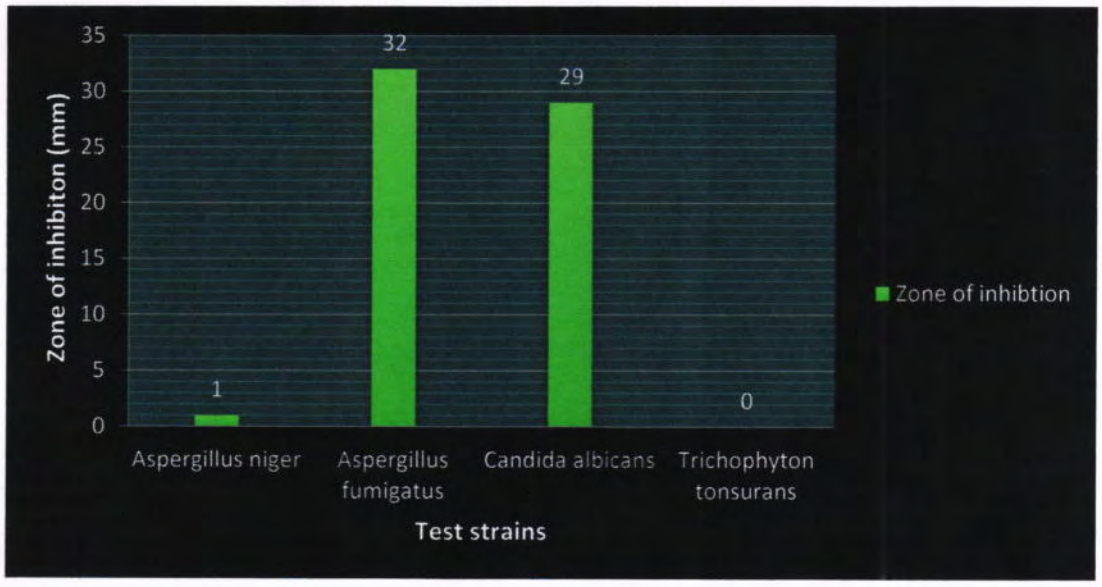

Fig. 3.17: Antifungal activity of *Pseudomonas aeruginosa*

3.6 Optimization of Culture conditions

After incubation when the nutrient agar plates were compared with each other and with the control plate, glycerol supplemented nutrient agar plate showed maximum growth of S17 isolates and maximum pigment production. On the other hand least growth was shown by the Copper sulphate supplemented nutrient agar plate (Fig. 3.18).

Fig. 3.18: Influence of different chemicals on pigment production of *Pseudomonas aeruginosa*

3.7 Solid state fermentation for the preparation of crude extract

Various seed media were tested and finally the best medium was selected in order to get maximum yield of the final desired product. After 7 day incubation of inoculated culture plates elevated growth of pigment produced by the isolated *Pseudomonas aeruginosa* strain was observed.

Bioactive compound produced by the bacterial isolate was isolated by the solvent extraction technique. In solvent extraction the released compound was infused in the solvent layer i.e. the organic layer or the ethyl acetate layer. This layer was collected in a separate beaker and evaporated at room temperature; dried residues were re-dissolved in DMSO. A total of 24.86 grams of crude extract infused in DMSO was obtained and collected in sterile vials and then was evaluated for the antibacterial activity.

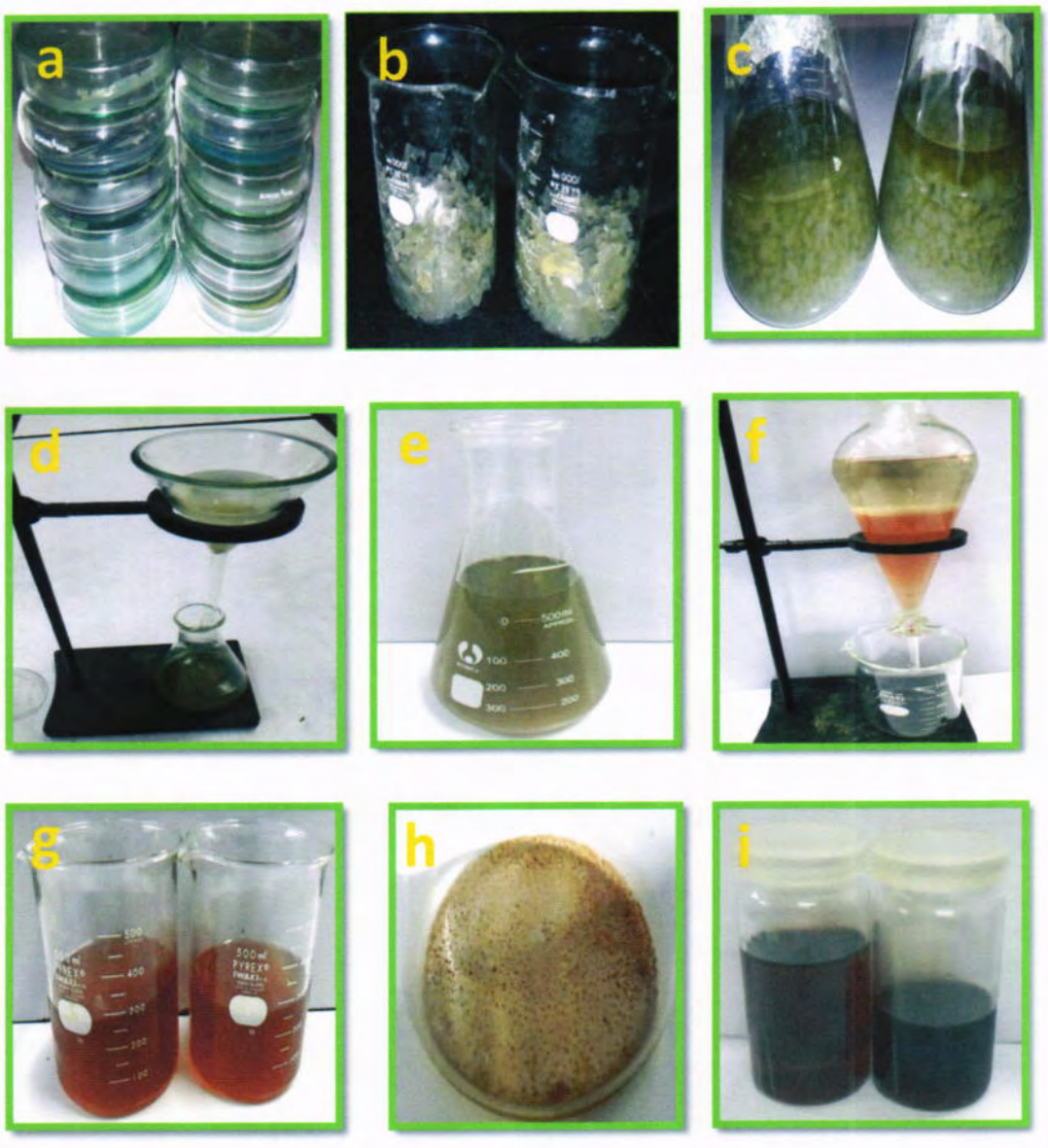

Fig. 3.19: Crude Extract Preparation steps

(a) Culture plates after 7 day incubation (b) Chopped culture media with 10gm NaCl (c) Culture media immersed in 80% acetone (d) Filtration of the fermented media to remove solid residues (e) Filtrate collected in conical flask (f) Solvent extraction through separating funnel apparatus. Aqueous and solvent layer can be differentiated clearly (g) Solvent layer collected in beakers (h) Dried residues after evaporation of organic layer (i) Crude extracts reconstituted in 100% DMSO

3.8 Determination of antibacterial activity of the crude extract

Antibacterial activity of the crude extracts was determined by the disc diffusion method against 5 gram positive clinical strains and 5 gram negative strains of bacteria.

Bacillus subtilis, Salmonella typhi and *Shigella flexneri* (Fig. 3.20(a)) were resistant to the compound, while Escherichia coli, Klebsiella pneumonia, Lactobacillus acidophilus and Listeria monocytogenes showed very weak zones of clearance. Bioactive compound from the isolated *Pseudomonas aeruginosa* produced 14 mm zone of inhibition against gram positive pathogenic bacteria *Staphylocoocu epidermidis* (Fig. 3.20(b)). Another gram positive strain of *Staphylocoocus aureus* showed intermediate sensitivity towards the crude extract of the compound with 28 mm zone of clearance (Fig. 3.20(c)). Extreme sensitivity was shown against the crude extract of the bioactive compound by a gram negative bacterium *Enterobacter aerogenes*, with zone of inhibition of 38mm (Fig. 3.20(d)). Calculated zones of inhibition against these strains are demonstrated in Table 3.8.

Fig. 3.21 is showing the graphical representation of the antibacterial activity of the crude extract.

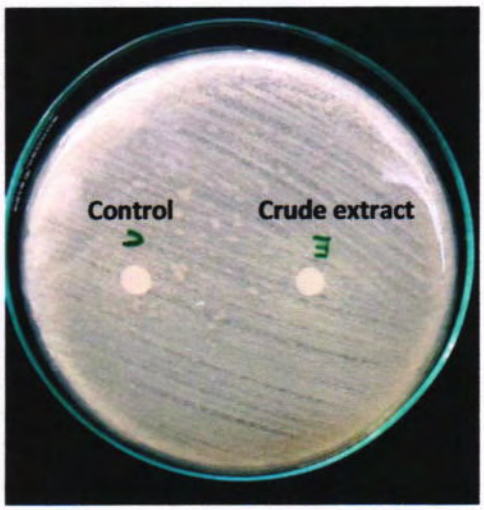

Fig. 3.20(a): Non sensitive *Shigella flexneri*

Fig. 3.20(b): Antibacterial activity against *Staphylococcus epidermidis*

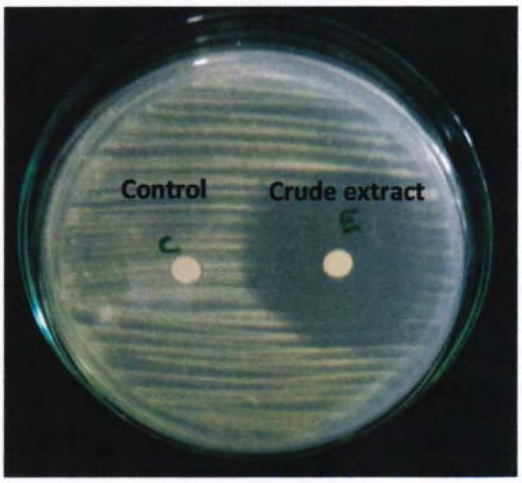

Fig. 3.20(c): Antibacterial activity against *Staphylococcus* aureus

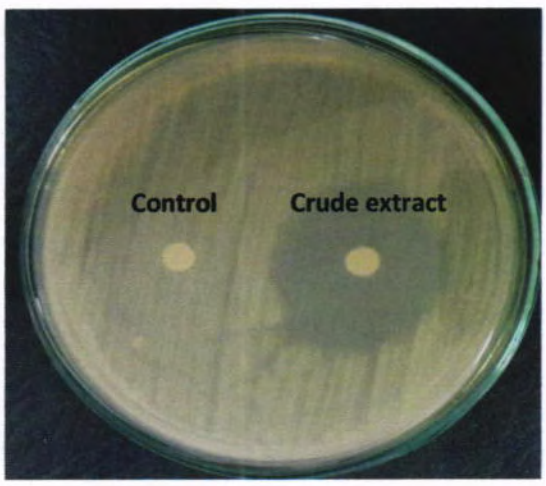

Fig. 3.20(d): Antibacterial activity against *Enterobacter aerogenes*

Table 3.8: Antibacterial activity of the crude extract against clinical bacterial strains

Test strain	Zone of inhibition	Sensitivity pattern
Escherichia coli	3mm	–
Staphylococcus aureus	28 mm	++
Shigella flexneri	0	–
Klebsiella pneumoniae	1mm	–
Enterobacter aerogenes	38 mm	+++
Lactobacillus acidophilus	2mm	–
Staphylococcus epidermidis	14mm	+
Salmonella typhi	0	–
Listeria monocytogenes	2mm	–
Bacillus subtilis	0	–

P > 0.1127

Non sensitive (-): For total diameter smaller than 10 mm

Sensitive (+): For total diameter between 11-20 mm

Very sensitive (++): For total diameter between 22-30 mm

Extremely sensitive (+++): For total diameter larger than 31mm

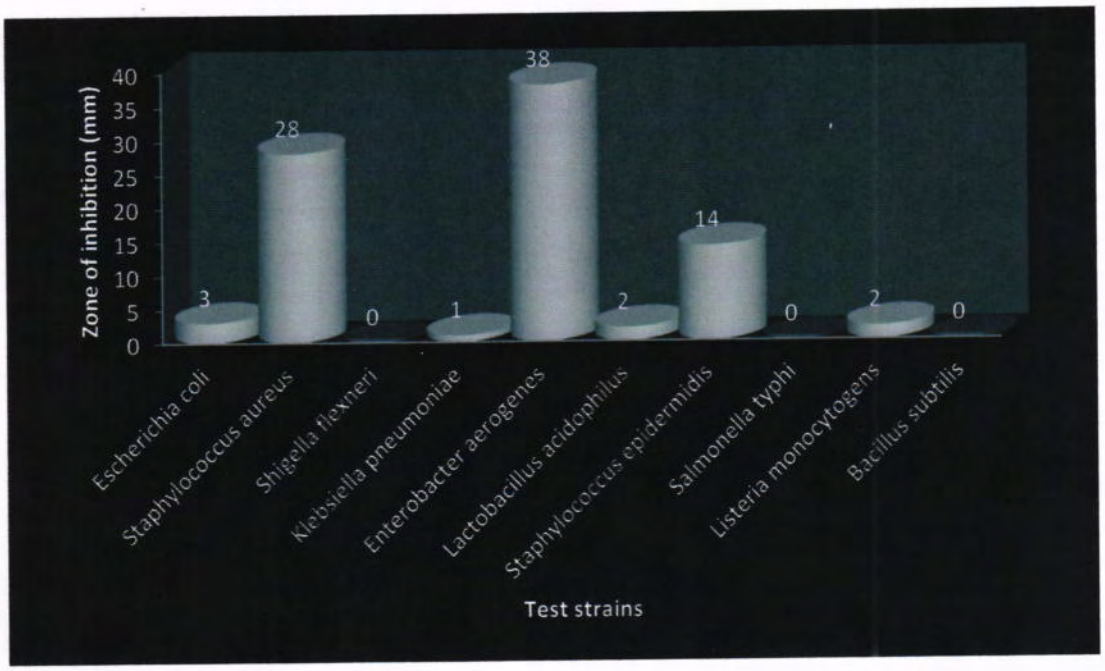

Fig. 3.21: Inhibition zones of the crude extract against 10 different bacterial test strains

CHAPTER 4
DISCUSSION

Antibiotics are the secondary metabolites which are produced by microorganisms or are synthesized chemically and they have the ability to kill other microorganism or inhibition of their growth. They are one of the significant biologically active antimicrobial compounds that are used for the treatment of contagious diseases. But nowadays, due to the emergence of multi drug resistant bacterial pathogens, crucial challenges for efficient treatment of diseases have appeared. As a consequence, there is an increased burden of resistant pathogens on the globe which has resulted in dire requirement of searching new effective antimicrobials from soil microflora (Abo-Shadi *et al.*, 2010).

It depends upon the development of effective antibiotics that would determine the future of modern medicine (So *et al.*, 2010). Between 1930's to 1960's, 20 new antibiotic classes were produced globally (Coates *et al.*, 2002; Powers and J.H, 2004; Coates *et al.*, 2011), from that time only two of the classes have been commercialized (Butler *et al.*, 2006). Simple and quick methods have been developed for exploring antibiotic producing microorganisms.

Soil is an ultimate reservoir of biologically active microflora, which provides nearly all of the clinical antimicrobial drugs used today (Demain and Fang, 2000; Foster and Woodruff, 2010). Nowadays, almost 500 antibiotics are found yearly and more than 80 percent of these are isolated from soil microorganisms (Falkinham *et al.*, 2009). Upper few inches of the soil are rich in these types of microbes (Demain and Fang, 2000; Rizk *et al.*, 2007).

Even though, soils are considered as an excellent source for the isolation of antibiotic producing microbes, at present, exploration of formerly ignored eco-systems is the emphasized (Cragg *et al.*, 1997; Ouhdouch *et al.*, 2001; Lee and Hwang, 2002; Prabavathy *et al.*, 2006). The unexplored Kashmir Himalayas are of great significance because of its richness in biodiversity, it can be explored for isolation and characterization of native microbes for biologically active secondary metabolites (Shah *et al.*, 2017). Hence aim of the present study was to isolate and identify the antibiotic producing bacterial or fungal strains from the red soils of Himalayan region of Pakistan.

Ten different red soil samples were collected randomly from the Kashmir region in this study. Serial dilution method and spread plate technique was used for the isolation of microbial strains from the sample; these techniques were also used by Shah *et al.*, 2017 during their research for isolation of microbes from soil sample. Total 21 bacterial and 2 fungal strains were isolated from the samples which were then subjected to the preliminary screening for their antibiotic production ability. Soil isolates were tested against target test strains for antimicrobial activity by agar well diffusion method (Kim *et al.*, 1994). After screening the strains exhibiting antibacterial and antifungal activity were selected for their secondary screening against known clinical bacterial and fungal strains following the same method of agar well diffusion with 100µl broth of the selected strain, same concentration was also used by Shah *et al.*, 2017. From the isolated bacterial strains, only one strain was found to inhibit bacterial and fungal growth. While none of the fungal strain was found to be antibacterial or antifungal.

The selected strain was characterized on the basis of its colonial and cellular morphology. Upon gram staining the isolate was stained pink and appeared as gram negative rods under 100x lens of microscope. Colonies were seen to be smooth, spherical and of medium size. A diffusible blue green pigment production by the colonies was observed after 24hrs incubation of the bacterial isolate. This type of colonial morphology was also reported by Mavrodi *et al.*, 2001; Mandryk *et al.*, 2007; Isnansetyo and Kamei, 2009 and the strain was identified as *Pseudomonas aerugionsa.* Isolated strain when grown on MacConkey agar differential media, colonies were observed as non lactose fermentors as reported earlier by Brodsky and Nixon (1973).

In this study when the antibacterial activity of the isolate was assessed against test strains, it was observed that gram positive *Staphylococcus aureus* was extremely sensitive to the isolated strain with a zone of inhibition of 42mm in diameter. *Enterobacter aerogenes* (gram negative) and *Staphylococcus epidermidis* were also sensitive to the isolated strain and produced zone of clearance of 26mm and 22mm respectively. It was estimated from the present study that the isolate has more bactericidal effect against gram positive pathogens.

Antifungal activity of the isolate named as S17 was tested again four different fungal strains, from which two of them were sensitive to the isolate including *Candida albicans* and *Aspergillus fumigatus,* similar antifungal study was reported by Kerr *et al.,* (1999).

Foe the isolation of biologically active antimicrobial compound from the selected strain, ethyl acetate extract was prepared from 9-10 days old fermented broth and culture of the active strain and was screened for icrobial potential(Shah *et al.,* 2017). Exploring biologically active compounds in nature is a multistep process which involves the assortment of appropriate source and testing procedures (Shah *et al.,* 2017).

Antibacterial activity of the crude extract prepared through solvent extraction was then subjected to the antibacterial activity with disc diffusion test. Ethyl acetate crude extract of selected bacterial isolate was evaluated for antibacterial activity against 10 different clinical isolates, among which seven strains showed non-sensitivity against it. Maximum activity was shown by *Enterobacter aerogenes* with 38mm inhibition zone, *S. aureus* and *S. epidermidis* also showed sensitivity to the crude extract of isolate. In a similar study by Rina *et al.,* (2015), it was reported that *Staphlococcus aureus* was inihibited by the supernatant of *P. aeruginosa* with a zone of 25mm in diameter. While here in this study the zone of inhibiton against *S. aureus* was measured as 28mm, so a slight difference in the diameter was observed. Sensitivity of *S. epidermidis* to the isolate identified as *P. aeruginosawas* also reported by Zhiqiang *et al.,* 2009.

4.1 Conclusion

This study shows that the bacterial isolates from the soil sample has the potential to act as a source of new antimicrobial compounds, against pathogenic microbes to humans. Here, we found that the Himalayan region is rich in biodiversity and has been sufficiently acceptable due to its vast microbial diversity. The extracellular substance produced by *Pseudomonas specie* may become effective as antibiotics if exploited and purified as those of the commercially available antibiotics. The isolated strain showed potent antibacterial activity against *Staphylococcus aureus, Staphylococcus epidermidis* and gram negative bacterium *Enterobacter aerogenes.* Antifungal activity was shown by two of the test strains; *Aspergillus fumigatus* and *Candida albicans.* This study also reveals that the crude extract of the isolated strain shows antibacterial effect, as reported earlier, against targeted test strains.

This analysis is a primary study therefore further investigations are required to determine the type of biologically active antimicrobial substance that is produced. To get pure antibiotic compound for the treatment of different pathogenic microbes, additional purification process is requisite.

CHAPTER 5
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