PROSPECTS OF MANAGING FEW MULTI DRUG RESISTANCES WORLD HEALTH ORGANISATION PRIORITIZED PATHOGENS

M.Sc. Thesis
2018

Submitted By
Central Department of Biotechnology
Tribhuvan University
Kirtipur, Kathmandu, Nepal

For partial fulfillment of M.Sc degree in Biotechnology

By
Manju Maharjan
Registration No: 5-2-553-1-2010

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ACRONYMS

AST	Antibiotic susceptibility test
ATP	Adenosine triphosphate
AA	Amino Acid
ABC	ATP Binding Cassette
ATP	Adenosine triphosphate
AMR	Antimicrobial Resistance
BCG	Bacillus Calmette–Guérin (BCG) vaccine
BLAST	Basic Local alignment search tool
Bp	Base pair
CNS	Central Nervous System
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EP	Efflux pump
g/l	gram per liter
HSCs	Hematopoietic stem cells
Kb	Kilo base pair
LB	Luria Bertani
Mg	Milligram
MHA	Muller Hinton Agar
Ml	Milliliter
NCBI	National center for Biotechnology Information
NOD	NO dioxygenase
OD600	Optical Density at 600nm
PDB	Protein Data Bank
RNA	Ribosomal nucleic acid
RBS	Ribosome Binding Site
SAM	S-adenosyl methionine
SAH	S-adenosylhomocysteine
Ml	Microliter
µM	Micromolar
WHO	World Health Organization

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ABSTRACT

Multidrug resistance (MDR) bacteria are considered as the most emerging problem in the world. Various kinds of antibiotics are produced for treatment of those bacteria but being resistance against them by various causes. The main cause is the misuse of the antibiotics by consumers which enhance the bacteria to develop different resistance mechanisms such as: lack of cell wall permeability, enzymatic deactivation of antibiotics, alters the target of antibiotics as well as efflux mechanism and many more. Most of the antibiotics are discovered by targeting the cell wall permeability and efflux pump. But antibiotics identified are not effective to act as bactericidal and bacteriostatic which may be due to the lack of knowledge related to the specific target of the bacteria.

Moreover, these kinds of antibiotics, have enhance in certain modification in the bacteria causing mutations in genetic sites and converting them into extensive resistance bacteria (XDR) and multidrug resistance bacteria(MDR).Therefore, for specific identification of sites as target and antibiotics for bacteria computational method should be used. This method is being used as the most effective method to identify target molecule and to know effectiveness of molecules against those bacteria without using lab equipment.

Some literature have reviewed that *Streptomyces* are being the most effective way to be treated against different bacteria by developing different kinds of antibiotics. Antibiotics produced were developed on the basis of targeting different sites of bacteria. Recently, *Streptomyces coelicolor* is found be the most studied Streptomyces which is found to be similar with Mycobacterium .So, instead of using BCG it is the most preferable one to act against *Mycobacterium tuberculosis* without effecting human.

For the development of new drug ring structure is being preferable which can produce biproduct from bacteria found in soil such as *Streptomyces*. In this study, target site was identified by using the computation techniques such as the tools namely protein databank, Discovery studio, pyrx etc. Target sites were taken as the protein TrmD, one of essential proteins for bacteria for methylation process. S-Adenosyl methionine (SAM) or S-Adenosine homocysteine (SAH) essential to carry out the activity of TrmD which was replaced by the derivatives of indole such as: indole,indurubin,isatin etc and binding energy was calculated. Higher binding energy indicates that it can replace original ligands and able to block the methylation of the genetic material of bacteria. Furthermore, modified media was prepared for the extraction of antibiotics in high concentration, and then AST was carried out. During this process Candeula extract were used as comparative sample. Thus, it is presumed that the ring structure phenolic compounds can potent for the antimicrobial activity and also act as enhancer for other antibiotics producer bacteria.

CHAPTER-1

INTRODUCTION

1.1. Background

Multidrug resistance (MDR) is the antimicrobial resistance shown to the multiple drugs used for managing their infections and the ability to grow in the presence of the chemicals which normally kills them (VizcarraCora, C R N IBelcherDulce, B S N, 2006).

MDR property is mostly seen in bacteria by the various mechanisms which are:

- No longer relying (dependent on) on a glycoprotein cell wall.
- Enzymatic deactivation of antibiotics
- Decreased cell wall permeability to antibiotics
- Altered target sites of antibiotics
- Efflux mechanisms to remove antibiotics.
- Increased mutation rate as a stress response.

These resistance mechanisms in bacteria are mainly through the accumulation of multiple genes coding for the resistance to the particular single drug in a single cell which eventually increase in population. The expression of these genes coding for the multidrug efflux pumps change the molecular structure of the target proteins, change the metabolic status, helping in complex structure formation which make difficult for the drugs to act against it(Piddock, 2006).

The MDR capacity in bacteria occur by different causes such as the accumulation resistance coding genes present in resistance plasmids and the efflux which pumps out drugs more than one. In addition, mutation in bacteria (Jose M. Munita and Cesar A.Arias, 2016) may cause the resistance to the different antibiotics such as Fluorquinolone due to the change in the target enzyme DNA. Fluoroquine susceptible bacteria containing additional resistant gene does not make it fully resistance to it and cannot be transferred into other through the plasmid but clonal selection can transfer the gene by the selective pressure (Hooper DC, 2000).

The gram negative bacteria are different from the gram positive bacteria by their different properties such as Gram-negative bacteria have what is referred to as envelope, consisting of three principal layers: the outer membrane, containing the (possibly fatal) lipopolysaccharide/endotoxin, the peptidoglycan cell wall with peptide chains, partially cross-linked, and the cytoplasmic or inner membrane. The bacteria are resistance due to cell permeability, enzymatic resistance towards the antibiotics, efflux mechanism and mutation (Silhavy, Thomas Kahne, Daniel Walker, Suzanne, 2010).

Recently, it has been known that most of the bacteria are resistance due to the mutation occurs in the genes involved in the functions as well as the interactions between the resistances mutations also helped in the emergence of the antibiotic resistance bacteria. Among all bacteria *Escherichia coli,* is found to be faster bacteria to carry out the single resistance mutation gene. Various antibiotics are produced for treatment of these kinds of bacteria but in some condition, Broader spectrum antibacterial or the combination of

the antibacterial therapy can be used for the treatment of the multidrug resistance bacteria when it exceeds the threshold in the community. Multidrug resistance bacteria mostly occur during the process of treatment and after the treatment of the microbial infection (Levy, S. B.,2002).

The multidrug resistance bacteria gets colonized by various indwelling medical devices such as urinary catheters, feeding tubes, endotracheal tubes, and vascular lines also the risk factors for the infections includes the immunosuppressed states such as solid organ or hematopoietic stem cell transplant recipient and other comorbid conditions such as renal failure.(van Duin, David Paterson, David L., 2016).Random use of the antibiotics in animals as it being a good carrier also contribute in persistence and spread of antibiotics resistant bacteria(CDC, Centers for Disease Control and Prevention,2013).

Centers for Disease Control (CDC)is one of the agency which is conducting various activities for prevention of multidrug resistant bacteria at the national, regional and local levels by providing the guidance to health facilities interested in better antibiotics use and to control all patients infection by infection control guidelines so it has conducted four main ways to prevent from the deadly infections are:

• Preventing the infections and preventing the spread of resistance.

• Tracking resistant bacteria

• Improving the use of today's antibiotics.

• Promoting the development of new antibiotics and developing new diagnostic test for resistant bacteria.

(States, United,2013)

1.2. Tracking Resistance Patterns

CDC is tracking cause of infection and analyzing whether it is infection or not in the people as well as providing protection and prevention related to infection and spreading of resistant bacteria (Frieden, Tom,2013). CDC's National Healthcare Safety Network (NHSN),electronically reporting infections ,use of antibiotics also helping in regular tracking and benchmark antibiotics resistance in all bacteria as well as track antibiotics usage(CDC, US Department of Health and Human Services,2013).

CDC is reference laboratory conducts antibiotics susceptibility test for the identification, isolation of sporadic cases and outbreak including the resistance pattern shown by the antibiotics resistance bacteria. NARMS provides the information about the way of resistance among enteric pathogens to stakeholders, including federal regulatory agencies, policymakers, consumer advocacy groups, industry and the public, to guide public health prevention and policy efforts that protect people from the resistant infection(States, United,2013).

It helps to prevent the antibiotics resistance by the tracking the infection and change in resistance; improving prescribing at national, regional and local levels; limiting or interrupting the spread of the infections. These techniques are similar to that of the

medical settings but the approach may differ due to the large population and different setting. Some techniques to prevent antibiotics resistance in communities are:

•Tracking community infections and resistance, helping in the identification of theoretical infections in the community and monitor resistance trends shown by the different bacteria.

•Active Bacterial Core surveillance (ABCs): It includes tracking infections caused by Neisseria meningitides, Streptococcus pneumonia, Groups A and B Streptococcus and Methicillin-resistant Staphylococcus aureus.

•Gonococcal Isolate Surveillance Project (GISP): Collecting isolates from gonorrhea infections to monitor resistance.

•National Tuberculosis Surveillance System (NTSS): National Electronic Disease Surveillance System (NEDSS)-based reporting of tuberculosis cases including resistance data.

•Healthcare-Associated infections-community interface (HAIC): Tracking infections with Clostridium.difficile and with multidrug-resistant gram-negative microorganism.

(Center for Disease Control and Prevention, 2013).

1.3 Antibiotic Stewardship: Improving Prescribing and Use

Tracking resistant bacteria and providing guidance for improvement of antibiotics and developing new antibiotics and diagnostic tests. Program called Get Smart , campaign has been carried out for prescription of antibiotics against bacteria published annually in November to raise awareness among patients, healthcare provides, hospital administrators and policy makers relating the antibiotics resistance and decrease the inappropriate antibiotics use (Centers for Disease Control and Prevention,2016).

It provides the public health message for the improvement of antibiotic use in healthcare setting and is working in the improvement of the use in antibiotics in healthcare (Centers for Disease Control and Prevention (CDC),2011).It provides local public health authorities with the messages and resources for improving the antibiotics use in outpatient settings and helping in the prescription for the treatment of diseases by identifying new approaches to improve the use of antibiotics (CDC, Centers for Disease Control and Prevention,2013).

It has carried out the various activities to control the spread of the antibiotics resistance infections such as: Contact tracing, vaccination, treatment guidelines, promotion of safe sex. It has also being working for the antibiotic resistance in food working with the state and local health departments and also with the U.S. Food and Drug Administration (FDA)which helps in the regulation of the antibiotics ,many foods, animal feed and other products as well as the U.S. Department of Agriculture(USDA) which regulates meat, poultry ,and egg products. It is being supporting the FDA’s plan for the improvement of the training curriculum for veterinarians use in animals and also to improve prescribing in humans because there is the linking between the antibiotics resistant infections in humans and the food-producing animals (Prevention, Centers for Disease Control,2011).

For prevention, it is conducting the activities for the treatment of the foodborne and other enteric infections by the following activities such as estimation, monitoring, investigating, educating, identifying the foodborne infections and also promoting the proper hand washing (Centers for Disease Control and prevention,2013).

1.4 Developing New Antibiotics and Diagnostic Tests

Antibiotic resistance is considered as the natural process which can be slow down but cannot stopped so new antibiotics should be develop continuously by new diagnostic test and by tracking development of resistance by bacteria.

Various steps are carried out for the treatment of various microorganisms which are in threat level and is urgent for the treatment which includes the *Clostridium difficile*, carbapenem-resistant Enterobacteriaceae ,Drug-resistant *Neisseria gonorrhoeae*,Multidrug resistant *Acinetobacter*, drug resistant *Campylobacter*, Extented spectrum β lactamase(ESBL), Vancomycin-Resistant *Enterococcus* (VRE), Multidrug-Resistant *Pseudomonas aeroginosa*,Drug-Resistant Non-Typhoid Salmonella, Drug-Resistant *Salmonella Typhi*, Drug-Resistant *Shigella* ,Methicillin-Resistant (MRSA), Drug-Resistant *Streptococcus pneumoniae*, Drug-Resistant *Mycobacterium tuberculosis*, Vancomycin-Resistant *Staphylococus aureus* (VRSA), Erythromycin-Resistant Group A Streptococcus, Clindamycin- Resistant Group B Streptococcus etc(CDC, Centers for Disease Control and Prevention,2013).

NIAID research is focusing in new drug discovery related to the *Mycobacterium tuberculosis* by studying their physiological and its interaction with the host. It has also concluded that the first line and the second line therapies related to the Tuberculosis should be reexamined and optimized to determine that whether it is causing the emergence of the new disease in the children, while doing the antiretroviral therapies and also with other comorbidities. Also, FDA –approved the antibiotics which are not currently used for the treatment of the diseases and is further tested whether they can contribute to treatment of the drug –resistant and drug-susceptible tuberculosis which will be very helpful for the avaibility of the drug related to global threat to MDR/XDR TB(Lowy et al., 2004).

The antimicrobial peptide can be one of the main target for the treatment of diseases related to MDR, carbapenem resistant bacteria etc. Late-stage clinical trial are being carried out in some derivatives of antimicrobial peptides such as pexigenan, omiganan and OP-145 which is related with disease such as diabetic foot ulcers ,rosacea and ear infection respectively. For the toenail fungal infections and MRSA other peptides are used such as Novexatin and Lytixar. Till now around 2000 natural and designed antimicrobial peptides are used for the treatment of the various kind of disease related to the gram –positive, gram-negative, fungal, Mycobacterial and protozoan pathogens (Centers for Disease Control and Prevention, 2014).

Antimicrobial peptides are the positively charged so they can be easily attracted by the bacteria due to the negative charge on the cell surface. The attraction of the peptide bond helps in binding to the bacterial cell surface causing death of the bacteria. The bacteria is killed by three different mechanisms which are toroid pore formation, carpet formation and barrel stave formation. The mechanisms difference but the main activity is the binding in the membrane and causing the leakage of cytoplasm causing the

ultimate death. Antimicrobial peptides activity can act differently such as metabolic inhibitors, inhibitors of the DNA, RNA and also the protein synthesis process. As well as it can also inhibit the cell wall synthesis and septum formation causing ribosomal aggregation and delocalize membrane proteins (Zhang, Gallo et al.,2016).

Most of the vaccines such as BCG acting against *Mycobacteria tuberculosis* help in cell mediated immune responses which causes the limitation of the bacteria growth but not the prevention. The latest update include that not only the T-cell (cytokines IFN-Y and TNF-a)but also the antibodies can also act as the protection for the body. Selection was done on the basis of proteolytic activity between Streptomyces. So, *Streptomyces lividans* were used instead of *S. coelicolor* (Glacomini,Remoli,Gafa et al.,2009).

Mycobacterium bovis and *Mycobacterium tuberculosis* has different lifestyle in comparison to *Streptomyces coelicolor* but showed high synteny and similarity in their individual gene sequence. DnaA is used for showing the central broken diagonal. DnaA is used for the for showing the central broken diagonal cross pattern formed by the synteny.So Streptomyces can be a potent gram positive bacteria to act against *Mycobacterium* (Prasanna and Mehra, 2013).BCG and other vaccines are related to the cell-mediated immune response which helps in the limitation and dissemination but cannot prevent the infection (Watanabe,Watari,.Matasunaga et al.,2006).

Phytochemicals are naturally present in many foods but it is expected that through bioengineering new plants which can make it easier to incorporate enough phytochemicals with our food (Surh, YJ, 2003).

More than 80% of the world population solely relies on medicinal plants for their primary health care needs. Some of these herbs are proven to provide symptomatic relief and assist in the prevention of the secondary complication of the diseases, while others are reported to help in regeneration of abnormal cells and in overcoming disease causing pathogens. Moreover, these natural substances are readily available, cheap and do not result in adverse side effects usually associated with synthetic drugs. Most of the side effects caused by phytotherapy methods of disease management are not as severe as those caused by conventional methods. The therapeutic effects of these medicinal plants can justifiably be attributed to, among others, the phytochemicals in them especially the flavonoids, alkaloids, sterols, terpenoids, phenolic acids, stilbenes, lignans, tannins and saponins. They cover a wide range of therapeutic indications with a great diversity of chemical structures. Therefore, these phytochemicals provide qualified lead for the development of new drug entities in drug design and discovery (Nyamai et al., 2016).

The session on phytochemicals included talks describing recent research achievements, with examples of successful agricultural use of various phytochemicals as antibiotic alternatives and their mode of action in major agricultural animals (poultry, swine and ruminants). Scientists from industry and academia and government research institutes shared their experience in developing and applying potential antibiotic-alternative phytochemicals commercially to reduce AGPs and to develop a sustainable animal production system in the absence of antibiotics.

Oleanic acid of triglycoside was first reported in Calendula which as the property of anti-oxidant and anti-inflammatory in nature which are able to protect cell damages caused by oxidation and inflammatory factor (Varnava, 2016).

Identification of *Streptomyces* was conducted by providing the sucrose as the source of carbon and by the basis of morphologically differences such as spore color, aerial and substrate mycelium formation and production of diffused pigment. Five Streptomyces which were isolated showed zone of inhibition towards *Staphylococcus aureus*, *Bacillus cereus*, *B. subtilis*, and *Pseudomonas aeruginosa* also showed antifungal activity against *Candida albicans* and *Aspergillus niger*. It has been found that the microorganisms of rhizopheric region of fertile areas showed the antibacterial and antifungal activity which represented that soil of that area can be site for identification of new antibiotics. As well as deserted soils can also be target area for the exploration of new bio products (Al_husnan, Latifa A.Alkahtani, Muneera D.F, 2016).

Computational technique can be effective method can be used for drug development .Structure and ligand based methods are mostly used in drug discovery field whereas proteochemometrics is emerging combinatorial techniques (Katsila et al.,2016).

1.5. Statement of Problems

Multidrug resistance is the most emerging fatal problem in hospital and health care center overall in world due to the lack of the antibiotics being effective against the bacteria it is being dangerous to conduct the medical procedures related to the surgery such as organ transplantation, caesarean sections, joint replacements. Multidrug resistance is mostly associated with the nosocomial infections (van Duin, DavidPaterson, David L, 2016).

Multidrug resistance bacteria are mostly possessed in the hospitals and nursing homes. The list of the bacteria which causes the severe and deadly cause of the infection such as blood infection and pneumonia. Some bacteria which is high and medium priority drug resistance bacteria causes common diseases such as gonorrhea and food poisonings causing by Salmonella (Tacconelli, E.Magrini, N., 2017).

Multidrug resistance organisms has created a challenging to care the wounds causing the shortage of the safe and effective antibiotics which created the chronic skin and soft tissue infection, osteomyelitis and in some cases leads to limb loss, sepsis and death (Defense, Office of the Secretary).

The causes of the multidrug resistance are the overuse and inappropriate consumption of the drugs causing the increase in the morbidity, mortality, length of hospitalization and healthcare costs. The cause of multidrug resistances are: 1.Natural causes (selective pressure), 2.Mutation, Gene transfer, 3.Societal pressure, 4.Inappropriate use, 5.Inadequate Diagnostics,6. Hospital use, 7.Agricultural use (Seebeck, T Maser, P, 2009).

1.6. Significance/Rationale

The emerging problem related to the multidrug resistance microorganisms is addressed by the extract produced by putative *Streptomyces* extracted from the soils collected from the different areas of Nepal by the different processes such as Antibiotics susceptibility test (AST) and Minimal Inhibitory Concentration. Being the Streptomyces a

good antibiotics producing bacteria which kills and inhibits the growth of other bacteria by blocking the different enzymology sites of the pathways which is essential for the bacteria for its growth .The targeted sites and the proteins were identified by the computational docking method.

Recently researchers found the Global Natural Products Social (GNPS) molecular network, database of mass spectra of natural products collected by researchers worldwide, which represents the goldmine for the drug discovery.

Most of *Streptomyces* are known to be saprophytic indicating that it can degrade lignin. Lignin degradation is known to release phenolic compound suggesting that Streptomycetes could potentially produce secondary metabolites consisting of phenolic ring and indole derivatives or indurubin upon phenolic stress and stress. Since, 5mM indole is reported to be toxic to bacteria.

Present work of computational docking model revealed that the indurubin core appears to bind at ATP binding pocket of the kinases with binding energy equal too or higher than native ligand ATP. It has been reported that some of Streptomycetes produce isatin which is reported to be precursor of indurubin, isatin and indole have been reported to have bactericidal effect.

1.7. Hypothesis

1.7.1. Null hypothesis:

Antimicrobial molecule will not be screened against WHO priorities bacteria.

1.7.2. Alternative hypothesis

Antimicrobial molecule will be screened against WHO priorities bacteria.

1.8. Objectives

1.8.1. Main Objectives

- To develop strategy for putative antimicrobial agent production**.**

1.8.2. Specific Objectives

- To rationalize interaction of kinase inhibitors with methyl transferase (TrmD).
- To isolate *Streptomyces* from soil samples collected from different area of Nepal.
- To confirm the character of putative Streptomyces by Polymerase Chain Reaction.
- To confirm the character of putative Streptomyces by Antibiotic sensitivity test and antioxidant test.
- To confirm the character of putative Streptomyces by Biochemical test and specific PCR.

CHAPTER- 2

LITERATURE REVIEW

2.1. Literature review on *Mycobacterium tuberculosis*

Mycobacterium tuberculosis is one of the fatal infectious agents with high mortality rate but effective drug has potential to prevent death. Due to emerging cause of multidrug resistance bacilli (Smith, 2003), mostly showing resistance to isoniazid (30%) and rifampicin (5%). they are mostly resistance to quinolone and three drugs used as second line drugs: Kanamycin, Amikacin or Capreomycin (Gillespie et.al., 2002), causing fewer option for the treatment and risk high mortality especially in HIV infected persons (Gandhi et al., 2006),so to overcome these limitations different tools are used. One of such attempts have been use of bioinformatics for the identification of specific genes and to develop the drugs against it and some have gone for chemotherapy trials (Fathima, Boopathi, Selvam.,2017). The intrinsic drug resistance is caused due to the highly impermeable mycolic acid and efflux shown by the bacteria (Jarlier and Nikaido., 1994).The acquired drug resistance is not transfer to other microorganisms due to the absence of plasmids and transfer of the gene is not reported yet (Ramaswamy, Dou, Rendon., 2004).BCG is being used for the treatment of the different stages of mycobacterium which is the live attenuated vaccine of Mycobacterium bovis.

As a vaccine BCG,main strategy include the use of live microorganisms as vector which can express the mycobacterium antigen, express long term immunological memory power after passing through the mucosal membrane and having low cost. Different non-pathogenic strains (*Mycobacterium vaccae*, *Mycobacterium microti*, *Mycobacterium habana*) of Mycobacterium are also used for the treatment but due to the slow grower and even recombinant.*Mycobacterium* is not used due to lack of better protection than BCG.Mycobacterium tuberculosis is one of the most complex disease causing bacteria, Tuberculosis, leading to the death and has serious complication when co-infection by HIV/AIDS (UN Aids, 2013).

In 2006, an estimated 500,000 individuals throughout the world developed multi-drug resistant TB which otherwise could have been easily treat with isoniazid and rifampicin as the first line of drug. In 2008, the Extensively drug –resistant bacteria (XDR),the strain that show the resistant to Isoniazid, Rifampin and any Fluoro quinolone and at least one injectable second-line anti-TB drugs are: Amikacin ,Kanamycin, or Capreomycin(A.Fauci,2008), was found in 46 countries and this type of MDR TB is difficult to treat .Thus,new line of drug is sought to manage the infection by these MDRs.

National Institute of Allergy and Infectious Disease (NIAID), component of National Institutes of Health (NIH) and World Health Organization (WHO) formulated the Global MDR-TB and XDR-TB Response Plan.The plan- conducted a program to increase surveillance, control, and treatment efforts, focusing in the prevention - for the halting of the threat of the drug-resistant TB. The initial, completely drug-resistant TB bacterium was reported from Italy where two HIV-negative patients had thee pathogen that was

resistant to all known different anti-TB drugs. Patients infected with the TB pathogen develop drug resistance due to the multiple factors that are:

•Primarily suboptimal drug concentrations and varying degree of non-adherence to therapy.

•Transmission of drug-resistant Mycobacterium Tuberculosis has been observed in countries with high numbers of patients co infected with the HIV (Solomon, Rutledge, and Boyd, 2009).

The burden of TB compounded with MDR TB and XDR TB is limiting management by available of treatment of the TB. To keep anti-TB drug discovery and development from disappearing completely, government-sponsored research organizations, philanthropic donors, and public-private partnerships have shouldered most of the responsibility to continue research and development efforts for new anti-TB agents.

The main objectives of the NIAID'S that can be applied to the adult and pediatric populations as well as persons with HIV infections:

I. To develop the technologies for diagnose and test of TB and to identify the drug resistance.
II. To define the most effective use of the existing second-line anti –TB therapies and other antimicrobials available to treat drug –resistant TB and development of new chemotherapeutic agents particularly against MDR/XDR TB.
III. To understand the epidemiology of M. tuberculosis, including host and strain characteristics,to help in the development of drug and prevention of spread of the MDR TB.
IV. To determine the influence of the overall immune status of the infected individual, other host factors and HIV co- infection on to understand drug resistance and the outcome of TB chemotherapy.
V. To develop effective chemo preventive and immune-preventive strategies for drug –susceptible and drug-resistant TB.

The program will complement the ongoing NIAID efforts in the most critical areas of TB research, understanding latency; developing surrogate markers of infection, disease, and response to therapy; identifying correlates of immunity and markers that signal transition from latent to active TB; and discovering and developing the new diagnostics, drugs and vaccines for TB that are relevant to all persons at risk, including children and those co infected with HIV (World Health Organization, 2017).

Furthermore, the NIAID helps in by focusing on leading and sponsoring research activities to create a foundation of knowledge for the discovery of new diagnostics, drugs and vaccines with the further thinking that it could be helpful for the drug companies, public-private partnerships for tools and approaches that help in the improvement for the quality improvement of TB infected patients (Peters,.Dixon,.Holland ,2008).

2.2. Literature review on Antimicrobial Resistance Bacteria

Antibiotics are the natural products produced by the microorganisms or the synthetic products designed with the help of the computational methods to act against the

bacteria (Magalhaes and Blanchard, 2009).However, the use of antibiotics is being compromised since the organisms against which they are used for the management and the treatment of the infection induced by these microorganisms are developing resistance against these drugs.

The main causes of antibiotics resistance in bacteria are thought to be: misuse of drugs, mainly because of patient failure to complete course of antibiotics and/or over dose prescription of antibiotics.

More alarming is emergence of multi-drug resistant (MDR) strains that show resistance against more antibiotics. Among large number of bacteria, the most common MDR bacteria are Vancomycin –Resistant Enterococci, Methicillin-resistant Staphylococcus aureus (MRSA), Extended-spectrum β-lactamase (ESBLs) producing_ gram-negative bacteria: Klebsiella pneumonia including carbapenemase (KPC) and multidrug resistant gram negative rods (MDR GNR) such as Enterobacter species, E.coli, Pseudomonas(Ventola et al.,2015).Some of the resistant bacteria have been categorized by WHO on the priority depending upon the need for new molecules to treat the infections (Lawe-Davies and Bennett,2017).They are:

• Critical priority

- *Pseudomonas aeroginosa*, carbapenem –resistant
- Enterobacteriaceae, carbapenem –resistant
- *Acinetobacter baumannii*, carbapenem-resistant

• High priority

- *Enterococcus faecium*, vancomycin-resistant
- *Staphylococcus aureus*, methicillin-resistant, vancomycin –intermediate and resistant.
- *Helicobacter pylori*, clarithromycin-resistant
- Several species of Campylobacter, fluoroquinolone-resistant

•Medium priority

- *Streptococcus pneumonia,* penicillin-non-susceptible
- *Haemophilus influenzae*, ampicillin-resistant
- Several species of Shigella, fluoroquinolone-resistance.

Scientists have given sufficient rational with the strong evidence that the Antibiotics used in the food-producing the animals produce harm in the public health by different events such as:

i. The use of the antibiotics in the animals have the ability for the suppressing or the death of the susceptible bacteria while thrive the antibiotic resistant bacteria.

ii. The resistance bacteria are transmitted from the food-producing animals to the humans through the food-supply.

iii. Infections can be caused by the resistant bacteria in the humans.

iv. Adverse health factors in humans are caused by the infection with resistance bacteria.

2.3. Literature review on mode of action of antibiotics.

There are two types of mechanisms by which the antibiotics act against most of the bacteria which are: bacteriostatic and bactericidal.

1. Bacteriostatic: Antibiotics which stop the growth or the replication of the bacteria are known as bacteriostatic antibiotics and they are mainly tetracyclines, spectinomycin, sulphonamides, macrolides, chloramphenicol, trimetoprim.

2.Bactericidal:Antibiotics that kill the bacteria and are penicillins ,cephalosporins ,fluoroquinolones(Ciprofloxacin), glycopeptides(Vancomycin),monobactams,carbapenems. (https://study.com/academy/lesson/types-of-antibiotics-bacteriocidal-vsbacteriostatic-narrow-spectrum-vs-broad-spectrum.html:Metzler et al., 2015).

The different sites of the bacteria are targeted by the antibiotics for the bacteriostatic and bactericidal effects according to their activities (https://amrls.umn.edu/antimicrobial-resistance-learning-site).

2.3.1. Inhibitors of cell wall synthesis: The inhibition of cell wall causes the inhibition and kill of the growth of plant cells and bacterial cells because the animal and humans do not have the cell walls so it can act as the selective drugs. Examples: penicllins, cephalosporins, bacitracin and vancomycin (Sun ,Cohen,and Mani,2002).

2.3.2. Inhibitors of cell membrane function: Cell membranes are the important part for all eukaryotes and prokaryotes that helps to create the specific structural barrier between the cell cytosol and outer membrane and keeping important solutes inside it the cells for the cell's survival. The antibiotics that act on cell membrane biosynthesis cause the leakage of the solutes in killing the bacteria. However, their uses are limited as they may be toxic in for systemic use in mammalian host.Most clinical usage is therefore limited to topical applications. Examples: polymixin B and colistin (Calvo and Martinez-Martinez,2009).

2.3.3. Inhibitors of protein synthesis: All the Enzymes and majority of cellular structures are primarily made of proteins. So, Protein synthesis is considered as essential process necessary for the multiplication and survival of all bacterial cells. For the synthesis of the proteins different ribosomes a such as 30S or 50S subunits are required and are the target of drug.The inhibition of different steps of protein biosynthesis helps in disruption of the normal cellular metabolism of the bacteria, and consequently leads to the death of the organism or the inhibition of its growth and multiplication. Examples: Aminoglycosides, macrolides, lincosamides, streptogramins, chloramphenicol, tetracyclines (Enris H,1965).

2.3.4. Inhibitors of nucleic acid synthesis: DNA and RNA are the key elements for replication and transcription in all living forms, including bacteria. Some antibiotics inhibit the process of DNA or RNA synthesis by binding to components involved in the process. This causes interference of the normal cellular processes which ultimately leads

compromise in bacterial multiplication and survival. Examples: quinolones, metronidazole, and rifampin (Hayashi, Okutomi and Suzuki.,1983).

2.3.5. Inhibitors of other metabolic processes: Other antibiotics act on selected cellular processes essential for the survival of the bacterial pathogens. For example, both sulfonamides and trimethoprim disrupt the folic acid pathway, which is a necessary step for bacteria to produce precursors important for DNA synthesis. Sulfonamides target and bind to dihydropteroate synthase, trimethophrim inhibit dihydrofolate reductase; both of these enzymes are essential for the production of folic acid, a vitamin synthesized by bacteria, but not humans (Cornish-Bowden and Cardenas,2001).

2.4. Literature review on modes of resistance

Staphylococcus aureus (MRSA) are not only the methicine resistance but also resistance to the multiple drugs such as aminoglycosides, microlides, tetracycline, chloramphenicol and lincosamides, which is leading as the source of immerging hospital acquired disease. Similarly, *Pseudomonas aeruginosa* and *Acinetobacter baumanii* are also emerging resistance to drug mainly through low cell wall permeability and multiple efflux mechanisms(Munita,Arias,Unit,2016).There are several mechanisms underlying for resistance to antibiotics.Some could be described as below:

2.4.1. Enzymatic inactivation of the drug

Drugs (aminoglycosides, acetylation and β-lactams adenylation emerge from the natural source is resistant by the enzymatic activity of the bacteria (Cattoir,2016).

Amino glycosidase is inactivated by the change in polycationic charge of the antibiotics. The major source of antibiotics resistance is the production of proteins involved in the deactivation of the antibiotics by the microorganism for their survival .Also different genes play the important role in the amino glycosidase inactivation AAC(3)-II,aminoglycosidase acetylase acts on position 3 of substrate and belong to the second phylogenic grouping among these enzymes. APH(3')-I, aminoglycoside phosphorylase is coded by the gene in the plasmid and is present in 46% of Gram –negative bacteria and related to the gene from the Streptomyces fradiae in chromosomal gene. In addition AAC (3)-II, enzyme coded by gene in plasmid is common with more than 60% of aminoglycoside- resistant found in Gram-negative bacteria (Klen and Rezanka, 2008).

Pharmaceutical companies have tried to overcome resistance by developing natural and/or synthetic substrates which are not the inactivating enzyme.

The problem of β-Lactams inactivation and resistance exhibited by the Staphylococcus aureus was initially solved by the invention of methicillin and similar compounds that are resistant to the enzymatic hydrolysis shown by lactamase.But the TEM- β-lactamases are also reported in gram –negative bacteria containing multiple –drug resistant R plasmids. The lactamases are divided into three different classes according to their activities: class A, includes Staphylococcus aureus and TEM enzymes; classB, includes metalloenzyme causes hydrolysis of carbapenem efficiently and class C includes chromosomally coded enzymes (Decuyper.Jukic and Sosic, 2018).In addition, presence of

efflux pumps renders these bacteria resistant since methicillin and its relatives are pumped back by the multidrug efflux.

Management of these pathogens were complicated and the first generation antibiotics were not effective but the third generation antibiotics cephalosporin was effective against them because it inhibit the induction of the AmpC enzymes, and is also resistant to hydrolysis by AmpC(Jacoby,2009).However,the selective pressure results in selection of the plasmid which produce the mutant of these plasmid containing enzymes such as TEM or its relative SHV able to hydrolysis the third as well as fourth generation cephalosporin and it is commonly called ESBL(extended spectrum β –lactamases).The greater complexity arises with ESBL enzymes when they posses CTX-M component that has been derived from gram-negative bacterium Kluyvera and is transferred to R plasmids. This process has occurred many times so it is wide spread through the R-plasmid containing pathogenic bacteria (Paterson and Bonomo,2005).

The use of β -lactam with new nucleus can be effective to the bacteria but it may give rise to different enzymes which may hydrolyze these compounds. Another mechanism of resistance is the plasmid coded with ciprofloxan resistant gene (Malloy and Campos,2011).

2.4.1.1. Acquisition of Genes for Less Susceptible Target Proteins from Other Species.

One of additional mechanisms of resistance has been loss of susceptibility of target proteins and the production of the mosaic proteins was the cause of the penicillin resistance. This was observed among penicillin resistance among Streptococcus pneumonia, which can import the foreign DNA and can be naturally transformed to build resistance in this mechanism and has been also found in the Neisseria meningitides.

The methicillin and penicillin binding protein (PBP) combine to form PBP-2A' or 2' found in MRSA and can be induced in the presence of methicillin.The gene acquires large areas of the DNA region of the bacteria that also contain other resistant genes,thus induction of this gene simultaneously also express other genes.However,S.aureus is not known to be easily transformed naturally and it is not clear how the transformation could have taken place (Nikaido,2009),and open new avenue to further understand resistance mechanisms.

2.4.1.2. Bypassing of the Target

Vancomycin, fermentative product of the Streptomyces, binds to the lipid-linked Disaccharide penta peptide precursor of cell wall peptidoglycan instead of inhibition of enzyme like that shown by β-lactam ring containing antibiotics. However, nowadays, *Enterococcus* is resistant to vancomycin that is found in hospital environment and colonizes the patients making it difficult to treat.

The resistance mechanism appears that vancomycin binding site of the peptide bond is replaced by the ester bond that prevent binding by vancomycin and causes the resistant to the vancomycin.(Bolla,Albert-Franco and Handzlik,2011)

2.4.1.3. Preventing Drug Access to Targets

The decrease in influx across the cell membrane of gram negative bacteria also cause resistant towards antibiotics.(Nikaido,2001).Such as in mycobacterium tuberculosis and gram negative bacteria it is found that they are able to resist presence of toxic compounds by combining two mechanisms as effective permeability barrier and by pumping out drug molecules.As well as they are able to interact specifically with outer membrane channels and accessory proteins,forming multisubunit complexes that extrude drug molecules directly into medium and by passing out membrane barrier.

2.4.1.4. Local inhibition of drug access

Some proteins like Tet(M) or Tet(S)binds to heribosme and changes the ribosomal conformation causing prevention of binding tetracyclines to ribosomes. Qnr proteins coded in plasmids is predicted to protect DNA topoisomerase from fluoroquinolones(Boiteux,Vorobyov,andFrench,2014).

2.4.1.5. Drug-specific efflux pumps

Efflux was discovered in the tetracycline resistance gram negative bacteria in the presence of the protein named TetA which catalyze a proton–motive–force-dependent efflux pump and pumps outward the tetracycline-Mg complex(Saier,.Paulsen and Sliwinski,1998).

2.4.1.6. Nonspecific inhibition of drug access

The mutation in the porins of bacterial species of Enterobacteriaceae (Enterobacter aerogenes, Klebsiella pneumonia) can also reduce the permeability for the influx of the nutrients through the cell membrane and also it reduces the permeability to bulky β-lactams without affecting smaller nutrient molecules(McGovern,Helfand and.Feng.,2003).

2.4.2. Sources of the resistance genes

There are so many resistance genes that rely on many different mechanisms from where they come are (Tuma, and Prattl., 2013)

2.4.2.1. Producing Organisms

The aminoglycoside-resistant genes found in Enterococci is found to be homologs to the gene of Streptomycetes producing vancomycin.inducing the resistant trait could have emerged from horizontal gene transfer,most probably through homologous recombination.

2.4.2.2. Microorganisms in the Environment, Especially Soil

The antibiotics produced by the microorganisms in soil are being used by the different bacteria as a nutrient source causing the resistance to the antibiotics by the evolutionary origin for the resistant genes.

2.4.3. Assembly, maintenance, and transfer of resistance genes

R plasmids contain many resistant genes and also can be transferred to the other neighboring bacteria which are susceptible to that specific drug (Jiang,Ellabaan,Charusanti,2017).

2.4.3.1. Assembly of resistance genes in R plasmids.

R plasmid transferred into susceptible bacteria by the single conjugation event. Many bacteria contain resistance genes for aminoglycosides, tetracycline, chloramphenicol, and sulfonamides. Sequencing for the identification of cluster in plasmid,revealed that the early-generation R plasmids, most of the resistance genes are components of transposons, which can deliver the genes to any piece of DNA which can be seen in the plasmid R100(Bennett,2008).

R plasmid R100 consists of Tetracycline resistance gene tetA as the transposon Tn10, chloramphenicol acetyltransferase (cat) as a part of Tn9, and sulfonamide resistance gene sul1 and an aminoglycoside adenyltransferase gene aadA1.Tn21 is a particularly remarkable example of large, complex, multiply composite transposons which contains mercury resistance genes.(Smale,2010)

The sulfonamide resistance and aminoglycoside resistance genes in Tn21 were assembled as described below:

The R-plasmid contain the unique 59-base 3'-sequence tag for the remarkable area called integron which contains the coding gene, and integrase which catalyzes the insertion at the downstream of resistance gene from the strong promoter. The high mobility causes the organized form of resistance gene into single operon with same orientation of transcription under strong promoter supplied by the intergon structure. An intergon may contain eight resistant genes in a plasmid (Huovinen, Sundstrom, Swedberg., 1995).

The use of integron such as tn21 helps to hop the resistance genes between different plasmids and chromosome, the similar assembly gene mechanism is seen also seen in Vibrio cholera.Many integron are found to be associated with downstream structure called ISCR which contains putative transposes gene helping in delivering resistance genes to the closely related integron structure (Domingues and Silva, 2012).

2.4.3.2. Maintenance of R plasmids in the host cells

The chance of losing the R-plasmid during multiplication is low also if they have low copy number because it gives a correct partition of copies to daughter cells. Some natural plasmids contain the killer elements containing stable killer protein or mRNA and an unstable inhibitor protein or antisense protein and upon loss of the plasmid causes the death of the host cell, hence, maintain the population of bacteria harbouring the plasmid. (Sengupta and Austin,2011)

2.4.3.3. Cell-to-cell transfer of R plasmids

The study of molecular mechanism in E. coli related to the cell-to-cell transfer was found to be homologs to the Agrobacterium tumefaciens transfer gene virB genes that transfer piece of bacterial DNA into plants nucleus.In addition.there is effector protein,known as Type IV Secretion System, injected in human and animal pathogens.This is cut by the VirD2/TraI enzyme. The complex is also known as relaxosome,due to the nicking one strand results in the relaxation of supercoiling of the plasmid DNA.Type IV secretion systems export pure protein,but not protein–free DNA.The VirD2/TraI protein attached is taken up by ATPase so called coupling (Sengupta and S.Austin,2011).

The original strands of R-plasmid when transferred in the cells get rolled by the replication mechanism so it exists in donor and recipient cells. The entry of the DNA of foreign origin is faciliated by the strain-specific restriction endonuclease of the recipient cells.The initial DNA piece that enter is single stranded and thus may escape this mechanism. Some R also contains the functions that antagonize the attack by restriction endonucleases (Burman, 1977).

A model of the mating-pair formation complex with the coupling protein (VirD4) has been postulated. The proteins are named after the VirB components in Agrobacterium tumefaciens, but homologs for most of these proteins are found in the conjugative R plasmid transfer systems (Fernandez, Dang and Spudich, 1996).

Some plasmids do not contain the genes for the construct of mating –pair formation complex but these R-plasmids are small and which can be mobilized by the transfer genes of other plasmids transferred efficiently into recipient cells. Conjugational mechanism is found to be the main mechanism for many groups of bacteria than the horizontal transfer (Schroder and Lanka, 2005).

Conjugative transposons, a large transposon of a special class which plays an important role in bacteria, use a λ-phage-like mechanism of transposition which generates a circular DNA as an intermediate. The intermediate can be transferred to the plasmid or chromosomal DNA in the previously determined location of the plasmid to other bacteria by conjugation (Schroder and.Lanka, 2005).

In these transposons, the drug resistance genes are the part of the integrated regulatory mechanism of conjugational transfer but not a passive passengers, conjugation may also occur between both bacterial types gram positive and gram negative bacteria(Munoz-Lopez andGarcia-Martin,2010) making genetic transfer in cross species, also.

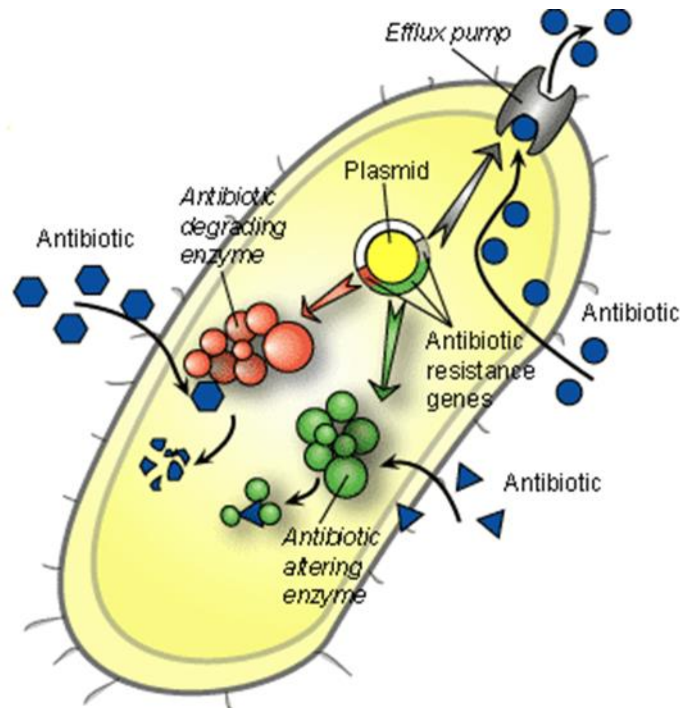


Figure2.1: Mainly three mechanisms occurred in the bacteria including inactivation of antibiotics, antibiotics resistance gene and assembly, maintenance and transfer of resistance genes (Todar, 2009).

2.5. Literature review related to efflux

Efflux is known to be important mechanism for the intrinsic and acquired drug resistance in numerous eukaryotic and prokaryotic organism which is been associated with the pathogenicity,virulence,biofilm formation and quorum sensing (J.Szumowski, K.Adams, P.Edelstein et.al,2013). *Mycobacterium* has high efflux activity among the microorganism which causes resistance towards drugs so also called as EP MDR. *Mycobacterium smegmatis* was use as the model for expression of the heterologous EP with large genome. LfrA is firstly characterized in *Mycobacterium*, expressed in multiply plasmid, confer low-level resistance to floroquinolones, ethidium bromide, acridine and some quaternary ammonium compounds (Silva, Groll, Martin et al., 2011).Similarly, other two EP were TetV for tetracycline resistance and Tap confers low resistance to tetracycline and aminoglycosides when over expressed in Mycobacterium smegmatis(Ramon-Garcia,.Martin, and Ainsa ,2006).

The active efflux is also one of the mechanisms to the resistance to the drugs such as tetracycline. The *S.aureus* strains which are resistant to the multiple cationic bacteriocides are emerging cause of hospital-acquired infections which coded with the multidrug efflux transporter QacA (or QacB), belonging to the Major Facilitator superfamily (MFS),first multidrug efflux pump identified in bacteria.

EPs is also known as the transporters classified in different five subfamilies: ATP-binding cassette(ABC),major facilator super families (MFS),resistance nodulation division(RND)as a secondary transporter which typically energized by proton motive force but among

them ABC is considered to be the primary transporter using ATP as energy source (Slipski,Zhanel and.Bay,2018).

The gene cluster drrA-Drrb-drrC, having similarity to ABC exporter of daunorubicin with *Streptomyces* species causes the resistance to broad range of clinically relevant antibiotics when overexpressed in *Mycobacterium smegmatis* (Braibant.Gilot, and Content, 2000). Similarly, ABC transporter coded by the genes Rv2687c-Rv2688c-Rv2688c increased minimal inhibitory concentration of ciprofloxan by eight fold while the operon was overexpressed but when only the gene Rv2687c increased by only four fold (Pasca, Guglierame, and Arcesi, 2004).

There are several ABC transporter but among them 12 putative EP genes were identified:Rv0194,Rv1218c-Rv1217c,Rv1348-Rv1349,Rv1456c-Rv1457c-Rv1458c,Rv1473-Rv1667c-Rv1668c,Rv1686c-Rv1687c,Rv1819,Rv2477,Rv2688c-Rv2687c-Rv2686 and drrA-drrB-drrC (Brabant,Gilot,Content ,2000). Rv0194 gene was found to be the novel gene through the molecular analysis of β-lactam antibiotics analysis, whose low expression causes the increase in resistance to many antibiotics including BCG (Danilchanka, Mailander and Niederweis, 2008).

Different genes are known to show resistance towards different antibiotics such as Tap protein and LfrA showed resistance to tetracycline and fluoroquinoloes,Rv1410c gene code the protein P55 showed resistance to the Streptomycin, tetracycline and also require cell surface.LprG lipoprotein to function properly which is require for the survival of Mycobacterium during infection (Lancioni,Li,and Thomas,2011).and also Mmr protein showing resistance to acriflavine,ethidium bromide and erythromycin while expressed in a multicopy vector. As well as when there is overexpression of mmpL7 gene in *Mycobacterium smegmatis* code for the RND transporter showing resistance to the isoniazid and can be reversed by the use of EP inhibitors.(Pasca,.Guglierame,and Rossi,2005).transporter present in MATE family(Multidrug And Toxic Compound Extrusion) that is *Escherichia coli* and Vibrio sps are not reported in *Mycobacteria*(Peracchi, and Mozzareli,2011).Rv 1258c and Rv1410 (R55) produce resistance to tetracycline and aminoglycosides.

The correlation between the resistance EP gene and the inducers are necessary to know for the invention of new antibiotics for the *Mycobacterium tuberculosis* (Aeschlimann, Dresser, Kaatz et al., 1999) . The Staphylococcus aureus does not produce 3’, 5’cAMP, which plays important role in physiological activity of bacteria may be competitive for S-adenyl-l-methionine causing the inhibition of the growth of the bacteria.

2.5.1. Multidrug Efflux Pumps Belonging to the Major Facilitator Superfamily

MFS is one of the largest families of transporters and contains many important efflux pumps. These pumps actively remove monocationic biocides and dyes, such as benzalkonium chloride, cetyltrimethylammonium bromide, ethidium bromide and also QcaA pumps out the dicationic biocides (pentamidine isethionate and chlorhexidine)(Kumar,.He,P,Kakarla et al.,2016).Trans membrane region contain the 14 trans membrane segments and residue of several acidic aminoacids.QacA act to remove out of the dicationic compounds depending on presence of an asparate residue in the TMS 10(Kumar, and Varela ,2012).

QacA pump is repressed by the QacR repressor, which remove the repression which results in expression of pump. It can bind two ligands to different minipockets of the binding site,TtgR family as QacR but regulating a transporter in the resistance –nodulation –division(RND)family was crystallized with several antibiotics and plant secondary metabolites which has larger binding pocket and some ligands utilize the hydrophobic area of pocket but the natural phenolic group known as phloretin has high affinity to bind to the deep valley within the pocket, with the help of H-bond interaction with the wide range of ligands which regulates the cognate RND pump(Sun,Deng and Yan,2014).

EmrB, a multidrug transporter which is being coded in the chromosomal gene such as carbonyl cyanide m-chlorophenyl along with the EmrA, a periplasmic protein which connect Tolc, the pump to outer membrane channel as described in the section on RND family pumps (Lomovskaya and Lewis, 1992).

2.5.2. MFS Pumps with 12 TMSs

NorA, chromosomally codes for resistance to fluororquinoloes, cationic dyes and cationic inhibitors including puromycin and tetraphenylphosphonium.*S.aureus* contains other homologus related to NorA ,NorB and NorC which produce similar type of phenotype and all these efflux pumps are inhibited by reserpine, sensitization of bacteria to substrate drugs in the presence of reserpine, so it can be used as the effective tool for the efflux process to resistance(Juarez-Verdayes,.Ortega, Rodrigrez et al.,2012).

Different MFS pumps had been studied among them some are LmrP of *Lactococcus lactis* pumps out cationic dyes, daunomycin, tetracyclines, and macrolides which can also act as a vaccum cleaner (Poelarends,Mazurkiewicz,and Konings,2002) as well as MdfA of E.coli is been studied but the deletion of it did not show any effect on drug susceptibility but it is seen that the deletion of the mdfA showed that they were hypersensitive to the alkaline pH,presumably because MdfA functions as a K+/H+ antiporter (Bibi,Adler,Lewinson et al.,2001).This is suggestive of the similar function for a chromosomally coded “monodrug efflux pump,”TetB(L),of Bacillus subtilis (We,.Bechhofer,2002).The gene EmrD of E.coli pumps out the uncouplers,quaternary ammonium biocides as well as sodium dedecylsulfate.The central cavity is lined with the aliphatic and aromatic substrate(Naroditskaya,Schlosser,Fang et al.,1993).

2.5.3. Multidrug Efflux Pumps of the Small Multidrug Resistance Family

Efflux pumps were found to be on chromosomes of gram-negative bacteria present in Small Multidrug resistance (SMR) family. These proteins press out the cationic compounds such as quaternary ammonium biocides or ethidium which represents the smallest transport protein containing only four TMA in a 110-residue sequence in EmrE of E.coli.Glu14 is found to be only one charged residue found in trans membrane segment with high unusual high Pka of 8.5.The residue become deprotonated when the transporter encounters the substrate as shown by the transporter solution in detergents. The common binding site is shared by the inward flux and outward flux of substrate. The structure is found to be symmetrical on the basis of biochemical and electron microscopy but some publication suggested it to be dimer one facing outside

and other facing inside which was done by the use of faulty software (Gottschalk,Soskine,Schuldiner et al.,2004).

2.5.4. Multidrug Efflux Pumps of Resistance-Nodulation-Division Family

Transporters of this family play, by far, a predominant role in the multidrug resistance of gram-negative bacteria.

Resistance-nodulation-division pump usually exists as a part of a tripartite complex (DIVIDED IN OR CONSISTING THREE PARTS)

Efflux pumps play an important role in converting gram negative bacteria into multidrug resistance bacteria due to the two classes of proteins:TolC of E.coli which is outer membrane factor of protein and adapter proteins found in periplasm such as AcrA of E.coli and MexA of P.aeroginosa which known as membrane fusion protein. Chemical Cross-linking and isolation is carried out to confirm the association of these all three proteins. The export of the drugs occurs into the external medium but not in periplasmic space. This may be a great advantage due to once exported in external space should again transfer through outer membrane to enter in the bacteria. The outer membrane barrier is the main cause for the most gram negative bacteria towards the lipophilic antibiotics as intrinsic resistance (Daury,Orange,Taveau et al.,2016).

Hence, the inactivation of the RND pump AcrB of E.coli makes the bacteria most susceptible towards the antibiotics the minimal inhibitory concentration (MIC) such as lipophilic penicillin; cloxacillin goes down from specific concentration 512ug/ml to 2ug/ml even when there is the presence of the outer membrane barrier(Daury,Orange,Taveau et al.,2016).

The model of the AcrB-AcrA-TolC tripartite complex exports drugs directly into the medium. The Trans membrane domain of the AcrB pump trimer is embedded in the cytoplasmic membrane, whereas its periplasmic domain is connected to the TolC channel (Hayashi, Nakashima, Sakurai et al., 2016).

Not only the antibiotics but also the dyes, detergents and even solvents are also pumps out by the Resistance-Nodulation-Division (RND) pumps .The aminoglycosides are also pump out but can only carried out by the homologs AcrD.Due to the regulatory responses or mutation causes the increment in the regulatory responses of the chromosomal genes causing the bacteria more resistant to the antimicrobial agents (Yu, Aires, and Nikaido, 2003).

Properties of some Resistance-Nodulation-Division (RND) multidrug efflux pumps

Some RND pumps can capture the drugs in the periplasmic space, among them one is being predicted that carbenicillin, which contain two carboxylate groups so cannot cross the inner (cytoplasmic) membrane, and is also a good substrate for these pumps, which allows the pumps not only to prevent the entry of drugs into the cytoplasm ,but also to extrude agents, such as β-lactams, that have targets in the periplasm.The RND tripartite pumps collaborate with the simple efflux pumps synergistically which helps in creating the specific shape under pressure through the periplasmic area (Venter,Mowla,Ohene-Agyei et al.,2015).

2.5.5. Regulation of RND pump expression

Sometime the substrate can also induce the efflux pumps such as MexXY found in the P.aeruginosa which is much complex, than the QacA whereas the repressor QacR is repressed by the binding of the inducer drug. Aminoglycosides does not inhibit the ribosomal function as inducer drug, so involve another protein(Morita,Tomida,and Kawamura,2012).

The efflux pumps is mostly over produced by the mutation in different chromosomal repressor genes such as mexR;nalD, NfxB repressor,MexT and also on different genes PA3721 and called nalC phenotype causes the overproduction of strains as well as instead of mexT gene mutation occurs in the gene PA2491;which codes the different enzyme like molecule in different operon such as MexAB-OprM The efflux pumps is mostly over produced by the mutation in different chromosomal repressor genes such as mexR;nalD, NfxB repressor,MexT and also on different genes PA3721 and called nalC phenotype causes the overproduction of strains as well as instead of mexT gene mutation occurs in the gene PA2491;which codes the different enzyme like molecule in different operon such as MexAB-OprM operon system, mexCD-oprJ operon system and MexEF-oprN operon(Morita,Cao,and Gould ,2006).

The expression of acrB operon repressed by the AcrR, but the small effector molecules which binds to the repressor is not known. MarA and SoxS are the exceptional small protein containing DNA-binding domain and regulation occurs at the level of production of these proteins. The MarA production is regulated by the presence of the repressor MarR, and become inactivated by the presence of salicylate and plumbagin.TransketolaseA is increased under the oxidative stress binds to MarR and relieves its repression of MarA production .Level of SoxS is determined by its repressor SoxR containing the Fe-S centers and become inactivated by the superoxide. Rob, constitutively expressed having large size similar to that of MarA and SoxS containing domain outside the DNA-binding domain.it gets inactive by the binding substrate like molecules, such as α, α′-dipyridyl, fatty acids, and bile salts (Duval,and Lister,2013).

Some RND-transporter such as MdtBC is regulated by the two component system, of BaeSR,; MdtBc which is up regulated by indole and AcrD but it not clear whether indole is sensed by the sensor kinase BaeS.The soluble receptor for the quorum sensing signal acyl-homoserine lactone, SdiA driven from plasmid helps in increased expression of AcrAB, but the presence of hexanoyl-homoserine lactone did not increased the acrAB operon (Du.Wang.and James ,2014).

2.5.6. Biochemical and crystallographic studies

By the study of the RND type efflux, the fact that the pumps functions as the multicomplex protein complexes spanning two membranes. β- Lactams when added to the E.coli cells, they diffuse to the outer membrane and periplasm which is either hydrolyzed by the β-lactamase of periplasmic or removed by the AcrAB-TolC pump. The efflux caused in the periplasm is obtained by the rate of influx and the hydrolysis rate. It is been confirmed by the use nitrocefin but did not show the positive cooperativity as shown by with cephalothin, cephamandole, and cephaloridine which was found to be much higher K0.5 values(Pradel et al.,2002).

Reconstitution made in purified RND transporter into the proteoliposomes, due to the substrate character which is lipophilic and can easily cross the bilayer, the assay used is the movement of the labeled phospholipid from the exporter –containing vesicles that do not contain the proteins. This study showed that the AcrB pump act as the proton antiporter which is also shown with the conjugated bile salts with the high affinity. Addition of the AcrA also increases the rate of transport strongly. AcrA is essential in producing the aminoglycoside pumping activity of reconstituted AcrD, where such association of vesicles is not needed and AcrA probably activates directly the pumping activity of AcrB. It is found that the homologus of AcrA whose structure is not known is found to be in C-terminal is necessary to interact with AcrB (Poulikakos P and Falagas ME, 2013).

The crystallographic structure of AcrB trimer, aproton antiporter is found to be solved by the (Murakami et al., 2002).The structure of MexA and AcrA are also observed in 1999, and in crystal structure it is found to be elongated proteins and has the tendency of packing to form hexamers and heptamers(Nehme and.Poole,2005).

Most important pieces of knowledge concerning the RND pump complex came from crystallography. Thus, the structure of the outer membrane channel TolC was solved in 2000, followed by the structure of its P. aeruginosa homolog, OprM. These proteins exist as a tightly woven trimer, containing a single 12-stranded β-barrel traversing the outer membrane and a remarkably long (~70-Å) periplasmic extension of the channel in the form of long α-helical bundles(Murakami et al.,2002) solved the crystallographic structure of AcrB trimer in 2002, which was a first for a proton antiporter. The periplasmic portion is at least as large as the transmembrane portion, and the top of the periplasmic domain, the TolC-binding domain, has a dimension that is similar to the tip of the α-helical bundle of TolC. This was followed by the crystallographic elucidation of the structures of the central portion (about two-thirds) of the adaptor proteins MexA and AcrA (Symmons, Bokma, Koronakis et al., 2009).

These are elongated proteins, as shown in 1999 and in crystals show a strong tendency to become packed side by side to form hexamers and heptamers. Thus, the structures of all three component classes of the tripartite assembly are known, and it is possible to propose how these proteins are assembled together. In such models, the coiled-coil domain of the adaptor is assumed to interact with the outer membrane channel protein, a hypothesis that is supported by biochemical data. Studies with chimeric constructs of AcrA homologs showed that the C-terminal domain, whose structure is not known from crystallography, is essential for interaction with AcrB. Mutant studies also suggest that the domain close to this end of AcrA, containing α + β structure, is the main site of its interaction with the periplasmic domain of RND pumps (Kim,Xu,.Lee et al.,2010).

Outer membrane component TolC and its homologs are synthesized by the signal sequence and exported in the outer membrane .The periplasmic adaptors either contains lipoprotein bound with the lipid at its N-terminus or hydrophobic N-terminal helix and thought to become bound to the outer surface of the inner membrane. The signal recognition and SecY apparatus for insertion into membrane is required for the RND pump which is the classic example (Kim.Jeong, Song et al., 2015).

AcrB also capture some substrates from the periplasm because it's trimer structure contains the small opening between the subunits at the bottom of periplasmic domain close to the external surface of membrane bilayer which causes the large central cavity in the trans membrane domain .So, it is predicted that the drugs diffuse through the vestibule is captured in the central cavity. It has been assumed that there is not the correct path to extrusion which was not clear in the crystal structure. The crystallization of AcrB showed the conformation mimicking that of a transient intermediate during the drug extrusion. The transmembrane domain of the AcrB is found to be similar in conformation to the one promoter named extrusion promoter (Yu, Aires, McDermott et al., 2005).

In binding promoter, the periplasmic domain has several aromatic residues in binding pocket which open between the binding pocket and vestibule.in extrusion promoter the binding pocket is narrower and the pathway to vestibule become closed. These observations suggested that the promoters within trimeric AcrB go through a cyclic conformational change, from the open conformation through the ligand-bound one to finally the extrusion conformer, whose Trans membrane domain shows signs of a disrupted network among proton-trans locating residues. The cyclic changes involve the opening and closing of the large external cleft in periplasmic domain by the forced closing of this cleft by the fast acting disulfide cross-linking agents stop the function of pumps instantaneously and gives the biochemical support for this hypothesis(Yao.Kimura,Murakami et al.,2013).

A giant gene containing three copies of AcrB genes which are connected with the linker sequences which showed that the inactivation of one promoter in the trimeric complex completely abolished the pumping activity, which support the functionally rotating mechanism. Cut out view of the binding protomer with the bound minocycline (in a ball-and-stick representation in green) (a) and the extruding protomer (b), both from Protein Data Bank file 2DRD. Molecular graphics images were produced using the UCSF Chimera package (Takatsuka and Nikaido, 2009).

The binding site in asymmetric structure is large and flexible. The two substrates which are co crystalized with AcrB is found to be minocycline and doxorubicin, which occurs different location of cavity which explain the wideness of the substrate of this pump. The pocket in AcrB favors lipophilic substrate to bind in the hydrophobic surface .The AcrB is found to be contain many electronegative atoms, which is homology to AcrD which pumps out the basic and hydrophilic aminoglycosides. It confirmed that it as a genuine substrate binding site in this transporter (Horiyama,and Nishino,2014).

Substrate binding pockets of AcrB and a homology-modeled AcrD are Residues facing bound minocycline (in a ball-and-stick model), i.e., residues 177, 178, 275–278, 610, 612, 615, 620, 626, and 628 of the binding protomer of AcrB in Protein (Du,van Veen,Murakami et al.,2015).

Finally, we note that inhibitors of RND multidrug efflux pumps have been developed.

I. "Natural" substrates for multidrug efflux RND pumps

MFS pump is also regulated by other more physiological functions than the exogenous chemicals one of them is Blt of Bacillus subtilis which detoxifies

spermidine.(D.Woolridge,N.Vazquez Laslop,P.Markham et al.,1997).The main function of AcrB of E.coli is to protect the bacteria from the bile salts, detergent which are found in the surviving environment of E.coli, especially in the intestinal tract of vertebrates.AcrAB-TolC system is reported to be the essential to the Salmonella spp but for the other bacteria which deals with different bile acids needs AcrAB pump for their survival.(J.Sun,Z.Deng,A.Yan,2014). In the Pseudomonas syringae, the inactivation of RND pump causes the decrease in the secretion of lipopeptide phytotoxins, but it was found to be modest (41% to 67%)(Stoitsova,Braun,Ullrich et al.,2008).

Some soil organism including Pseudomonas aeroginosa mostly uses the RND pumps to exclude the toxic compounds. The loss of RND pumps causes the loss of the invasiveness in the cell culture but I's mechanism is not clear till now (Dreier and Ruggerone,2015).

II.Contributions of RND efflux pumps to the resistance in clinical strains

The RND efflux has caused the increase in the intrinsic factor in bacteria causing resistance to the different bacteria causing decrease in the permeability.Fluoroquinolone resistance in Pseudomonas aeruginosa is mostly due to the mutational alternation of the targeted topoisomerase, high level resistance causing increase in efflux. Multidrug efflux also plays resistance to increasingly use of different disinfectants in household products like soaps, because such compounds may select for pump overproduction mutants (Blair and Piddock,2016).

III.RND transporters that pump out nondrug substrates

NodGHI protein is fused with the RND protein which helps in secretion of nodulation factors or lipochitin oligosaccharides by the Rhizobium. In the Niemann-PickC1 disease, human proteins a member of RND family while mutated causes the accumulation of cholesterol in some organelles which is involve in the transport in the intestinal epithelial cells(K.Pos, 2009).

There are many examples of RND transporters in the genome of M. tuberculosis. One of them (MmpL7) is involved in the export of complex, a polar lipid, phthiocerol dimycocerosate and another one (MmpL8) in that of 2,3-diacyl-α,α′-trehalose-2′-sulfate, a precursor of sulfatides.

RND transporters also play a major role in resistance of gram-negative bacteria against toxic metals. CzrA, an RND transporter in Ralstonia that pumps out Co2+, Ni2+, Cd2+, and Zn2+, has been studied intensively, and an early successful reconstitution of an RND pump has been achieved in this system (Bolhuis,Van Veen,Poolman et al.,1997).

IV.Other Multidrug Efflux Pumps Energized by Ionic Gradients

NorM of Vibrio parahaemolyticus,a protein with 12 trans membrane helices, pumps out the fluoroquinoloes and ethidium for the influx of Na+.Similarly, similar kind of pump is also found in Bacteroides fragilis(Braibant,Guilloteau,and Zygmut,2002).

V.Multidrug Efflux Pumps of the ATP-Binding Cassette Superfamily

Transporter of the ATP-binding cassette also plays an important role in multidrug resistance. The protein LmrA of Lactobacillus lactis is homologous to one-half of the

mammalian MDR1 protein.(Hurlimann,Corradi,Hohl et al.,2016).The biochemical studies to this pump have related the mechanism for the coupling of drug extrusion and ATP hydrolysis and to the discovery that the trans membrane domain of this pump can alone catalyze a proton-gradient-dependent flux of drugs ABC drug exporters of gram-positive bacteria is found to be BmrA of Bacillus subtilis and Sav1866 of Staphylococcus aureus which helps in study of ABC transporter(Orelle,Durmot,Mathieu et al.2018).

MacB in E.coli act as the structural gene for periplasmic adaptor protein MacA and resist the macrolides when overexpressed. ATPase activity of MacB is stimulated by MacA showing that the membrane fusion protein has the function of other than structural one(Lin,Bavro,Barrera et al.,2009).

2.6. Literature review on kinases

Kinase, enzyme helps to catalyze the substrate by transferring the phosphate group from the high energy ATP molecules and convert into the formation of the ADP which occurs during the glycolysis. The kinases help in various critical activities in metabolism, cell signaling, protein regulation, cellular transport, secretory processes and other cellular pathways.There are different types of kinases such as:

- Protein kinases.
- Lipid kinases.(https://en.wikipedia.org/wiki/Kinase)
- Carbohydrates kinases.
- Other kinases. (http://www.ebi.ac.uk/interpro/entry/IPR000577)

Figure2.2: Overview of the different kinases in the cell signaling pathways

2.6.1. Protein Kinases

Protein kinases are those that act only on to the protein in phosphorylating them at their serine, threonine, tyrosine or histidine residues. Phosphorylation helps in modification of the three dimensional structure of the proteins and subsequently increases or decreases protein's activity.This causes either stability or destruction of the protein while it interacts with the other proteins. It is one of the major studied kinase among all kinases, which plays a major role in conjunction with phosphatases for the protein an enzyme regulation in cell. Phosphorylation is the one of the reversible covalent modifications. The modifications in the protein can be made by the allosteric control (Nishi, Hashimoto, Panchenko., 2011).

Allosteric control helps in controlling the signals inside the cells whereas the phosphorylation control the activity outside the cell was proved by Edwiin Krebs and mostly the phosphorylation is carried out in eukaryotic cells in comparison to prokaryotic cells due to the complex cell type causing the wider array of signals (Manning.Whyte and Martinez, 2002).

2.6.1.1 Cyclin Dependent Kinases (CDK)

It is group of different kinases which helps in cell cycle that phosphorylates respective interacting proteins at the threonine or serine.But to perform this enzymatic reactions

the CDKs should first bind to cyclin protein for its activation (Malumbres,2014) hence called cyclin dependent kinases(CDKs). Activated CDK is critical to their activity so they are also activated by other kinases and phosphatases (Cheng,et al.,1999). The activated CDK helps in changing the activity of the other proteins which leads in the next stage of cell cycle. The most important function is to control the cell cycle including the transcription, metabolism and other cellular events (Barford,2001). It is found in the cancerous cells due to the controlling activity of cell division, mutations in it leads in uncontrolled growth of the cells.CDK mutation is mostly found in lymphomas, breast cancer, pancreatic, tumors and lung cancer. So, inhibition of CDK can be a target for treatment of some types of cancer (Malumbres, Manning and Whyte ,2014).

2.6.1.2.Mitogen-Activated Protein Kinases

It is the type of kinase of family serine/threonine in which the protein is used for its activation which helps in respond to the various kind of extracellular growth signals. There are various factors which is considered as mitogenic stimuli pathway such as, growth hormone, epidermal growth factor, platelet-derived growth factor, and insulin. Various kinases such as RasGTPase helps in exchanging GDP to GTP;Rafkinase which activates MEK(MAPKK).MEK activates MAPK (ERK) helping in regulation of transcription and translation it is a serine/threonine kinase whereas MEK is a tyrosine kinase(Robinson and Cobb,1997).

Varieties of mitogenic signals engage the MAPK pathway and promote cell growth and differentiation through a kinase cascade. MAPK can directly or indirectly regulates the transcription as well as the translation by the phosphorylation of S6 kinase in the large ribosomal subunits The major transcriptional subunits targets are ATF-2, Chop, c-Jun, c-Myc, DPC4, Elk-1, Ets1, Max, MEF2C, NFAT4, Sap1a, STATs, Tal, p53, CREB, and Myc(Mitogen-activated protein kinases: new signaling pathways functioning in cellular responses to environmental stress)(Cowan,2003).It can also phosphorylate components in the upstream portion of the MAPK signaling cascade including Ras, Sos, and the EGF receptor itself (Tena, et al.,2001).MAPK pathway is considered to be the one of the carcinogenic potent for the carcinogenic application(Plotnikov.Zehorai and.Procaccia ,2011).

This pathway helps cells in uncontrolled growth and tumor formation. Mutation in this pathway affects the activities in cells which helps in causing cancer such as cell differentiation, proliferation, survival, and apoptosis(Kennedy ,Davis and Roger J.,2010).

2.6.2. Lipid kinases

Lipid kinases help in the phosphorylation of the lipids in the plasma membrane as well as in the organelles membrane. Addition of phosphate group helps in change of reactivity and localization of the lipid and helps in transmission of signal.

There are different type of the kinases which helps in phosphorylation of different types of lipids such as:

- Phosphatidylinositol kinases.
- Sphingosine kinases

(Loo.Wright, and Zylka, 2015)

2.6.2.1. PhosphatidylInositol Kinases

While the insulin binds in the receptor there is the formation of PI3 kinase which dock in the membrane where it can phosphorylate PI lipids. Phosphatidylinositol kinases phosphorylates phosphatidylinositol species to create the species such as phosphatidylinositol3,4-bisphosphate(PI(3,4)P2) , phosphatidy linositol3 ,4,5 tri s phosphate (PIP3) and phosphatidylinositol 3-phosphate(PI3P).The kinases phosphoinositide-4,5-bisphosphate 3-kinase, include Phosphoinositide3-kinase and phosphatidylinositol-4-phosphate 3-kinase.The phosphorylation of the phosphatidylinositol plays a significant role in the cellular signaling such as insulin signaling pathway ,endocytosis, exocytosis and other trafficking events (Carpenter,1996).So, mutation in the P13K can cause cancer or insulin resistance.

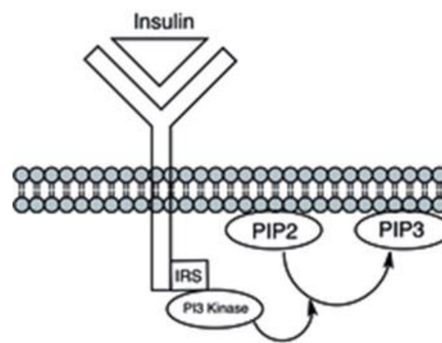


Figure2.3: The kinases used for the phosphorylation of the PI lipids after binding of the insulin.

The kinase helps in increase the rate of reaction by making the inositol hydroxyl group more nucleophilc, also by the use of the chain of the amino acid residue for the production of the general base and deprotonate the hydroxyl which as shown below. In figure phosphatidylinositol react with the adenosine triphosphate (ATP) and generates adenosine diphosphate(ADP) that helps for the proper orientation of the ADP molecule as well as the inositol group to carry out the reaction faster as well as metal can also be used for the enhancement of the reaction (Gehrmann,and Heilmeyer,et al.,1998).

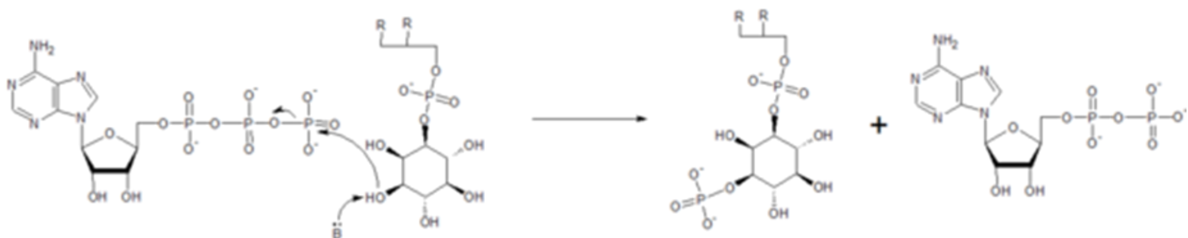


Figure 2.4: Mechanism of the ADP formation due to the of reaction phosphatidylinositol with the ATP

2.6.2.2 Sphingosine Kinases

Sphingolipids are found all over in the membrane of the cells to convert the sphingosine into sphigosine-1-phosphate (S1P) sphingosine kinase plays a vital role. Upon the activation the sphingosine kinase migrate from the cytosol to the plasma membrane and then transfer the γ phosphate from ATP or GTP to sphingosine.The S1P receptor is also known as GPCR receptor ,which is the combination of large protein family that helps in detection of molecules outside the cells and also helps in the signal transduction that then helps in regulation of G-protein signals (Hansen,Kasper,2000).The generated signal can activate the intracellular effectors like ERKs ,RhoGTPase,RacGTpase,PLC and AKT/P13 K.S1P showing direct inhibition of the histone deacetaylase activity ,class of enzyme that remove the acetyl group (O=C-CH3) from an ε-N-acetyl lysine amino acid on a histone, allowing the histones to wrap the DNA more tightly.DNA is wrapped around the histone by the acetylation and deacetylation (Zhang, Williams, Huang et al.,2001).

The apoptosis is carried out by the cells due to dephosphorylating of the sphingosine so it is important to understand the regulation because of the role in cell development and differentiation(http: //groups. molbiosci. northwestern. (Edu /Holmgren /Glossary /Definitions/Def-C/cell_fate.html). Some research had shown that Sphingosine kinase may sustain the cancer cell growth due to their enhancement for cell-proliferation and specific Sphingosine kinase (SK1) found to be in high concentration in certain cancer cells (Maceyka,Payne,and Milstien,2002).

SK1 and SK2 are the two kinases found in the mammalian cells among two kinases SK1 is more specific than SK2 as well as their expression pattern is also different from each other.SK1 is found in lung, spleen, and leukocyte cells whereas SK2 found in kidney and liver cells. They can be included as the target for the chemotherapeutic therapies due to their activities in cells survival, proliferation, differentiation and inflammation.So,for the specific site target on its pathway computational tools are introduce for treatment of cancer(Ozbayraktar and Ulgen,2010).

2.6.3. Carbohydrate kinases

The carbohydrate, oligosacchrides which is used as a food source for the different animals need to be converted into the monosaccharide which is easy to enter in the metabolism by the use ATP. In this process, the kinase plays an important role in metabolism activity. During glycolysis the formation of the 1,3-bisphosphogylcerate is one of the unstable compound formed which requires the 1,3-bisphosphogylcerate kinases as well as ADP for yielding 3-phosphoglycerate and ATP .In the final step the pyruvate kinase helps in transferring the phosphoryl group from phosphenolpyruvate to ADP and generates ATP and pyruvate (Zhang et al.,2011).

Hexokinase one of the enzymes which helps in conversion of glucose into glucose-6-phosphate by transferring gamma phosphate of an ATP to the C6 position(Phosphorylation of C6- and C3-positions of glucosyl residues in starch is catalysed by distinct dikinases)(Ritte et al.,2006) is also considered to be the one of the important steps of glycolysis. It helps to trap the glucose inside the cell due to the negative charge on the cell membrane. Mutation in hexokinase can lead into hexokinase deficiency which can lead to anemia (Bianchi et al.,1997).

In addition, phosphofructokinase catalyzes the fructose-6- phosphate to fructose-1-6-bisphosphate and is important regulation of glycolysis. High level of ATP, H+ and citrate inhibit the phosphofructokinase. This is because that indication of the high citrate level means glycolysis is working in an optimal rate (Bianchi et al., 1997). High level of Adenosine Monophosphate (AMP) stimulates phosphofructokinase. The mutation on the phosphofructokinase gene causes reduce in its activity causing disease named Tarui's disease (Maughan and Ron, 2009).

Carbohydrate kinases use the ATP for the phosphorylation of the different sugar substrates.These enzymes includes different kinases such as L-ribulokinase (EC:2.7.1.16) (gene *Ara*B); Erythriol kinase (EC:2.7.1.27) (gene *Ery*A); L-fucolokinase (EC:2.7.1.51) (gene *Fuc*K); gluconokinase (EC:2.7.1.12) (gene *Gnt*K); glycerol kinase (EC:2.7.1.30) (gene *Glp*K); xylulokinase (EC:2.7.1.17) (gene *Xyl*B); L-xylulose kinase (EC:2.7.1.53) (gene *Lyx*K), D-ribulokinase (EC:2.7.1.47) (gene *Rbt*K); and rhamnulokinase (EC:2.7.1.5)(Zhang, Zagnitko, and Rodionova , 2011).

There are different kinases that are under the carbohydrate kinase which are:

- FGGY carbohydrate kinase,
- Pentulose kinase,
- Gluconate kinase
- Glycerol kinase
- L-fuculokinase,
- Putative glycerol kinase 5,FGGY,
- Ribulokinase,
- Xylulokinase
- **Autoinducer-2 kinase**

Autoinducer-2 kinase: All the bacteria have the ability to separate from the signal produced by the specific bacteria by the cell-cell communication (Cell-Cell Communication in Bacteria: United We Stand) (Bodman,Wiley Diggle,2008). During this process the 1st operon is induced by AI-2(Ng,Bassler ,2009). These are the signal molecules which helps in quorum sensing (Miller,Bassler,2001) , the chemical communication of bacteria for the increase in cell population number and continuity of the behavior . One of the auto inducers is 4, 5-dihydroxy-2,3-pentanedione(Regulation of autoinducer production in Salmonella typhimurium) which is produced by different bacteria and is the important autoinducer element (AI-2)(Guo et al.,2013) and this is carried out by autoinducer-2 kinase.

2.6.4. Other kinases

Kinases also act on other molecules except proteins, lipids and carbohydrates such as nucleotides (DNA and RNA) which involve the nucleoside-phosphate kinases and

nucleoside-diphosphate kinases. Other substrate of kinases includes creatine, phosphoglycerate, riboflavin, dihydroxyacetone, shikimate, and many others.

A. Riboflavin kinase

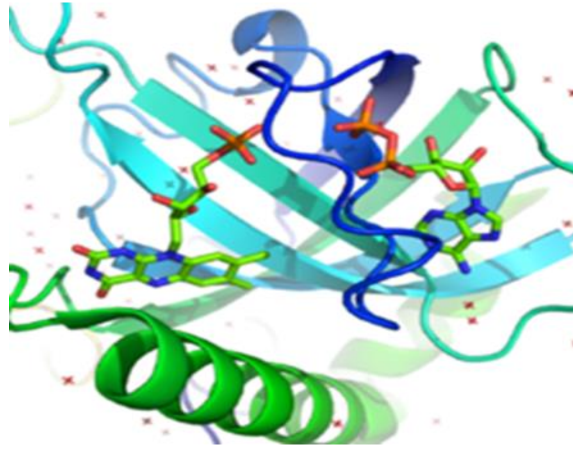


Figure 2.5: The active site of riboflavin kinase bound to its products--FMN (on left) and ADP (on right).

Riboflavin kinase helps in the phosphorylation of riboflavin to create the flavin mononucleotide.At first, Riboflavin must bind to the Kinase before it bind to the ATP molecule.Divalent cations help in coordinate the nucleotide as depicted in the schematic diagram below:

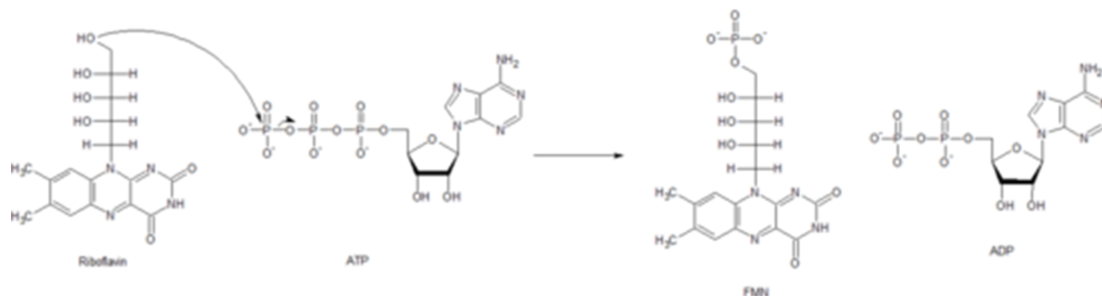


Figure 2.6: Schematic diagram of mechanism of riboflavin kinase.

The flavin mononucleotide formed by Riboflavin kinase act as the important cofactor and also acts as the precursor for flavin adenine dinucleotide (FAD), a redox cofactor used by the different enzymes involved in metabolism(Friedmann.1974) (Flavin-adenine Dinucleotide) and electron transfer(A flavin-based extracellular electron transfer mechanism in diverse Gram-positive bacteria)(Hartshorne et al.,2009). Riboflavin can be taken as the target for the prevention of the stroke and also be used for the future treatment (Giancaspero, Locato, and De Pinto, 2009).

A. Thymidine kinases

Thymidine kinases are the kinases which are responsible for the phosphorylation of the nucleoside.It produces the thymidine monophosphate (dTMP) by the phosphorylation of the thymidine. During the phosphorylation the ATP is used for the supply of the phosphate group as shown below: The transfer of phosphate occurs from one nucleotide to another by the thymidine kinase or from one nucleoside to another that helps in the control the level of different nucleotides.

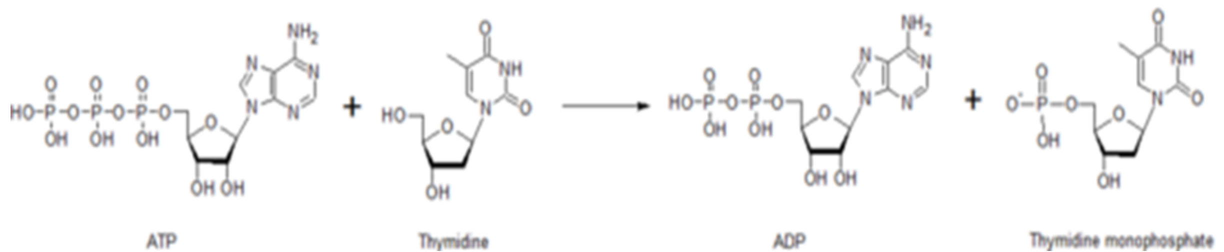


Figure 2.7: Overall reaction catalyzed by thymidine kinase

Thymidine monophosphate (dTMP) after formation could be further acted by the thymidylate kinases in the formation of diphosphate form dTDP.Nucleoside act on for further formation Thymidine triphosphate (dTTP)for biosynthesis of DNA molecules. So, the thymidine kinase gene has a close correlation between the cell cycle and is taken as the tumor marker in clinical chemistry for prediction of the prognosis of the patient. Mutation in the thymidine kinase gene causes the depletion syndrome which leads in the death in the children (Perez-Perez, Hernandez, and Priego, 2005).

B. Glycogen synthase kinase-3

Glycogen synthase kinase-3(GSK-3), mediates the addition of phosphate group in the serine and threonine amino acids. It helps in regulation of diverse range of cellular functions:

1. Glycogen synthase
2. Signaling pathway

It is an attractive therapeutic target to treat numerous human diseases including cancer, type II diabetes, inflammation, stroke, bipolar disorder and Alzheimer’s disease and other neurodegenerative diseases (Weber, Deeney and Beaudreau, 1973).

The serine/threonine kinase glycogen synthase kinase-3 (GSK-3) is taken for regulation of glycogen (storage form of glucose formed from carbohydrates) synthesis and its regulation which helps in cellular processes (Yang et al., 2006). Many abnormal activities are regulated for human pathologies including bipolar depression, Alzheimer's disease, Parkinson's disease, cancer, non-insulin-dependent diabetes mellitus (NIDDM) and others (Jope et al., 2007).

As reviewed in McCubrey, Steelman and Bertrand (2014), supression of GSK-3 activity by phosphorylation of AKT and other kinases were found to enhance cancer process where it can be presumed to act as cancer supressor. However,in some other instances oncogenic property of GSK-3 observed with stabilizing components of beta-catenin

complex.Different signaling pathways are affected by GSK-3 alpha and beta.Pathways which are deregulated by GSK3 in cancer are the PI3K/PTEN/Akt/mTORC1 and Wnt/beta-catenin pathways. Furthermore can interact with mTORC1 to regulate p70S6K (p70 ribosomal protein S6 kinase) helping in the regulation of AKT indirectly (McCubrey, Steelman and Bertrand, 2014).

In addition, GSK3 is known to exhibit its potential with interaction of both AKT and TSC2 to regulate mTORC1. Upon GSK3 phosphorylation by AKT, it further phosphorylate TSC2 showing the negative effect on mTORC because when GSK3 is phosphorylated it enhances activity of TSC2 and inhibits mTORC1, causing anti-proliferative effect (Buller et al., 2008).

Moreover, p70S6K helps in regulation of different cell activities such as cell regulation and proliferation.Deregulation of kinases can lead to type 2 diabetes, obesity, aging, and cancer (Shin et. al., 2011)

GSK-3 interacts with Wnt/beta-catenin pathway in some conditions. It inhibits the activity Wnt/beta-catenin pathway by phosphorylation of beta-catanin, resulting in proteasomal degradation of the protein. Furthermore, in some condition it promotes pathway by phosphorylation of LRP5/6 resulting in stabilization of beta-catenin. (Wu,Pan,2010).Nevertheless,GSK-3 also interacts with the Raf/MEK/ERK, Hh, Notch, and other important regulatory pathways. (Harwood,2001).Due to these contradictory roles of GSK-3 the use of GSK-3 inhibitors remains controversial though several inhibitors have been developed.(Mancinelli et al.,2017)pathways.

While many inhibitors to GSK-3 have been developed, their use remains controversial because of the ambiguous (uncertain) role of GSK-3 in cancer development. Different signaling pathways are affected by GSK-3 alpha and beta .pathways which are being disregulated in cancer are the PI3K/PTEN/Akt/mTORC1 and Wnt/beta-catenin pathways. Interaction with both Akt and TSC2 to regulate mTORC1 whereas when GSK3 is phosphorylated by Akt it cannot further phosphorylate TSC2 showing the negative effect of Akt.However,GSK-3 has negative effect on mTORC when it is phosphorylated it enhance activity of TSC2 and inhibits mTORC1,causing anti-proliferative effect . In this review, we will focus on the diverse roles that GSK-3 plays in various human cancers, in particular in solid tumors. Recently, GSK-3 has also been implicated in the generation of cancer stem cells in various cell types (McCubrey, Steelman, Bertrand et al., 2014).

There is no specific activity of GSK-3 because it can interact with mTORC1 to regulate p70S6K (p70 ribosomal protein S6 kinase) helping in the regulation of AKt indirectly. p70S6K helps in regulation of different cell activities such as cell regulation and proliferation ,disregulation of this kinases can lead to type 2 diabetes, obesity, aging, and cancer(Shin et al.,2011) .Moreover, GSK-3 interacts with Wnt/beta-catenin pathway in some conditions it inhibits the activity Wnt/beta-catenin pathway by phosphorylation of beta-catanin resulting in proteasomal degradation and in some condition it promotes pathway by phosphorylation of LRP5/6 resulting stabilization of beta-catenin.

GSK-3 also interacts with the Raf/MEK/ERK, Hh, Notch, and other important regulatory pathways. It is intriguing to consider why inhibiting GSK-3 does not have dire consequences as so many pathways will be affected which will lead to activation/suppression of various signaling pathways. However, the GSK-3 inhibitor

lithium has been used to treat patients for many years now without apparent increases in the cancer incidence. GSK-3 is a kinase with many biochemicals, biological and neurological effects. Understanding of these effects may lead to improved therapies for certain diseases and conditions.

GSK-3 inhibitors overexpression can only be effective when it as a promoters of tumor. The inhibitors exerted their anticancer activity through a complex mechanism which involved the promotion of apoptotic signals (caspase-8, caspase-3 and TP53 activation) and of protective signals (JNK activation)(GSK-3 as potential target for therapeutic intervention in cancer).It is has been found that signal transduction pathways, are deregulated in Acute Myeloid Leukemia(AML)by GSK3 and components of Bcl-2 family proteins by interplaying with each other(Ricciardi et al.,2017) .Disregulation causes leukemogenesis,promote drug resistance and favor leukemia stem cells.

AKT kinase is activated by mutations inn PI3K,PTEN,RAS and other upstream which helps in cell proliferation and survival.In this condition two isoforms of GSK-3 activation helps in destruction of oncogenic proteins such as beta Catenin,c-MYC and MYC-1 .So,in some leukemias GSK3 can act as tumor suppressor whereas GSK-3 kinase act as a promoter. An example is GSK3 targeting p27Kip1 in AML with MLL translocation (Ruvolo, Peter P, 2017).

Inhibition of GSK-3 leads to neuroprotective effects, decreased β-amyloid production, and a reduction in tau hyperphosphorylation, which are all associated with AD(Alzheimer disease).Some it has been a target and many inhibitors are developed such as:Lithium chloride,Maleimide Derivatives,Staurosporine and Organometallic Inhibitors,Indole and derivatives,Paullone Derivatives, Pyrazolamide Derivatives,Pyrimidine and Furopyrimidine Derivatives,Oxadiazole Derivatives,Thiazole Derivatives.All inhibitors are identified on basis of interaction between ATP binding pocket and inhibitors it self.Different inhibitors were found to be developed against GSK-3 among them lithium is one of them but the specific activity is not yet known. The effectiveness were further analysed by computational technique by identifying the binding sites bound with different aminoacids(Kramer,ThomasSchmidt,BorisLoMonte,Fabio,2012).

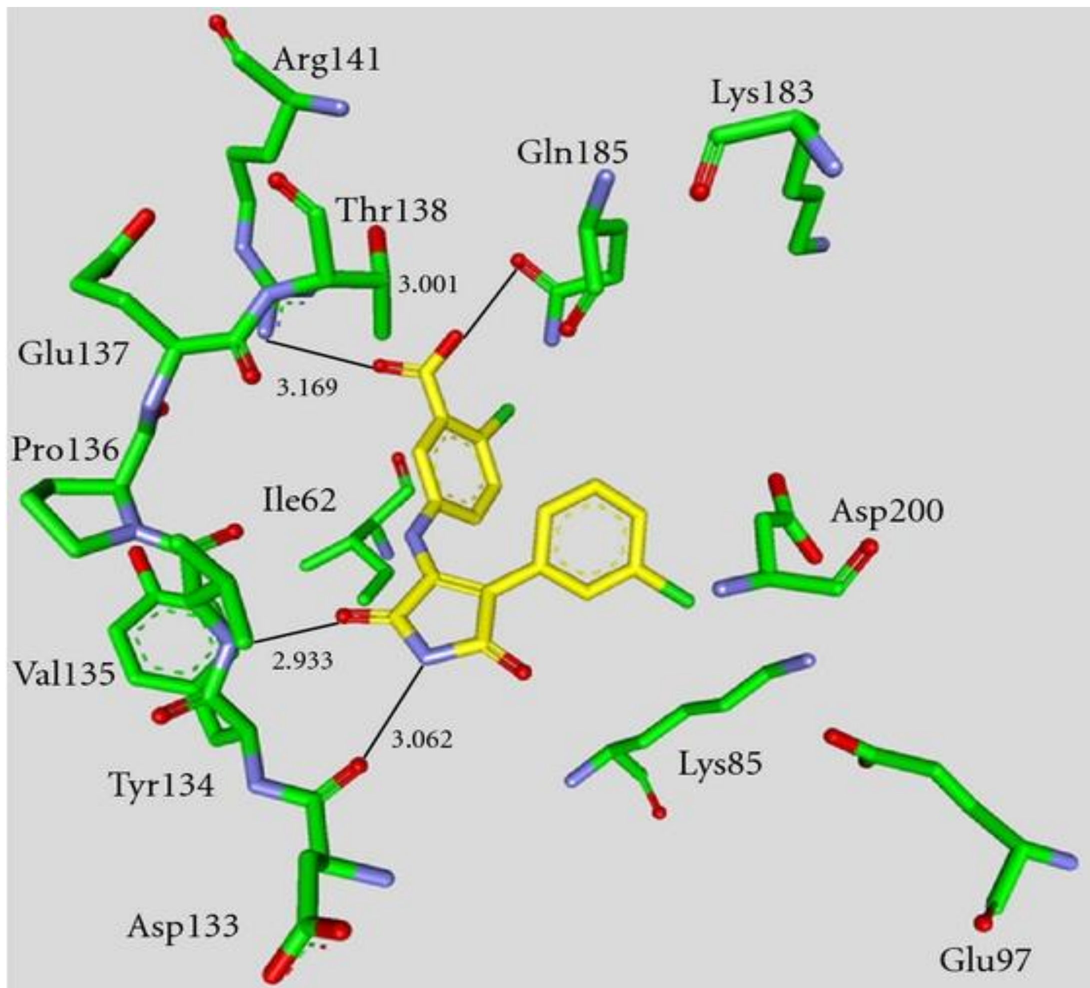


Fig2.8: Binding site of GSK-3 surrounded with aminoacids and inhibitors at ATP binding site (Bertrand et al.,2003)

Similarly colorful compound named indurubin likewise derivatives of indole and its analogue indigoids were found to be inhibitors of GSK-3 and cyclin-dependent kinases.these property was found in eleven plant extract by Chinese drug prescription Danggui Longhui Wan,which were traditionally used for treatment of chronic diseases but synthesis is limited due to limited precursor isatins and O-protected indoxyls(Wu,Aryal,et al.,2005).Among all the indurubin test the most effective was found to be indurubin-3'-monoxime inhibitiong CDK1 and CDK5.

Indole is the key factor which can convert it into indurubin in the presence of cytochrome P450 (CYP) 2A6 found in liver and CYP2A13 lungs helping in detoxification of various kinds of toxin, nicotins etc.Whereas, Cytochrome P4502A6, member of cytochrome P450 which involve in metabolism of xenobiotic in body. CYP2A6 helps in the oxidation of nicotine and cotinine but its catalytic activity is inhibited by flavone and flavanone which is found in different types of extracts of plants. The inhibition of the human CYP2A s enzymes helps in smoking decreasing the appetite and reduces exposure to the carcinogenic NNK and also decrease risk of carcinogenesis development.

The mechanisms of synthesis of indole derivatives can be easily seen in the given pathway

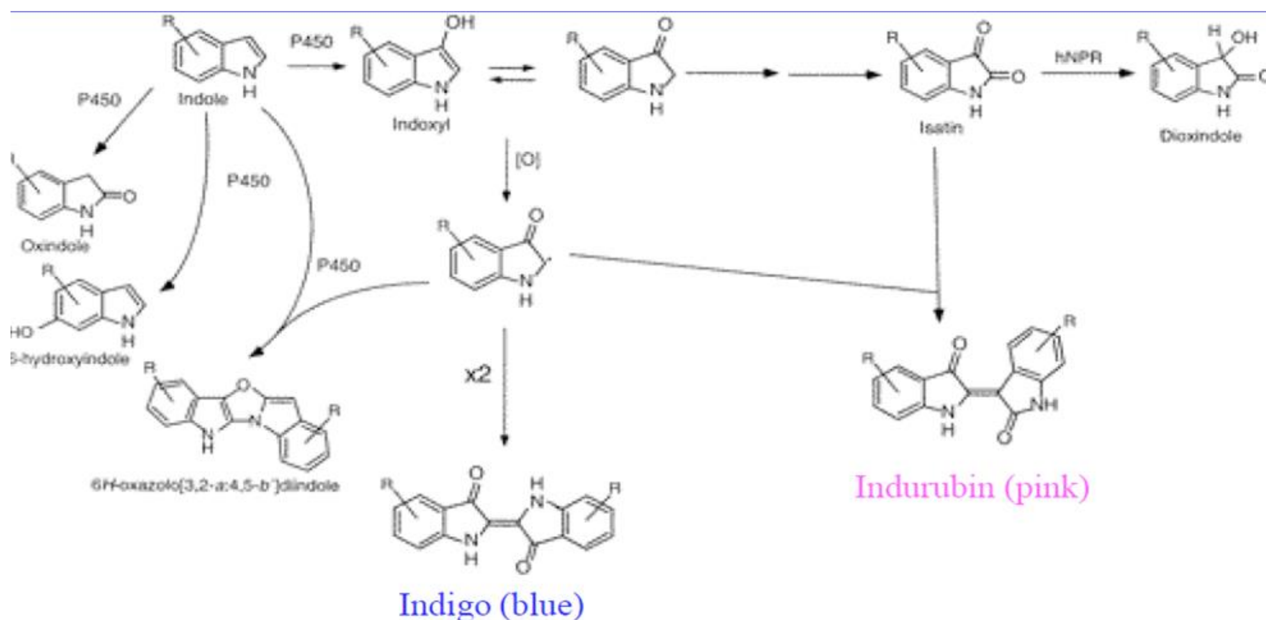


Figure2.9: Pathway of cytochrome P450 2A6 mediated metabolism of indole(Gillam et al.,2004)

The cytochrome P4502A6 helps in converting the indole into different colour forming materials such as indigo (blue) and indurubin (pink). The indole is converted into Dioxindole by the oxidation process during the process there is the reversible pathway which can convert into indoxyl.During the irreversible process the reaction with the nascent oxygen helps to convert the indole into the indigo (Blue).The isatin formed by the indole when react with the nascent oxygen can cause the formation of the indurubin(Mansilla,Maria Cecilia,and Diego de Mendoza,2000;Turnbull and Surette,2010;Shatalin et.al.,2011;Becerra et.al.,2013).

Lithium carbonate has been used to treat mania patients since 1871. It was approved by the FDA in 1970 and it is currently used to treat many different types of patients with depressive disorders [276]. Early clinical studies indicated that lithium had effects on HSCs and other hematopoietic cells [277-279]. Lithium increased circulating HSCs and peripheral blood counts [280]. In 1996, it was proposed that lithium could mobilize HSCs for BM transplantations [281]. An important consequence of suppression of GSK-3 is stimulation of both the Wnt/beta-catenin and PI3K/PTEN/Akt/mTORC1 pathways. However, GSK-3 has many biological effects [275,282,289-299]. Development of GSK-3 inhibitors for CNS diseases is complicated by the importance of GSK-3 in glucose metabolism and pancreas function and the possible effect of GSK-3 inhibition to be oncogenic. Further development of GSK-3 inhibitors for clinical trials should be approached with caution.

2.7. Literature review related to the indole and its derivatives

Indoles are bicyclic organic compound consisting of six –membered benzene ring fused with a five –membered nitrogen-containing pyyrole ring.It is product formed by degradation of tryptophan, aminoacid.which occurs naturally in human feces causing intense fecal smell.(https://pubchem.ncbi.nlm.nih.gov/compound/indole)

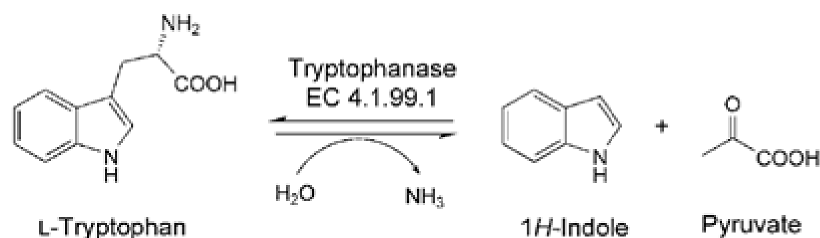


Fig2.10: Synthesis of indole through tryptophan

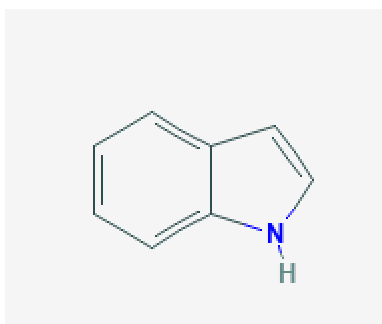


Fig 2.11: Structure of indole

Indoles being inhibitors of GSK-3 α/β it can also act as a bactericidial and bacteriostatic. Different types of indoles were tested against different bacterias among them some showed different properties such as indigoid production, cytotoxic at high concentration and non-substrates.

Indoles has also effect on healthspan in worms and flies in presence of arylhydrocarbon receptors(AHR),conserved detector of xenobiotic small molecules,they are able to express the gene which is seen in young age.In aged animals.the genes are related with the oogenesis and, accordingly, extends fecundity and reproductive span.This has proven that small amount of indole can also promote to healthy aging and can be developed as a therapeutics to extend health span such as motility,tolerance to stressors , but donot have effect on lifespan.Commensal bacteria acts as essential thing which utilize nutrients and indole to create intenstinal epithelial barrier,enhance host immunity, and limit pathogen colonization.*Caenorhabditis elegans* was taken as the biosensor, commensal bacteria mostly *Escherchia coli* induce hormetic protection against stressors in worms, an effect mediated by factors controlling innate immunity and lifespan.Indoles are found in plants which can regulate as virulence in pathogenic bacteria, protect hosts from infection, and limit colitis induced by pathogens or chemical stressors (Sonowal, et al., 2017).

Indole alkaloids which are found in different parts of flowers.The aerial part of one flower named *Kopsia fruticosa* showed new alkaloids, kopsifolines which was able to act as the antifungal and antimicrobial which was tested against different cell lines to observe the cytotoxicity (Long et al., 2018).

Table 2.1: Lists of the 1H-indoles which were tested against the bacteria (Wu, Aryal, Lozach, et al., 2005).

Substrates for indigoid production	Cytotoxic at high concentration	Non-substrates
5-Methyl-1H-indole	5-Bromo-1H-indole	1H-indole-5-amine
7- Methyl-1H-indole	6-chloro-1H-indole	5,6-Dimethoxy-1H-indole
5-Nitro-1H-indole	4-Bromo-1H-indole	1H-indole-5-carboxylic acid
6- Nitro-1H-indole	6-Bromo-1H-indole	7-Aza-1H-indole
7- Nitro-1H-indole	7-chloro-1H-indole	1H-indole-4-amine
Methyl-1H-indole-5-carboxylate	5-chloro-1H-indole	5-(Benzyloxy)-1H-indole
1H-indole-5-carbonitrile	4-chloro-1H-indole	1H-indolol
7-Ethyl-1H-indole	5-Iodo-1H-indole	1H-indolyl acetate
4-Fluoro-1H-indole	7-Bromo-1H-indole	1H-Methyl-1H-indole
5-Fluoro-1H-indole		5,6-Methylene dioxy-1H-indole
6-Fluoro-1H-indole		Methyl 1H-indole-6-carboxylate
7-Fluoro-1H-indole		
1H-Indole-5-carboxaldehyde		
4-Methoxy-1H-indole		
5-Methoxy-1H-indole		
6-Methoxy-1H-indole		
6-Methyl-1H-indole		
4-Nitro-1H-indole		
Methyl-1H-indole-4-carboxylate		
5-Methoxy-4-methyl-1H-indole		
4-Methyl-1H-indole		
1H-Indole-7-carboxaldehyde		
7-Methoxy-1H-indole		
1H-Indole		

These indoles were found to be toxic when expressed in bacteria so when they are overexpressed it can be a potent therapeutic agent which can be used against bacteria.It is predicted that during the biosynthetic pathway, adding a single indole compound to the system should produce one indigo and one indurubin, both containing two moieties with same substitution group. Two indole compounds should result in three indigos and four indurubins as major products.The production of indigoids and isatin can only in the presence of human cytochrome P4502A6.

Many kinds of modification were made in indole and its derivatives to act as a inhibitors against GSK3α/β and CDK/p25.Among 45 commerical indoles,33 cell extracts were taken because they were able to convert to indigoids and could be oxidized,from them 6-nitro-1H-indole showed highest inhibition in CDK5/p25 assays.

During the experiment the *E.coli* without tryptophanase were prepared which was able to provide advantage as a host for P4502A6L240C/N297Q expression in formation of disubstituted indigoids from indoles.Modification in the chemical structure in two indurubin and indigoids were able to show high activity and specificity for GSK-3 and

CDK showing relationship between kinase inhibition and indigoids (Wu, Aryal et al., 2005).

As we know cytochrome P450 plays a significant role in metabolism of indole to indigoids and isatin these similar type cytochrome is also found in Streptomycetes named cytochromes p450(CYP).Around 18 novel CYPs were found in *S.coelicolor* and 33 CYP in *Streptomyces avermitilis*(Xu,et al.,2009).Furthermore,Streptomycetes contains cytochrome P450-dependent monooxygenases ,superfamily of heme which involve in NADPH/NADH and O_2-dependent reactions .they have also developed in metabolism of xenobiotic drugs and toxic chemicals as well as other endogenous compounds such as sterols,fatty acids and prostaglandins(Lamb,et al.,2007).

2.8. Literature review in Phytochemical

Many multi drug resistance bacteria has been emerged and there is been the lack of new antibiotics for treatment of human disease and animal diseases. There is also a limited number of drugs for human and livestock to work against these bugs phytochemicals can be considered to be one way(Lillehoj et al.,2018).Phytochemicals are naturally occurring non-nutritive plant or secondary metabolities or chemicals which helps to protect, color odor ,flavor to plants (http://www.aicr.org/reduce-your-cancer-risk/diet/elements_phytochemicals html). They are non-essential nutrients which isnot required for the human to sustain their life but research has shown that it can also act as the protection to different kinds of human diseases.The well-known phyto chemicals are lycopene in tomatoes,isoflavones in soy and flavonoids in fruits.Most of foods which we consume contains phytochemical except refined foods such as sugar or alcohol.Phytochemical consists of flavonoids,phenolic acid,stilbenes,ligans,tannins and saponins,which are able to treat communicable and non-communicable diseases.Different phytochemicals have different chemical structures so it can act in various way to disease control.Some structures can be slightly modified to increase effectiveness of these compounds.

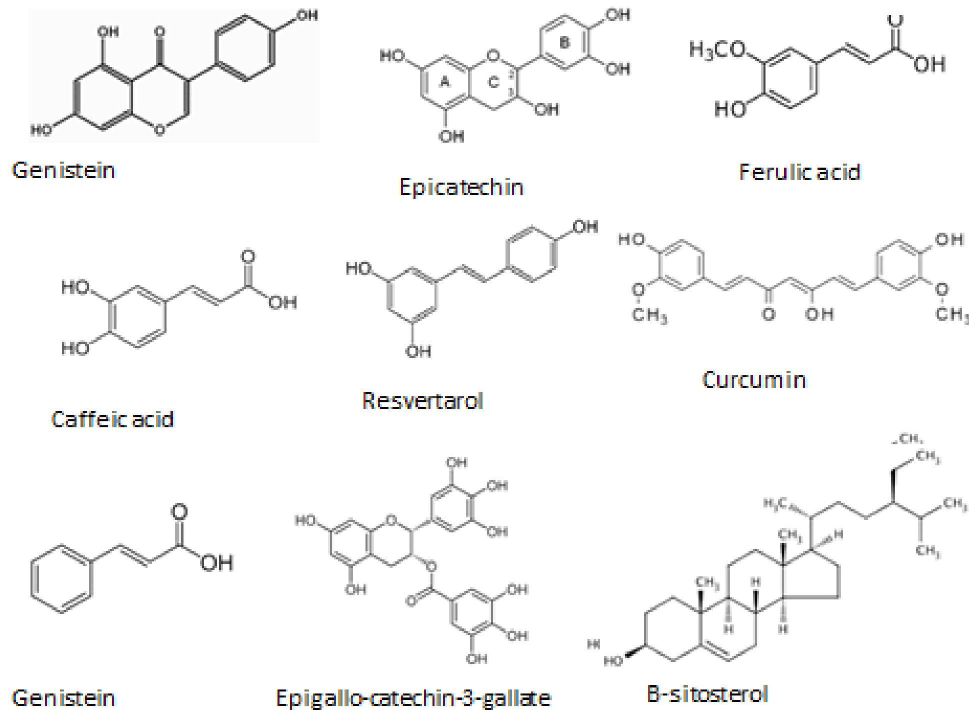


Figure2.12: Different structures of Phytochemicals (Nyamai et al.,2016).

There are various kinds of activities carried out by the phytochemicals such as:

- Antioxidant: It helps in developing the resistance to oxidative damage and development of cancer.Examples:allyl sulfides (onion,leeks,garlic) ,carotenoids(fruits,carrot), flavonoids (fruits ,vegetables), polyphenols(tea,grapes).
- Hormonal action: Isoflavones (soy) reduce human estrogens and reduce menopausal symptoms and osteoporosis.
- Stimulation of enzymes: Indoles found in Cabbages, helps to make estrogen less effective and decrease the breast cancer protease inhibitor (soy and beans) and terpenes(citrus fruits and cherries).
- Interference with DNA replication: Sponins found in beans helps in interference in replication of cell DNA which can cause the cancer and also Capsaicin,found in hot pepper helps to protect from DNA carcinogens.
- Anti-bacterial effect: allicin of garlic has anti- bacterial activity.
- Physical action: binding physically on cell walls prevents adhesion of pathogens to human cell walls.Proanthocyanidins are responsible for anti-adhension properties.it also helps in reduction of urinary tract infection and improve dental health (Surh, YJ,2003).

Different foods contain different types of phytochemicals and have their own benefits which are represented below:

Table 2.2: Phytochemicals with plant source and its benefits

Phytochemical(s)	Plant Source	Possible Benefits
Carotenoids (such as beta-carotene, lycopene, lutein, zeaxanthin)	Red, orange and green fruits and vegetables including broccoli, carrots, cooked tomatoes, leafy greens, sweet potatoes, winter squash, apricots, cantaloupe, oranges and watermelon.	May inhibit cancer cell growth, work as antioxidants and improve immune response.
Flavonoids (such as anthocyanins and quercetin)	Apples, citrus fruits, onions, soybeans and soy products (tofu, soy milk, edamame, etc.), coffee and tea	May inhibit inflammation and tumor growth; may aid immunity and boost production of detoxifying enzymes in the body
Indoles and Glucosinolates (sulforaphane)	Cruciferous vegetables (broccoli, cabbage, collard greens, kale, cauliflower and Brussels sprouts)	May induce detoxification of carcinogens, limit production of cancer-related hormones, block carcinogens and prevent tumor growth
Inositol (phytic acid)	Bran from corn, oats, rice, rye and wheat, nuts, soybeans and soy products (tofu, soy milk, edamame, etc.	May retard cell growth and work as antioxidant
Isoflavones (daidzein and genistein)	Soybeans and soy products (tofu, soy milk, edamame, etc.)	May inhibit tumor growth, limit production of cancer-related hormones and generally work as antioxidant
Isothiocyanates	Cruciferous vegetables (broccoli, cabbage, collard greens, kale, cauliflower and Brussels sprouts)	May induce detoxification of carcinogens, block tumor growth and work as antioxidants.
Polyphenols (such as ellagic acid and resveratrol)	Green tea, grapes, wine, berries, citrus fruits, apples, whole grains and peanuts	May prevent cancer formation, prevent inflammation and work as antioxidants.
Terpenes (such as perillyl alcohol, limonene, carnosol)	Cherries, citrus fruit peel, rosemary	May protect cells from becoming cancerous, slow cancer cell growth, strengthen immune

function, limit production of cancer-related hormones, fight viruses, work as antioxidants

(www.aicr.org/reduce-your-cancer-risk/diet/elements_phytochemicals.html)

From the characters and benefits shown by the phytochemicals it can act as a powerful which act as a drugs. Among all phytochemicals, naringenin acts as antioxidant, free radical scavenger,anti-inflammatory chemical,carbohydrate metabolism promoter, and immune system modulator and also inhibit the cytochrome P450 enzyme in liver.

There are two terminologies such as phytonutrient and phytochemical.Phytonutrients has only the positive effect whereas phytochemical has both effect so phytonutrient can be included in phytochemicals but not viceversa.Phytochemicals also includes cocaine, codeine, oxycodone, and nicotine which has negative effect to our health (Edward Group, 2016).

It has been found that the diet rich in fruits and vegetables containing biologically active plant secondary metabolities are effective against the cancer.It includes various kinds of mechanisms such as detoxification and increase excreation of carcinogens, the suppression of inflammatory processes such as cyclooxygenase-2 expression, inhibition of mitosis and the induction of apoptosis at various stages in the progression and promotion of cancer.All the phytochemicals arenot absorbed by human body,but still foods such as berries,brassica vegetables and tea should be further research for confirmation and quatify their benefits.It can also reduce chemotherapy by using the isolated compound from plants but need to be analysed properly the biological effect too(Johson IT,2007).

Regular consumption of fruits and vegetables helps in reduction of risk of different types of diseases suh as cancer,cardiovascular disease,stroke,Alzheimer disease,cataracts and other disease related to ageing.It has been found that the use of the whole foods can be the most effective way incomparsion of consuming pills or extract(Liu,2003).Not only fruits but also flowers contains the phytochemicals which includes four flavonols, three flavones, four flavanols, three anthocyanins, three phenolic acids and their derivatives which helps in benefits of health.they can act as anti-oxidant,anti-inflammatory,anti-cancer ,anti-obesity and neuroprotective effect.

Different parts of flowers were analysed for phytochemical analysis such as flower,leaf and barks in which presence of Saponins, steroids,flavonoids,phenolic and tannins compounds were found(Behera et al.,2016).Some edible flowers which can be used for therapeutic activities are:Sambucus nigra,Hedysarum coronarium,Malva sylvestris and also marigold.Marigold has been used for the treatment of various kinds of diseases from long time ago.The flower consists of fragnance which helps to develop perfumes and is also useful for extraction of oils(Swamy,2002).

Marigold is an annual herb with pale-green leaves and yellow or orange flower it has a strong but unpleasant odour.The palnt consists of different phtyochemicals such as cadinol, carotenoids, isorhamnetin, saponins, triterpenes, sesquiterpenoids, scopoletin,

flavonoids, quercetin, kaempferol.These phytochemical can act as anti-inflammatory and antispasmodic properties.It can be used in various purposes such as treat bruises, wounds, eczema, skin disorders, haemorrhoids and burns.Furthermore it can also be used for the treatment such as pain,swelling and remove warts.not only this,it can be for treatment of gastric ulcer and infections in mouth and throat as well as it improve the stimulation of digestion by enhancing bile production.However,it also helps in curing menstrual cramps,liver disease and constipation(Lee et al.,2017).

2.9. Literature review on Computational

All these antimicrobial peptide design were constructed as a computational language-modeling. Antibiotics resistance is one of the most immerging problems which can cause the death of above 10million annually by 2050 if no new drug is developed. So, antimicrobial peptide is considered to be one of the main target for the solving the problem of MDR because the innate immune is one of the most important factor for the innate immune system for their evolutionary origin and widespread prevalence across many forms of life(Tashi,Ullah,.Watanabe et al.,2018).

Many datas are being collected in the focus for biomedical discoveries ,so large data has been collected.So to identification and mechanism of action cost-effective method is required for this context information technologies and informatics tools are preferred as powerful toolbox.As well as it can also help in target identification,discovery and optimization of drug candidate molecules.Moreover,it also helps in studying the dynamic nature of drug,binding chacterization of binding sites also the molecular entities and their optimization(Katsila et al.,2016).

Drug design is a rational drug design process by which new medicines are known on the basis of biological target. Drug being organic small molecules which are able to inhibit function of biomolecule such as protein helping in therapeutic benefit of patients. Drug design is mostly focused in shape and charge of molecules which helps in interaction and bind to it which is known as computer-aided drug design. And the drug designed on the basis of the three-dimensional structure of biomolecular target is known as structure-based drug design. Computational methods are mostly been effective to observe the affinity, selectivity and stability of protein based therapeutics (shahinozzaman M et al., 2018).

During development of drugs two points should be focused that is focus in modification of target causes modification in disease and should be capable of binding small molecules.Computer-aided drug design is one of the most effective method for finding molecule for binding the target and their intermolecular interaction between small molecule and biological target.It is the effective method for predicting the affinity to compound,saving time and cost but present computational methods isnot perfect .There are two types of drug design that is ligand-based and Structure-based.Ligand based is also known as indirect drug design because biological target is formed and based on its knowledge new molecular entities are developed.Quantitative Structure-activity relationship (QSAR),correlation between experimentally determined biological acitvity and the calculated properties of molecules are derived and also helps in finding activity of new analogs(Besalu et al.,2002).

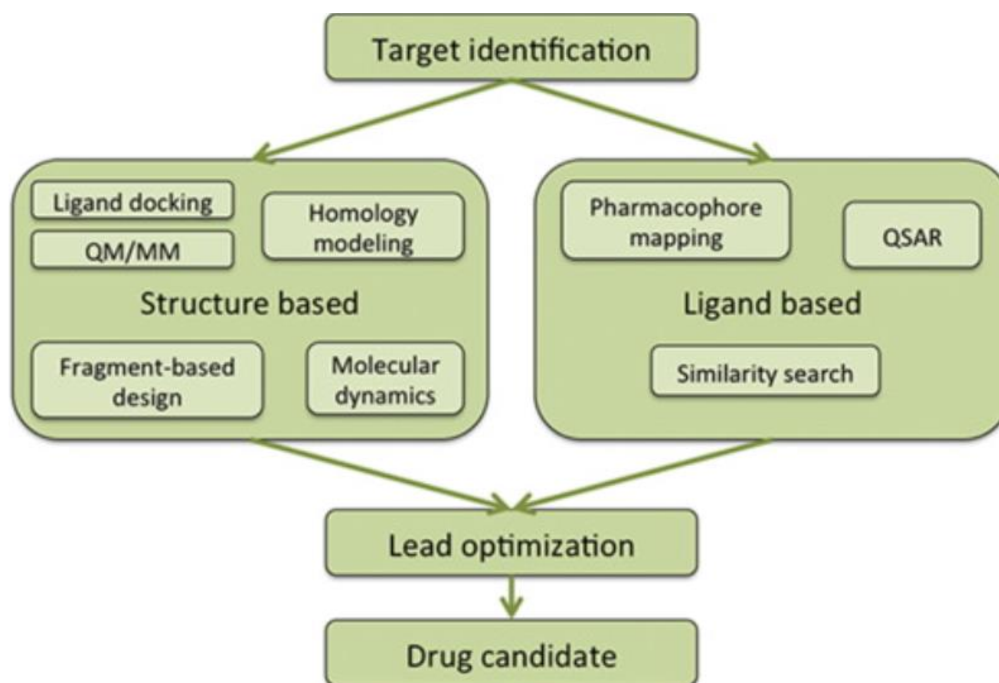


Figure2.13: General computer aid techniques for drug design (Yao.Evans et al., 2010).

The binding site is first priority on identification of Structure based drug design which is considered to be direct design due to knowledge of three dimensional structure obtained by the X-ray crystallography or NMR spectroscopy. It can be divided in three category such as virtual screening,denovo design of new ligands and optimization of known ligands by evaluating proposed analogs with respect to binding cavity.The binding energy calculation is based on empirical score

$\Delta Gbind=\Delta G0+\Delta Ghb\Sigma h\text{-bonds} +\Delta G_{ionic}\Sigma_{ionic\text{-}int}+ \Delta G_{lipophilic}lA\ l+ \Delta G\ rot\ NROT$

Where: ΔG_0 – empirically derived offset that in part corresponds to the overall loss of translational and rotational entropy of the ligand upon binding.

ΔG_{hb} – contribution from hydrogen bonding

ΔG_{ionic} – contribution from ionic interactions

ΔG_{lip} – contribution from lipophilic interactions where |Alipo| is surface area of lipophilic contact between the ligand and receptor

ΔG_{rot} – entropy penalty due to freezing a rotatable in the ligand bond upon binding

A more general thermodynamic "master" equation is as follows:

$\Delta G\ bind = - R\ T \ln\ K\ d$

$$K\ d = \frac{[\ Ligand\ ]\ [\ Receptor\ ]}{[\ Complex\ ]}$$

$\Delta G_{bind} = \Delta G_{desolvation} + \Delta G_{motion} + \Delta G_{configuration} + \Delta G_{interaction}$

where:

desolvation – enthalpic penalty for removing the ligand from solvent

motion – entropic penalty for reducing the degrees of freedom when a ligand binds to its receptor

configuration – conformational strain energy required to put the ligand in its "active" conformation

interaction – enthalpic gain for "resolvating" the ligand with its receptor)it has also been known for identification of molecular pattern and identifying the effectiveness of drug cocktails ,analyzing the genetic overlap between disease s and predicting alternative drug use(Yao.Evans et al.,2010).

Computer aided drug discovery (CADD) tool is being used as vitual shoetcut ,assisting in the expendition of long process and cost reduction of research and development.Human genome project have provided the sequences are available which helped in development of drug design.Quantitative structure-activity relationships (QSAR) is based in the correlation activity of target drug interactions with various molecular description.It gives mathematical data about activity response to the target and ligands.it helps in prediction of activity of new drug molecule analogs(Leelananda,Lindert,2016).

Long short-term memory (LSTM) language model is carried out for the understanding arrangement and frequencies of amino acids residues which is known as antimicrobial peptide sequences for the treatment of the multidrug and carbapenem resistant bacterial pathogens.10 peptides synthesized by the computational method was checked in the known pathogenic bacteria among them only 2 showed the effective results towards multidrug resistant bacteria isolated from the clinical samples which are *Escherichia coli, Acinetobacter baumannii, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus,* and coagulase-negative Staphylococci (CONS) strains. High activity was seen in the extended-spectrum beta-lactamase (ESBL), methicillin–resistant *Staphylococcus aureus* (MRSA) and carbapenem –resistant strains was observed. The peptide interacted with the bacterial cell and disrupted the bacterial cell membranes and caused the secondary gene-regulatory effects which were well folded as well (Yang, and Kim, 2018).

American and Russian computer scientists identified the novel variants of the known antibiotics in a few hours by the help of the algorithm called VarQuest which was found to be peptidic natural products and help to Global Natural Products Social (GNPS) (Yan and .Zhan, 2010). VarQuest algorithm should help to the Global Natural Products Social (GNPS) to enhance the searches and help to meet the promise (Olivier, K N, 2013).The antibiotics produced by the microbes are much more diverse than assumed .PNPS has the record of all the pharmacology also includes the antimicrobial and anticancer agents including as" antibiotics of last resort", Vancomycin and Daptomycin. (Spice Byron, 2018)

As computational models helps in identification of the actual space and temporal dynamics of antibiotics at the location of infection which helps in reduction of space to design and cost which may be costly as well as trial in pre-clinical and human clinical trials(Geris,Guyot,Schrooten et al.,2016). Identification and ranking of host mechanisms help in identification of potential target for immunotherapy. Immunotherapy was aimed

to boost macrophage activation during the treatment. Host mechanisms, bacterial load and drug permeability should be considered for immune modulation for antibiotics treatment of TB because bacterial load and drug penetration into the site of infection is considered to be indicators of reactivation during anti-TNF treatment(Abate and Hoft,2016)The granuloma was created and functioned by the computational method use of models for the plasma pharmacokinetics, tissue pharmacokinetics and pharm dynamics because as we know that the site of infection of Tuberculosis is granulomas and its infection in animal can be problematic(Crouser,2016).

The relation between the sputum and the granuloma is still unknown but the sputum is used as the confirmation of tuberculosis. Antibiotics exposure helps in treatment of the disease and increase in dose of antibiotics exposure enhance the treatment outcomes. The toxicity can be limited by oral administration and by use of nanoparticles. It can be targeted to specific site by reducing the systemic toxicity (W.De.Jong, P.Borm, 2008).

The concentration gradient accelerates the development of the bacterial populations; the bacteria exposed to suboptimal concentration contribute to development of drug-resistant bacteria. The computational tool helped in examines TB by different ways which includes following steps:

•It can be used to predict the model of the intra-granuloma activity by the new antibiotics and can be observe the improvement of antibiotics after which helps in identification of the promising invitro and poor invivo activity.

•It can test large number of antibiotics and the combination of antibiotics and also the dose of the drug with the guide of new combinations and regimens to be testes in costly animals and human trials.

•It helps in exploration of the immune therapeutic strategies while inclusion in human with alone or combination with antibiotics.

•It helps in predicting the efficacy of new drugs with the help of complementary system pharmacology which may cost high and difficult to check in the combination with the antibiotics and length during the clinical trials (Pienaar,Clifone et al.2015).

Computational model helps in optimization of the production process by stimulating dynamics of phage population growth and production in a two-stage, self-cycling process. Different parameters are calculated by the help of computer such as ordinary differential equations, allowing application to a setup with multiple reactors. This model helps in calculation of product with lowest cost, flexible (Krysiak-Baltyn. K, Martin .G, Gras .S.,2018).

Rational designing is carried out for antimicrobial peptide formation in Pexiganan, leucine-lysine repeats, tryptophan-leucine-lysine repeats, tryptophan-arginine repeats, and structurally nanoengi-neered antimicrobial peptide polymers (SNAPPs) which is formed by the repetition of hydrophobic and hydrophilic amino acid motifs with minimal computational input. The computationally constructed peptides were not further able to carry out beyond the early stages of experimental validation. Due to the limitation in antimicrobial peptides characterization and algorithm used at time, computational approaches are being hindered. Rapid growing of new antimicrobial peptides database

has helped formation of new generation antimicrobial peptides. The problem known to be computational language-modeling which is related to antimicrobial peptides (Lam, Brien-Simpson, Pantarat et al., 2016).

The arrangement and frequencies of amino acids residue within peptides are calculated with the long short term memory (LSTM) model .The lead molecule (NN2_0018) was identified from 10 antimicrobial peptide sequences which showed in vitro and in vivo antimicrobial activity against carbapenem-resistant bacteria named *A.baumannii* in a mouse model of peritoneal infection.The model displayed no mortality, hepatotoxicity, or nephrotoxicity at therapeutic doses. Different analysis where carried out to find the structure and mechanisms of model such as Circular dichroism,Nuclear Magnetic Resonance(NMR),scanning electron microscopy ,and microarray gene expression.(Nagarajan,Nagarajan,Roy et al.,2018).

2.10. Literature review related to tRNA methylation (TRMD)

TrmD (tRNA methylation), the biologically important organic chemical reaction in which methyl group transfer from one compound to another compound. The recovery of methionine from homocysteine which is requires the vitamin B12 and folate. The active folate (methyltetrahydrofolate) delivers the active methyl form to the vitamin B12,essential vitamin for human formed by complex form named Uroporphyrinogen, required for methylation of homocysteine .Elevation of the homocysteine causes the cardiovascular(Zorumski,Nagele,Mennerick et al.,2015).

TrmD is a highly conserved tRNA methyltransferase found in all three domains of life that converts Gly-37 into m^1G37 by transferring the methyl group from AdoMet to a subset of tRNA species. This modification on tRNAs is essential for maintaining the correct reading frame during protein translation. Abolishing the function of TrmD increases +1 frameshift events during protein translation and growth defects have been observed in its absence in bacteria and yeast.

TrmD containing amino acids where substrate binds S-adenyl-l-methionine which can be replaced competitively by the 3', 5'cAMP causing adaptive evolution to raising change in adenylate cyclase.In bacteria uses S-adenosyl-l-methionine (AdoMet) as a methyl donor for the catalyzation of the N1-methylguanosine (m1G) modification at position 37 in the transfer RNAs (tRNAs) with the 36GG37 sequence. The modification in the ribosome, m1G37 of the tRNA prevents the +1 frame shift errors related disease and link to the metabolic syndrome(Gamper,Masuda,Frenkel-Morgenstern et al.,2015). tRNA(guanine37-N1)-methyltransferase is an enzyme with systematic name S-adenosyl-methionine:tRNA –methyltransferase. This enzyme catalyzes the following chemical reaction

S-adenosyl-L-methionine + guanine37 in tRNA S-adenosyl-L-homocysteine + N1-methylguanine37 in tRNA

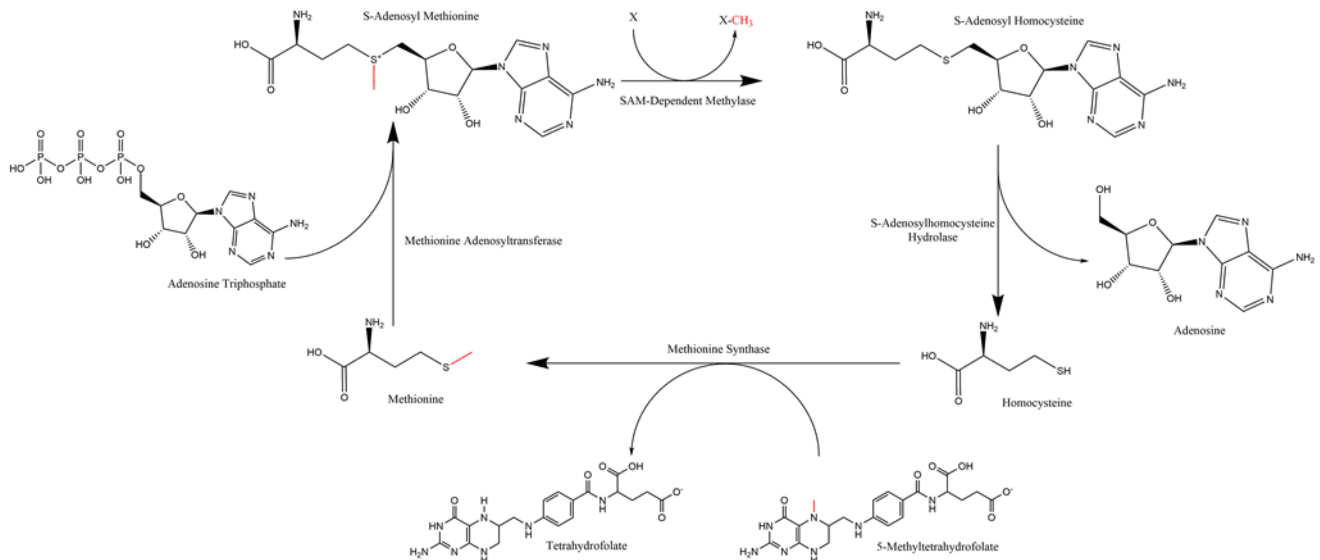


Figure 2.14: Recovery of methionine from homocysteine by transmethylation depicted in reaction 4.The trans methylation depiction in reaction 1-4.

The trmD gene helps in the methylation of N1 methylguanosine position of G37 in tRNA.The mutation in trmD leads in the growth defect and is also associated to increase of the +1 frame shift errors (C.Fagan, T.Maehigashi, J.Dunkle et al.,2014).The SPOUT MTases helps in the methylation at the N1 position of G37 in cases where the third anticodon letter, 5′ adjacent to G37, is also guanosine, G36 . The monomer of the trmD consists of the two globular domains that is N-terminal domain and the C-terminal domain, the N-terminal domain is also named as "SPOUT domain," because it harbors the SPOUT (SpoU-TrmD) fold(Dunkle,Dunham,2015; Ito,I.Masuda,K.Yoshida et al.,2015).

SpoU (also known as the trmH) catalyses the 2'-o methylation of guanosine at the 18th position in tRNA.type II methylase, methylates only a subset of tRNA species.(http://www.uniprot.org/uniprot/P0AGJ2)(Watanabe,Nureki,Fukai et al.,2005).

The structure of TrmD is determined by Ado Met bound to trefoil-knot belong to the RNA methyltransferease (MTases) helping in tightening and loosening of knot. This helps in binding of methionine moiety of AdoMet and helps in interaction with the specific tRNA at G37 and G36 resulting in synthesis of m1G37-tRNA.The SPOUTMTase helps in methylation of base or ribose moiety of transfer RNA (tRNA) or ribosomal RNA (rRNA) in three domains of life producing methylated RNA and S-adenosyl-l-homocysteine(AdoHcy).The helix located in carboxyl terminus of deep trefoil knot is involve in dimerization if the homodimeric SPOUTMTases is present(Ito.T,MasudaI,Yoshida.K et al.,2015).

The trmD has quiet similar dimer to Nep1 domain structure but showed different intersubunit orientation with distinct elements added (Zhang .T, et al.2015).It shows the chaperon function without showing methylation function. They contain similar SPOUT domain structure but contain different intersubunit orientation.TrmD only bind recognize the D, the anticodon arm and variable region and lacks any contacts with acceptor branch,formed by the acceptor and T arms in tRNA.Subunit B of interdomain linker forms helix upon tRNA binding and participate in interaction with substrate tRNA(T.Ito,I.Masuda,K.Yoshida et al.,2015).

At different positions of tRNA molecule there is variety of modified nucleosides, mostly in anticodon region among them few modified nucleosides conserved in domains is 1-methylguanosine,present in next to the 3'end of the anticodon(position 37) in tRNAs from *Eschericia coli* and *Salmonella typhimurium* reading CCN($tRNA^{pro}$)(N,any of the four major nucleosides)and CGG(tRNAArg) codons in tRNA (Persson,Olafsson,.Lundgren et al.,1998). trmD is the structural gene for the tRNA (m_1G^{37}) methyltransferase, in *E.coli* and Salmonella typhimurium which affect the elution profiles of Leucine.The m_1G deficiency cause suppression of certain +1 frameshift mutations (Hagervall, Tuohy, Atkins et al., 1993).Incase of $tRNA^{pro}$, m1G deficiency induces a frameshift by allowing the unmodified G-37 to interact with a C as a fourth base, resulting in a quadruplet translocation. The specific function of m1G is to prevent frameshifting and maintain reading frame.

tRNAs from both $trmD^+$ and $trmD_3$ were compared and showed that $tRNA^{pro}$ is fully aminoacylated in both $trmD^+$ and $trmD_3$, so the lack of m_1G does not influence the level of Pro-$tRNA^{pro}$ in vivo so the $trmD_3$ mutation does not affect the efficiency of aminoacylated $tRNA^{pro}$.$trmD_3$ mutation does not affect the leu_{ABCD} operon which suggested that m_1G deficiency does not effect in $tRNA_2^{Leu}$ and $tRNA_3^{Leu}$ influence the charge of tRNA (Vartak.Liu,and Wang,1991).

Modification in tRNA helps in the synthesis of different aminoacids, through which the possible influence of m_1G deficiency can be determined. Different responses to various analogs of the same amino acids (for instance, the proline analogs) were observed, probably because these analogs have different targets. Various plasmids were introduced in the trmD gene containing the trmD operon or parts, into strains GT874($trmD^+$)and GT875(trmD3).The changed sensitivity of strain GT875 to different analogs was complemented but plasmids that expressed the $trmD^+$gene but not by the vector (pBR322)or by plasmid pMW123 ,which has disrupted trmD gene.Thus,the observed responses to the amino acid analogs 3,4-dehydro-DL-proline,5,5,5-trifluoro-DL-leucine,and NT of strain GT875 are caused by the trmD3 mutation and not by some other unidentified mutation(Bjork, and Nilsson,2003).

The trmD3 mutant is slightly more sensitive than trmD+ strain to leucine analog trifluoroleucine, which implies that the translational elongation rate for the m1G-deficient $tRNA_{2,3}^{Leu}$ is unaffected, which is consistent with normal level of β-isopropyl malate dehydrogenase in trmD3 mutant. Deficiency of ψ in his T mutant caused a 2.8-fold depression of leuB gene, and accordingly, this mutant is also more resistant to trifluoroleucine (Bjork, Durand, Hagerval et.al, 1999).

The mechanism by which m1G deficiency mediates changed sensitivity to different amino acid analogs, we isolated spontaneous suppressors.NT was taken as analog which gave the largest difference between the $trmD_3$ strain and the $trmD^+$ strain.$trmD_3$ mutant was unable to grow at 37°c but trmD+ grew normally on citrate minimal plate with 215 μg of NT per ml.

In Salmonella strains, m_1G is present in different species; three $tRNA^{pro}$ (CCNcodons), three of five $tRNA_{Leu}$ species (CUNcodons) and in one of the $tRNA_{Arg}$ (CGG codons).The hisT mutant, which lacks enzyme that converts the uridine into pseudouridinne in anticodon region has reduction of 23% cgrp (Allaudeen.H, Yang. S, et al., 1972). The hisT

mutation depresses the leu operon 2.8 fold and renders cells resistant to leucine analogs we did not observe any increased expression of the leucine operon in the trmD3 mutant compared with wild type. Furthermore, the $trmD_3$ mutant was not resistant to leucine analogs (Molina L., Udaondo. Z, Duque.E, et al., 2016).$tRNA_{2,3}^{Leu}$ lacking ψ in anticodon region is enough to depress the leu analogs. The ψ lac kind $tRNA_{2,3}^{Leu}$ depress the leu operon several fold but whereas m_1G-^{37} deficiency of the $tRNA_{2,3}^{Leu}$ leads to an unchanged expression, implying an affected step time for these leucine tRNAs.The average step time caused by m1G deficiency is probably due to the other five tRNA species, which can increase average time of $tRNA_{1,2,3}^{Pro}$, $tRNA_3^{Arg}$, and $tRNA_1^{Leu}$(Kirino,Yasukawa,Ohta et al.,2004).

There is presence of different modified nucleotides especially in the anticodon site of tRNA among them the modified nucleotide conserved from all domain is the 1-methylguanosine (m_1G) present in the 3'end of the anticodon (position 37) in tRNA from the *Eschericia coli* and Salmonella typhimurium reading the sequence CCN (tRNA $^{pro}_{1,2,3}$)(N,any of the four major nucleosides) and CCG($tRNA_3^{Arg}$) codons. Mutation in trmD gene in *E.coli* and in *S.typhimurium* which is the structural gene for tRNA (m_1G^{37}) methyltransferease,affect the elution profiles of these leucine tRNAs which concluded that the m_1G is present in $tRNA_{1,2,3}^{Leu}$(Bjork,et al.,1995).

The +1 frameshift is the error reading of ribosome by binding four codon instead of binding only three codons for a specific aminoacids which is mostly seen when the tRNA are shifting from 5'to 3' direction on mRNA causing aberrant protein production(Fagan et al.,2014).The m_1G deficiency can suppress certain +1 frame shift mutations but in case of the tRNA ^{pro}, m_1G deficiency induces a frame shift by allowing the unmodified G-37 to interact with a C as a fourth base, resulting in quadruplet translocation. The one specific function of m_1G to prevent frame shifting and thereby maintain the reading frame. The m_1G deficiency induces the strong pleiotropic effects such as reduction in growth rate and polypeptide chain elongation rate (cgrp) in vivo. The presence of m_1G is important for the efficient translation, in a tRNA-dependent manner (Li, and Bjork, 1995)(https ://pdfs. semanticscholar. org/ 9ffa /d18f50d650cd6227d7fed97c523e16e8f8d5.pdf).

Several metabolic enzymes of $trmD_3$ mutant, show altered responses to several amino acid analogs and enhanced ability to oxidize certain carbon sources compared with the wild-type strain (Bjok, Wikstrom and Bystrom, 1989)indicating this could be a drug target.

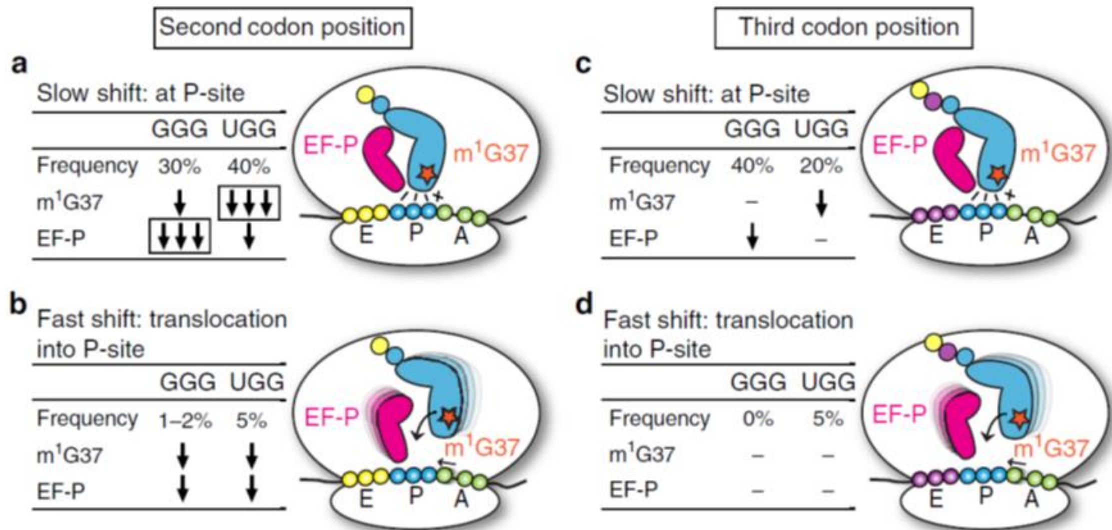


Figure2.15: A model of +1FS on CCC-C by GGG and UGG $tRNA^{pro}$.Frequencies of +1FS on CCC-C are shown for GGG or UGG tRNApro based on kinetic data for the G37-state of each.(a)In the post-translocation complex where CCC-C is placed at the second codon position, the high frequency(b)The low frequencies of fast shifts at the second codon, due to tRNApro shifting en route to the P-site, are suppressed by both m1G37a nd EF-P for each tRNA.(c)When CCC-C is placed at third codon position, the high frequencies of slow shifts are suppressed by m1G37 for the UGG tRNA and by EF-P for the GGG tRNA. (d) The low frequencies of fast shifts in the early elongation phasearenot effectively suppressed by m1G37 or EF-P. Open arrows indicate suppression of error frequencies, whereas boxed arrows indicate suppression of both frequencies and kinetics of error formation. One arrow indicates a reduction of 2-3 fold, two arrows indicate 3-30 fold, three arrows indicate greater than 30-fold and'-'indicates less than 2-fold effects. Percent frequencies are rounded up to the closest approximation.

During the translation process ,first step is selection step for binding to ternary complex (EF-Tu GTPaminoacyl-tRNA) of ribosomal site in which m_1G plays important role and in the absence of it may affect selection of proline and arginine tRNAs but is not affected on selection of $tRNA_2^{Leu}$ and $tRNA_3^{Leu}$ causing quantitative impact.The selection error of proline instead of other aminoacids by ribosome leads in +1 frameshift by forming tRNApro to reduce it m1G37 should be methylated of tRNApro on 3'side of anticodon and at translation factor EF-P which can cause effect on the protein synthesis (Gamper,Masuda.frenkel-Morgenstern et al,2015).

In actinomycetes there is the different highly conserved sequence of aminoacids in TrmD protein.It could be a main target for the translation process and can be used as a good drug target .Adenosine and methionine fragment of Ado Met inhibit TrmD MT after screening with high nanomolar affinities which resulted it as a good drug target for *Mycobacterium tuberculosis* also (Lahoud et al, Hill et al).Being analogus to Ado Met and contain a Tyr/Phe residue at position 86 and also it binds with the high affinity to TrmD from *S.aureus* as well as in mycobacterium and could also a inhibitors to the bacteria Firmicutes so it can be use as a target(Zhang, et al.,1997).

As known that TrmD as an essential and important enzyme for bacteria it cann be the target to develop drug against it.In human also there is a presence of Trm5 which act as

similarly as that by TrmD, so it can be targeted but due to lack of specific strategies related to it. It is being difficult to the pharmaceutical companies to develop drug against it.Now it has been found that the TrmD consist of trefoil knot which binds AdoMet which is based on the initiate substrate signaling to catalyze methyl transfer. The trefoil knot binds the AdoMet gives unusual novel shape which can be a target for drug design.Furthermore,divalent ion at active site can also be target for drug design,disruption of divalent can be effective drug target at active site for antimicrobial activity of HIV intergase such as Mg^{2+}(Hou.K,et al.,2017).

2.11. Literature review related to S-adenyl-l-methionine, Carbon Catabolities repressor and 3', 5'cAMP

S-adenyl-L-methionine is a co-substrate which helps in transfer of the methyl group and also involve in trans sulfuration and aminopropylation. This is produced by anabolic process in whole body and consumed in the liver. Methyl group is transferred from SAM to different nucleic acids, proteins, lipids and secondary metabolites. It is bound by SAM riboswitch which regulates genes involved in methionine or cysteine biosynthesis (https://examine.com/supplements/s-adenosyl-methionine).

The cycle of SAM consists of different enzymes which includes as follows.In the first step, SAM-e is used as the substrate and by the use of SAM-dependent methylases (EC 2.1.1) it is converted to the S-adenosyl homocysteine as a product. S-adenosyl homocysteine is a strong negative regulator of nearly all SAM-dependent methylases despite their biological diversity, which is hydrolyzed to homocysteine and adenosine by S-adenosylhomocysteine hydrolase (EC 3.3.1.1) and homocysteine recycled back to methionine through transfer of a methyl group from 5-methyltetrahydrofolate, by one of the two classes of methionine synthases This methionine can then be converted back to SAM-e, completing the cycle. In the rate-limiting step of the SAM cycle, MTHFR (methylenetetrahydrofolate reductase) irreversibly reduces 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate.

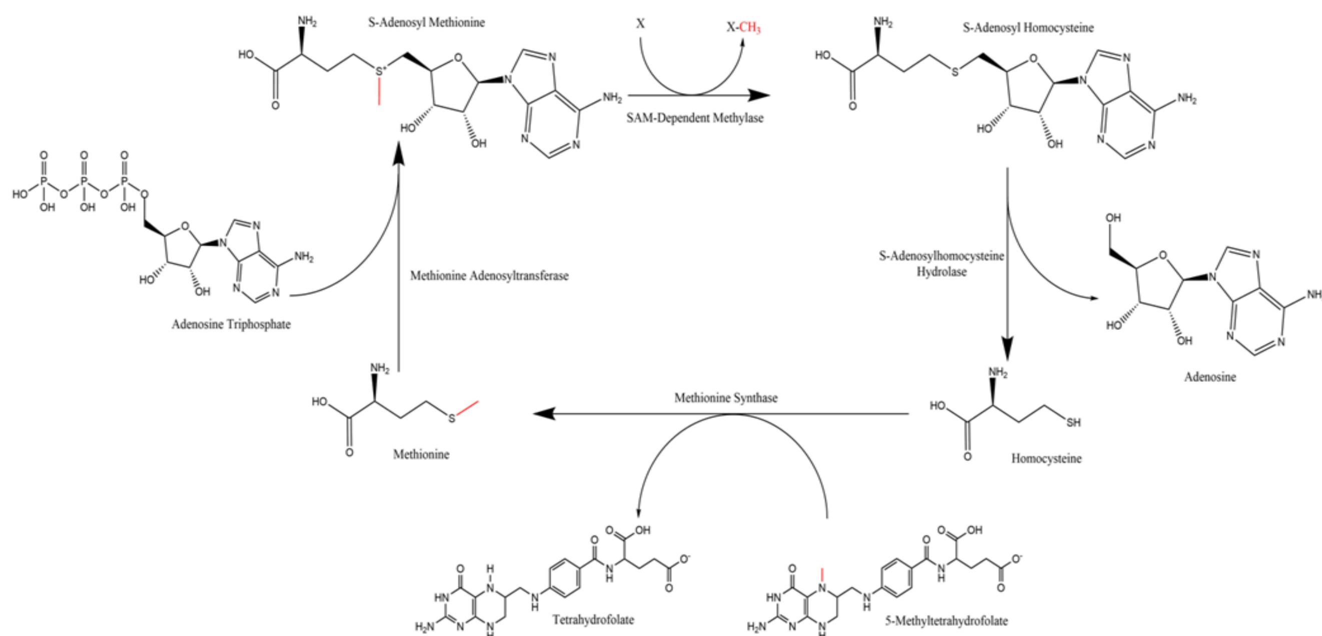


Figure 2.16: Cycle of the S-adenosyl methionine by the use of various enzymes.

Carbon catabolites repression helps to know catabolism of carbon sources in comparison to the secondary carbon sources (BruckneR. r, Titgemeyer,F. Stulke J. et al,2002).3’, 5’cAMP and catabolities repression protein combine to form active transcriptional factor activate the gene for catabolism of secondary carbon sources after glucose get exhausted.it is synthesis in presence of ATP by the enzyme named Adenylate cyclase(AC)(Steer.R, 1975).

Carbon catabolities repression is regulatory mechanism found in bacteria which helps in quick adaptation for carbon sources and energy source from the different sources. The mechanism helps to inhibit the enzymes involved in the catabolism of carbon sources other than the preferred one(Stulke and Hillen,1999).Furthermore it also enable to increase fitness by optimizing growth rates in natural environments provided with complex nutrient.Various kinds of mechanisms can be seen in different bacteria(Stulke,Hillen,1999).

This mechanism can be easily seen in *Escherichia coli* in the presence of the glucose and lactose under which the production of the different enzymes is controlled by the carbon source (Kremling, Geiselmann, Ropers et al, 2015). Especially the β-galactosidase enzyme which is produced by the *Escherichia coli* in the presence of the glucose source. In presence, of enzyme named Enzyme II A (EIIA) when it is grown in the mixture media of glucose and lactose it used glucose as the main source and then lactose by high accumulation of the cAMP by binding the catabolite activator protein and also the promoter sequence of the lac operon which indicate that the presence of the lactose by the production of β - galactosidase enzyme for lactose metabolism (Kaempfer and.Magasanik, 1967).

3’, 5’cAMP is secondary metabolites compose of adenosine and one phosphate group and is form by hydrolysis of ADP or ATP, which plays an important physiological activity. 3’, 5’cAMP play important role in physiological activity of bacteria ranging from caused by catabolite repression helping by the enzymatic activity in eukaryotes. (https://en.wikipedia.org/wiki/Cyclic_adenosine_monophosphate)(Serezani,Ballinger,Aronoff et al.,2008).3’,5’cAMP ,a secondary metabolites which is found in three domains it works in bacteria by carbon catabolities repression method and in human it act for the hormonal production(Yan,Gao,Cui et al.,2016).

As well as it also has been seen in the bacteria *Bacillus subtilis*, having a cAMP-independent catabolite repression mechanism contribute to catabolism of catabolites control protein A(CcpA) which negatively repress other sugar operons they are off in the presence of glucose(M.Jr,M.Saier,1996).

There are 5 different classes of Adenylate cyclase which have different functions .Among them Class III and CYTH proteins are named after the type IV AC. CyaB from *Aeromonas hydrophila* and human thiamine triphosphatase, which is inorganic tripolyphosphatases and subsequently evolved to contain other enzymatic activities such as adenylate cyclase, mRNA triphosphatase, and thiamine triphosphatase activity. CYTH proteins contain a characteristic and highly conserved EXEXK amino acid motif at their N terminus and have a conserved fold with eight β-sheets forming a tunnel-like structure; the amino acids having their side chains into tunnel which coordinates with different phosphates for the involvement of enzyme catalysis which is found in all three domains, Class I, Class

II and Class IV are present in bacteria only. Similarly, the Class V and VI is found in only two bacteria *Prevotella ruminicola* and Rhizobium etli(Tellez-Sosa,Soberon,Vega-Segura et al.,2002). The level of 3′, 5′cAMP was decreases when grown on the presence of nitrate under the aerobic condition of *Bacillus subtilis* (Shih,Lin,Hung et al.,2013).

In *Staphylococcus aureus*(*S. aureus*) ArcR,a member of the CRP/FNR family was found which plays in CCR process by inducing the arginine deaminase operon genes arcABDC under anaerobic conditions (Korner,Sofia et al.,2003).TrmD protein is a highly conserved tRNA which converts Gly-37 into m1G37 by transferring the methyl group from S-adenosylmethionine (ADoMet) to subset of tRNA. In *E. coli* and *Mycobacterium tuberculosis* the trmD do not bind the 3′, 5′-cAMP (Ahn, Kim,Yoon et al.,2003). Evolutionary adaptation is required for 3′, 5′-cAMP to act as signaling molecule to bind in the AdoMet-binding proteins such as TrmD in organism which doesnot contain 3′, 5′-cAMP such as *Staphylococus aureus (*Zhang, Agrebi, Bellows et al.,2017).

Similarly analysis were carried out in which *Klebsiella pneumonia*,which was incubated in glucose rich medium in which it showed that capsule polysaccharide (CPS) by reducing cyclic AMP (cAMP) levels.Deletion of cyaA or crp which codes for adenylate cyclase and cAMP receptor protein remarkably increased the production of CPS.This indicated that biosynthesis of CPS is directly related with cAMP- dependent Carbon catabolities repressor.The effect of glucose and CCR proteins on CPS biosynthesis resulted in bacterial resistance too serum killing(Lin et al.,2013).

To determine the specific binding of nucleotides in trmD different competitor nucleotides 3′,5′-cAMP, 3′,5′-cGMP, c-di-GMP, and c-di-AMP were used.Among them, the classic nucleotide 3′,5′-cAMP showed high affinity binding. The result showed that there is no any relation between *S.aureus* ArcR and transcriptional factor CRP of *E.coli* (Soberon-Chavez, Alcaraz, Morales et al., 2017).It is found that chemical structure of Adomet and nucleotide 3′, 5′-cAMP is similar which contains ribose and adenosine structure so they compete with each other for binding with trmD protein (Umitsu, Nishimasu, Noma et al., 2009).

In one experiment ,it is found to develop that *Serratia marcescens* were able to form biofilm in presence of glucose but in case of *Eschericia coli* and other enteric bacteria it inhibits the biofilm formation.There is inversely mediated intracellular cyclic AMP(cAMP) by help of adenylate cyclase (cyaA) activity,which helps in fundamental activities such as motility,carbon utilization and storage ,pathogensis and cell division in many bacteria(Kalivoda et al.,2008).Different computational techniques like multiple sequence alignment, PBLAST and pymol was used for specific identification of specific site through the amino acids with the structure of protein(Miller,Bromberg,Swint-Kurse,2017).

The biochemical analysis, bioinformatics showed that try-86 play a vital role in *E.coli* and *Mycobacterium* for the discrimination of native Ado Met and the 3′, 5′-cAMP (3.Tuma, J.M., et al.2013).The further phylogenetic analysis helps in identification of amino acids which went under the evolutory adaptation. The major function of 3′, 5′-cAMP in bacteria is the carbon catabolite repression by modified pathway in which the 3′, 5′-cAMP is not present (Warner and.Lolkema, 2003).

2.12. Literature review on *Streptomyces*

Actinomycetes are found to be intermediate of bacteria and fungi (Swaby, R J,1949).They are gram-positive ,free-living ,saprophytic bacteria containing high G+C content in DNA(Barka et al.,2016) helps in tolerance of environmental stress and are found everywhere. Mostly found in soil, water and colonizing condition in plants but abundantly in sediments, composts and fodder, aquatic buildings. In buildings it act as an indicator of moisture and microbial damage(Rintala Helena et al. ,2003).They produce slender,branched filaments which further develops in mycelia that may be long or short. In addition, it also possesses aerial mycelia that produce asexual spores called conidia (Jeya, Kiruthika, Veerapagu, 2013).Furthermore, some of them can also cause *invivo* and *invitro* inflammatory responses which can cause adverse health effects.

Streptomyces griseus , the largest cluster of genus mostly found in soil and some are reported in marine environment which produce many secondary metabolites .So for analysis of antimicrobial property, *Streptomyces griseus* and other members such as *Streptomyces albidoflavus* ,*Streptomyces cyaneus* and *Streptomyces coelicolor* were found to grown in indoor condition . For the further confirmation (Helena et al 2003), 16s rRNA sequence pcr was conducted in which proper identification of Streptomyces were done.Streptomyces plays a diverse role against pathogens (species of Corynebacterium, Mycobacterium, Nocardia, Propionibacterium, and Tropheryma), soil inhabitants (e.g., Micromonospora and Streptomyces species), plant commensals (e.g., Frankia spp.), and gastrointestinal commensals, Bifidobacterium species (Helena et al. ,2003).

Moreover,Streptomyces has high metabolic activity and high adaptative capabilities they are linked towards organic matter and convert to energy due to which they are able to form bioactive metabolites.As mentioned before Streptomycetes are found in all sites but there is limited number which may be due to low pH value.More actinomycetes were also found in area where there is absence of root which may due to more organisms in that site helping in degradation of organic compounds.But large number of actinomycetes were found in rhizospheric part of birch incomparision to pine (Golińska,Dahm,et al.,2011).

It has been found that Actinomycetes has been characterized with earthy smell and is ecologically important for turnover of organic material. It also shows a symbiotic relation between plants, fungi and animals.It has also been found that the secondary metabolities are formed by the interaction between other organisms. However, in some cases it can act as parasitic such as *Streptomyces somaliensis* and *Streptomyces sudanensis* effect humans .But can be beneficial and growth promoting in some cases for many insects, plants and marine animals. (Seipke et al.,2012).

Streptomyces is being used for antibiotics from long time which can be also observed in the given flow chart.

Figure 2.17: Chart of antibiotics discovered from different species of *Streptomyces*

(Lima Procopio, Silva, Martins et al., 2012).

Streptomyces species ,family Actinomycetes also known to be saprophytic in nature having the multiplication of three to nine days in medium which produce secondary antibiotics(Gamper,Masuda.frenkel-Morgenstern et al.,2015) and are being used for the treatment of various kinds of diseases in human and for the veterinary(Federation of Infectious Diseases Societies of South Africa,2011).It is a gram positive bacteria with high GC content which helps in tolerance of environmental stress.

There are different structures which are formed by *Streptomyces* which can be used for antibiotics formation and can be used in combination to act as antibiotics.

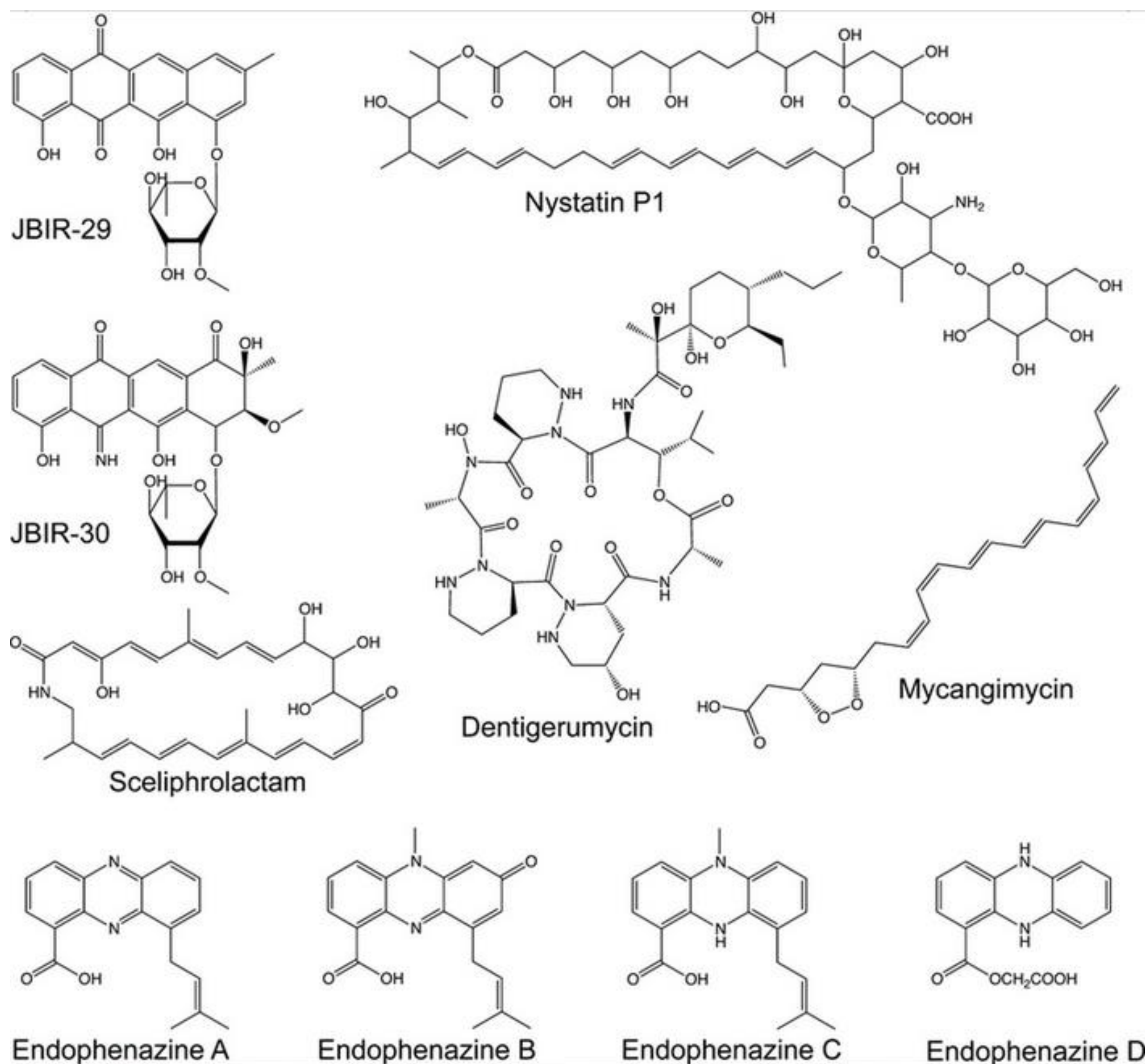


Figure2.18: Structure of novel antibiotics structure formed with combination of Streptomyces and insects.

From the above novel antibiotics structure formed by *Streptomyces* with combination of insects, JBIR29 and JBIR30 were isolated from *Streptomyces* spp. associated with a marine sponge (Khan et al., 2011). Nystatin P1 was isolated from a Pseudonocardia species associated with attine ant *Acromyrmex octospinosus*. Dentigerumycin was isolated from a Pseudonocardia species associated with the attine ant *Apterostigma dentigerum* (Oh et al., 2009);a mycangimycin was isolated from a Streptomyces species associated with the fungus-growing pine beetle *Dendroctonus frontalis* (Oh et al., 2009b); sceliphrolactam was isolated from Streptomyces species associated with mud dauber wasps (Oh et al., 2011; Poulsen et al., 2011) and the endophenazine compounds A–D were isolated from Streptomyces endosymbiont species taken from four different arthropods (Gebhardt et al., 2002).

Among them *Streptomyces lividans,* is important due to its non-filamentous bacteria, its genetic manipulation as well as it is able to produce large amounts of extracellular protein and for further processes such as cloning, expression and heterologous protein (Cruz-morales, Vijgenboom, Iruegas Bocardo et al.,2013).

Streptomyces coelicolor and *Mycobacterium tuberculosis* was found to be from same ancestors by analysis from comparative genome having similar type of surface protein (Bentley,.Chater,.Cerdeno-Tarraga et al.,2002). Streptomyces was used as the live vector in mice for protection from *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* AF2122/97 .Both bacteria has high similarity at level of individual genes sequence sharing several membrane proteins (Morris,Nguyen,Gatfield et al.,2005).

There was about 50% similarity between *Mycobacterium bovis* and *Mycobacterium tuberculosis* as well as in *Streptomyces coelicolor*. *Streptomyces lividan* is mostly used for DNA cloning in Streptomyces because *Streptomyces coelicolor* lacks methylation dependent restriction and also used in cloning, expression and secretion of heterologous protein (McGuire, Weiner,Park et al.,2012).

High serum antibody titer confirmed induced humoral response by body against *Mycobacterium* antigens by the cross reactivity induced after administration of *Streptomyces*. The comparative analysis carried out between the BCG and *Streptomyces*, suggesting it to be a good vector for the production of potent foreign protein for treatment of *Mycobacterium* (Verma and Jain, 2007).

For effectiveness of secondary metabolities produced by *Streptomycetes lividans* some experiment was carried out.It was carried out by Lima et al.,2012 by utilizing extract by inoculating in mice through intra peritoneal for the confirmation of non-pathogenic activity and to observe most effective route for immunogenic.After inoculation by peritoneal cavity it was found to be high induction of IgG as well as cross reactive recognize BCG proteins sharing similar type of epitopes present in bacteria. The cross reactivity after immunization of *S. lividans* produce was high in comparison to BCG and proteins helps in causing cross reactivity. The humoral response shown by the mice indicate that it is both organism that share several surface protein which can generate the cross immune response against Actinomycetes (Lima, Silva, Martins et al., 2012).

The viable bacteria after immunization with *S. lividans* and BCG was compared which showed decrease in bacteria by *S. lividans* incomparision to BCG.It was found that $CD4^+$ T-cells helps in development of immunity by enhancing secretion of the cytokines like IFNϒ and TNFἀ helping to induce macrophage with bactericidal products such as nitric oxide and reactive oxygen.$CD8^+$ where also evaluated by the major Th-1 type cytokine IFNϒ which helps in strong cellular immune found to be low after immunization of BCG incomparision to the Streptomycetes.Lymphocytes from the Streptomycetes were found to have similar type of IFNϒ and IL-10 as that of BCG(Glacomini,.Remoli,.Gafa et al.,2009).

Cell mediated immunization may be the sole mechanism for protection which controls by controlling the mycobacterial replication. *Mycobacterium* could be eliminated by the cross reaction antibiotics produced located in the mucosal surface which could help in efficient prevention of infection in host cells. The cell-mediated immunity in early lung bacilli is occurred due to inoculation of *S.lividans* should be noticed helping in the activation of the T-cells and macrophage (Sprott.W, Patel.Y. and Krishnan.K,2003).

Streptomyces are grown in ISP media with different carbon composition such as ISP1 to ISP9 which helped in identification of *Streptomyces* on the basis of their pigmentation,

growth and morphology (Taddei.P,2006) Different ISP which are designed for streptomyces are: ISP1 which contains yeast extract, pancreatic digest of casein, ISP2 contains yeast extract, malt extract, dextrose and ISP6 contains peptone, yeast extract and iron in media among them yeast extract was found to be common in all three media, because it helps nitrogenous compounds,carbon,sulfur,trace nutrients vitaminB and other growth factors essential for growth of bacteria (https://www.Sigmaaldrich.com/analytical-chromatography/microbiology /microbiology -products.html?TablePage=8657623).

The experiment was carried out by (Undabarrena, et al., 2017) in which mycelia was found to be white in color and grayish spores after incubation for 14 days in respective medias. Similarly, when Streptomyces sp. H-KF8 was grown on ISP3 containing outmeal, ISP4 containing soluble starch and inorganic salts, ISP5 containing glycerol and asparagine and ISP9 containing glucose media, in these media creamy mycelia was observed with white spores at surface area. Whereas incubation of Streptomyces in ISP7 showed low growth which contain tyrosine as medium (Undabarrena.Z, et al., 2017).

Among all the media prepared for Streptomyces ISP2 is considered to be the best media because it was able to generate bioactive metabolites and antifungal activity in submerged fermentation (Cunha et al., 2009).Large zone of inhibition was seen in Bacillus subtilis ATCC6633 when cultured in the presence of Streptomyces present in plant roots (http://www.scielo.br/pdf/bjps/v52n2/2175-9790-bjps-52-02-00265.pdf). Antimicrobial agents were produced by *Streptomyces* against *Aspergillus flavus* and *Aspergillus terreus* in ISP2 incomparision to ISP6 which showed good zone of inhibition but growth of bacteria and fungi were more promoted for growth(Rashad.K et al.,2015).

For antimicrobial activity different medias such as ISP1, ISP2, ISP6 were found to be effective. In which zone of inhibition against bacteria was easily seen.It was found that as incubation period of Streptomyces increases it helps to increase zone of inhibition which concluded that time of incubation also plays a vital role for inhibition activity (Undabarrena,et al.,2017).

Streptomyces species were able to form the different compounds such as gibberellic acid, indole acetic acid, abscisic acid, kinetin and benzyladenine.Among them, indole acetic acid was also found to be generated by marine Streptomycetes as phytohormones by utilizing shrimp shell waste as mono component medium which can be benefit for controlling agro-ecological condition and as biocontrol agent for management programs (Rashad et al.,2015).

Different *Streptomyces*, bacteria as well as fungi were isolated from rhizospheric region of Eucalyptus. Among them,Streptomycetes were taken as preferred one.Moreover three streptomycetes were taken as important which were EUSKR2S82 and EUSNT1H43 (isolated from rhizosphere soil), and EURKR1S17 (isolated from the root) found to be member of Streptomycetes capable of producing Indole-AceticAcid, siderophores and solubilise phosphate(Himaman, et al.,2016).Many research paper had reported that *Streptomyces* inhibits most of plant pathogens including fungi and bacteria (Golińska and Dahm, 2013, Kobayashi et al., 2012, Mingma et al., 2014).

Lignin,a phenolic heteropolymer which helps in thickening of cell-wall for mechanical strength which is formed by flavone tricin, native lignin polymer(Eloy et al.,2017) formed

during pulp formation(Puziy et al.,2018).Lignin polymer was formed by combination of lignin and [2-(methacryloyloxy) ethyl] trimethyl ammonium chloride (METAC) for the removable of anionic dyes from wastewater which is independent of PH.As well as inorganic salts also plays a significant role in removal of dyes(Fang et al.,2010).

Different *Streptomyces* characterize by filaments are found to degrade lignin(Fernandes et al.2014).Three enzymes (laccase, LiP, MnP) are found to play important role in biodegradation in lignin which are found in different species of Actinomycetes such as S. *coelicolor*, *S. griseus* and *S. psammoticus* (Niladevi, Sheejadevi and Prema 2008; Le Roes-Hill, Rohland and Burton 2011).As well as,different species of actinomycetes such as *S. cinnamomeus* which has enzyme laccase and LiP activity(Jing and Wang 2012) and *S. ipomoea* contain laccase activity which helps in degradation of lignin.(Niladevi, Jacob and Prema 2008; Molina-Guijarro et al.2009; Fernandes et al.2014).These bacteria helps in degradation of lignin of soil.

Fungi are also saprophytic nature which helps in degradation of lignin and helps in removable of organic matter in biosphere(Kuhad and Singh 2007; Huang et al.2013).Different saprophytic fungi are divided into three main groups: (i) white rot fungi, (ii) brown rot fungi and (iii) soft rot fungi helps in lignin degradation(Blanchette 1995; Liers et al.2011).After the utilization of lignin degradation by white rot fungal, production of CO_2 and H2O(Blanchette 1995).It was found to be produce multiple isoenzymes for white rot fungal but doesnot produce laccase(Deacon.W 2006).

Lignin degrading gene named LiP activity was seen in some bacteria such as as*Acinetobacter calcoaceticus* and *Streptomyces viridosporus* (Dashtban.R et al.2010).The gene LiPs are lignin attacking polymers but are non-specific to substrates but can oxidize different phenolic and non-phenolic compounds with many organic molecules (Wong 2009).The LiP has a globular structure composed of eight major and eight minor α-helices with limited β components which forms active site by the help of heme. (Choinowski et al.1999).As well as it contains two glycosylation site,Ca^{2+} binding sites and four disulfide bridges to form three-dimensional stable structure of enzyme(Sigoillot.L et al.2012).

Beside a suite, of oxidative enzymes that could modify lignin for breakdown by hydroxylation or demethylation, one interesting example, a gene encoding a non-heme iron dioxygenase fused to a carbohydrate-binding domain was found in *Streptomyces* sp. SirexAA-E (Bianchetti.P et al.2013).

SilA gene was found in *Streptomyces ipomoeae* CECT 3341 which was analysed by (Blanquez et al., 2017) in which the soluble form of lignin was extracted from wheat straw by Solid State fermentation. Moreover, it has been found that laccases from *Streptomyces* such as ScyA laccase and *Streptomyces cyaneus* and SLAC laccases from *S.coelicolor* were found for degradation of lignin (Blanquez.K et al., 2017).

2.13. Literature review on Carbon Catabolites Repression

Soil ,matrix of heterogeneous bacteria and other small components which helps in survival through ,metabolic activity.In this activity carbon sources is required and response shown by microorganism is studied by terminal restriction fragments length polymorphism (TRFLP) analysis, which helps in indication of enrichment of structurally

similar carbon sources for same type of bacteria. The analysis indicated that bacteria first utilize glucose or benzoate and transferred to primary carbon source. Leucine can be used as a selective challenge which can replay concept made by acetate. It is found that soil from different areas used same type of source for carbon which showed that multiple enrichments should be focused instead in diversity of bacteria (Wawrik .Met al., 2005).

In our environment, different organic carbon source compounds are found in different forms. Among them, bacteria can easily use organic compound which are in dissolve form which helps to enhance growth of bacteria but on other side, it also affect metabolic activity mostly in aquatic environment. Metabolic activities includes biomass production(BP),bacterial respiration(BR) and bacterial growth efficiency(BGE).On metabolic activity, negative interaction was seen only on BP whereas positive interaction was seen on both BR and BGE(Fonte.L et al.,2013).

For analysis of different Carbon sources for bacteriopankton ,it was grown in media containing leucine agar where colonies of bacteria was grown which helped to conclude that bacteria can utilize L-amino acids, glucose, acetate and pyruvate except glycolate.Whereas,colonies size and shape was found to be different while different source of carbon were used, which indicated that carbon source and environment had adverse effect on bacterial growth.It was observed that γ-proteobacteria, a Vibrio and α-proteobacteria found to be dominate towards glucose whereas one α-proteobacteria was found to be dominate towards glycolate.It indicated bacterial growth can be controlled by carbon sources and environmental condition.

Also, the utilization of carbon was also analysed by DNA extraction by result obtained. It was found that sharp band which represented that it may be due to utilization of single carbon.Similarly, second band was found to be normal or weak utilizing large source for carbon sources and third bacteria did not show the band which indicated that they did not participate in utilizing different carbon source or other specific carbon .All these activities were quantified by the pcr and microscopy by using probe named FISH (Lindh.L,2007).

Not only this but also carbon source can be used for the production of antimicrobial substances against some plant pathogens. In this context, antibacterial products produced by *Malikia spinosa* and UPMKB4 ,grown in different derivatives of carbon sources such as glucose, fructose and glycerol were used against *Fusarium oxysporum* and *Colletotrichum gloeosporioides* which showed positive result.So, modification in carbon source can enhance production of antimicrobial against them (Farhana.R et al.,2011) .Furthermore ,Carbon source utilization tests helps in taxonomical identification of bacteria(Snell, Lapage,1973).

Different derivatives of glucose such as maltose,sucrose and soluble starch were tested for observing CCR.Among them,dextrose was found to be best source for *Streptomyces kananmyceticus* giving high yield in which pH played important role but sucrose and soluble starch is mostly used by bacteria during cell growth. $(NH_4)H_2PO_4$and yeast extract were used as nitrogen source there is no any relation between number of bacteria and antibiotic production (Pandey.Y, Shukla.G, and Majumda.Mr, 2005).

Glycerol can be carbon source for *Gluconacetobacter xylinus* ATCC 53524 to gain high production of high labeled cellulose (Mikkelsen et al.,2009).

Antibiotics are secondary metabolities which have low-molecular weight and not essential for bacteria for growth but can be useful to other organisms and human too. Mostly they have unusual structure and generated from intracellular part of the cell and condensed to complex form through metabolic pathway. Some studies have shown that carbon has effect on production of antimicrobial activities through fermentation process it also causes the negative effect on bacteria for antibiotics production. Recently, it has been noticed that carbon source regulation should be well known for production of antibiotics in molecular and biochemical levels. Proteins and protein complex plays important vital role in antibiotics synthesis. The use of genetic analysis, understanding the CCR process could help the research area great profit also will be easily to select the secondary metabolite overproducing strain. Both strain and nutritional modification helps to lead in factory size (Sanchez et al., 2010).

Carbon catabolite repression (CCR), relates to utilization of carbohydrates helps to control metabolism of different carbon sources in bacteria and it depends on genes which is regulated by specific genes(microbiology letter).Diauxic growth (two type glucose,lactose) of bacteria is also under control of bacteria (Kremling et al.,2015).Catabolite gene activator protein(CAP) is considered as global control protein and also known as cAMP receptor protein or catabolite control protein(CcpA) with low GC content.CCR helps in sugar utilizing ability of their metabolic capacity.

The main achievement gain by CCR in bacteria is utilization of carbon source from a mixture in growth medium and to utilize carbon sources on the basis of metabolic capacities. During these processes regulatory response differs on the basis of strength and choice of carbon will on basis of sugar specific induction. Furthermore, it also limit the utilization of carbon source by auto regulatory process on basis of plentiful consumption by cells.(Bruckner,2002).

In *Eschericia coli*, there is presence of gene named Catabolite Repression gene (cat) found between pyrC and pur B genes. Mutation in these genes did not give any effect on carbon catabolite repression (Tyler et al., 1969).When bacteria was grown in media containing Xylulose, arabinose and lactose preferred carbon source was found to be sucrose and arabinose is preferred for transcriptional activity.In some condition cAMP also act in regulation of non-glucose sugars and it was found to be true through research. As well as arabinose and xylose also repress lactose gene.

For the carry out of glucose-lactose diauxie AMP and CRP complex are necessary for bacterial growth.In this type of condition a specific promoter are required and in the presence of glucose β-galactosidase synthesis does not takes place due to lack of cAMP which is controlled by phosphor transferase system(PTS) by reducing level of cAMP. So, lag phase can be reduced by adding exogenous cAMP in growth medium.

In condition of diauxine inducers play an important role, cAMP-CRP play a vital role by activating transcription of glucose transporter gene. But as cAMP increases there is decrease of β-galactosidase but increase in lag phase.Beside inducer,CRP can also reduce expression of β-galactosidase,cAMP in cells grown in lactose is comparatively

high incomparision to glucose one which help in detection of phosphorylated enzymeII ^{Aglc} in lactose-grown cells incomparision to glucose-grown cells.

In *Bacillus,*different transcription factors are found which are CcpA also coeffectors HPr and Crh which are phosphorylated by HprK kinase.Primary mediator of catabolite repression and activator.Regulation which is CcpA-dependentis fully regulated by HprK and partially regulated by serine –phosphorylated (HPr)(Lorca et al.,2005) genes which are mediated by nitrogen or phosphorus metabolism and which act on stress is related to CcpA-dependent glucose control.Sugar transported by phosphotransferase system (PTS) causes strong repression whereas Crh also causes repression.The phosphorylation state of HPr are found to be in ots His 15 and PTS activity.

However, the phosphorylation state of HPr at its His15 residue and PTS transport activity have no impact on the global CCR mechanism, which is a major difference compared to the mechanism operative in *Escherichia coli*. Our data suggest that the hierarchy in CCR exerted by different substrates is exclusively determined by the activity of HPrK/P (Singh et al.,2008).

2.13.1. Literature review on Carbon Catabolite Repressor in Streptomyces

Streptomyces coelicolor consists of glucose kinase which is necessary for Carbon Catabolite Repressor (CCR) exerting though glucose and other carbon which can be seen by monitoring cellular activities of enzyme. Purified glucose kinase by keeping protein stable for several weeks and used for polyclinical antibodies.Purified glucose kinase helped to relate protein-protein interaction by surface plasmon resonance indicating glucose kinase interacting with protein (Mahr et al., 2000).

Streptomyces lactamdurans depends on glucose for negative regulation on biosynthesis of cephamycin C. When there is excess glucose it cause decrease in synthesis of cephamycin precursor(α-aminoadipy1)-cysteinyl-valine which is due to repression on ring-expanding enzyme deacetoxycephalosporin C synthase by glucose.Whereas,Isopenicillin N synthase and Isopenicillin N epimerasse arenot repressed by glucose but activity of isopenicillin N synthase was inhibited by glucose-6-phosphate.As well as Deacetoxycephalosporin C synthase activity was inhibited by inorganic phosphate,glucose 6- phosphate,fructose 2,6-diphosphate and fructose 1,6-diphosphate.cAMP decreases as growth of bacteria increases and doesnot play any role in controlling glucose during cephamycin biosynthesis(CORTeS et al.,1986).

Streptomyces clavuligerus, which is regulated for production of Cephalosporin.Some carbon sources such as glycerol and maltose suppress production of antibiotics whereas poor carbon sources such as α-ketoglutarate and succinate, helps in production of cephalosporins (Aharonowitz et al.,2006).

The mutant *S.coelicolor* A3 (2) M145 grown in non metabolizable glucose analog –deoxyglucose. During this test glycerol kinase and agarose were relized on different carbon tests even when there wasnot carbon sources.It indicated that catabolite repression isnot only regulated by flux through glucose kinase and protein but has its own carbon catabolite repression.Over expression of glucose kinase results in catabolite repression and repress signal which is due to binding sites on DNA promoter regions and

indirectly by competing for binding of another regulatory proteins (Kwakman et al., 1994)?

CCR can also effect on synthesis of secondary metabolities and utilization of alternative carbon sources.In different *Streptomyces*, glucose kinase (Glk) helps in phosphorylation and regulatory protein for CCR which was analyzed in repressed and non-repressed conditions. Gene glk of *S. coelicolor* M145was found to be similar to *Zymomonas mobilis* (Zmglk) which isnot sensitive to CCR but can grow in glucose.Transcriptome of classical model of CCR was studied in *S. coelicolor* to observe the effect of glucose or ScGlk in regulatory mechanism. Glucose plays important metabolic and transcriptional changes in organism. It plays an important part in glycolysis, pentose phosphate pathway and gluconeogenesis. Glucose stimulate CCR by repressing transporter systems and transcription of enzymes for secondary carbon sources utilization.hence,it was found that glucose and ScGlk-regulated pathways and a putative regulatory proteins may be involved in control of CCR.(Romero-Rodríguez, 2016).

CCR mechanism controls the expression of genes involved in the uptake and utilization of alternative carbon sources in *Streptomyces* and is mostly independent of the phosphoenolpyruvate phosphotransferase system (PTS). All Secondary metabolites are not controlled by CCR in Streptomycetes. CCR also affects morphological differentiation and the synthesis of secondary metabolites, although not all secondary metabolite genes are equally sensitive to the control by the carbon source. When the product is same but essential mechanisms can be differ (Romero-Rodríguez et al., 2017).

CHAPTER-3

MATERIALS AND METHODOLOGY

3.1. Search for drug target and compound acting against it

Selection of target protein for molecular docking

The docking process is one of computational techniques carried out in the development of drugs for drug designing process. For docking process, different technical activities were carried out such as Pyrx, protein database.

On the basis of essentiality of SAM and SAH in pathway with tRNA methyltransferase D (TrmD) protein selected as lead protein of most prokaryotes such *Eschericia coli*,*Pseudomonas*, *Salmonella* etc.

3.1.1. Finding and obtaining the crystallographic structure.

Different derivatives of TrmD structure bounded with S-Adenosylmethionine (SAM) synthetase and S-Adenosylhomocysteine (SAH) were identified of different bacteria and crystallographic crystals were obtained from Protein Data Bank (https://www.rcsb.org/).

3.1.2. Selection of the control compounds and ligands.

S-Adenosylmethionine synthetase and S-Adenosylhomocysteine are the natural ligands for the protein which was taken as the reference compounds. Binding energies were calculated by replacing the different ligands obtained from ZINC database(http://zinc.docking.org) and the ligand giving high binding energy in comparison to the natural ligands were considered to be the most potent one for that protein.

3.1.3. Redocking of the natural ligand

Binding energy of the natural ligand was calculated by placing the natural ligands in the specific protein, which is known as Redocking process. This process is carried out for the comparative study of the binding energy with the other ligands derivatives of indole,Such as indole,isatin,indirubins etc.

3.1.4. Structure-based virtual screening

The structure –based virtual screening was carried out by using Auto Dock Vina achieved for the improvement of the accuracy of the binding mode predictions (Cosconati et.al., 2010). PyRx (Sawyer2007) is the easiest application which helps in finding the binding energies.

3.2. Selection of test organisms

Different bacteria were collected.Among them some were American type culture collection of *Eschericia coli* ,*Pseudomonas aeroginosa* and carbapenem resistant *Klebsiella pneumoniae* and *Pseudomonas aeroginosa* which were collected from Nutan

Thakur and Krisha Sthapit and further tested by standard disc diffusion techniques mainly carbapenem antibiotics.

3.3. Screening of *Streptomyces* from different areas of Nepal for antimicrobial production

Nepal is country of diversity on basis of organisms due to its diverse environmental conditions. Due to this diverse condition it is predicted that there is high number of microbes including *Streptomyces* expecting for novel antimicrobial products from them mainly targeting to emerging antibiotics resistance bacteria.

3.3.1. Sample Collection

Soils were taken as the samples from different locations of Nepal (including all regions such as upper, central and lower) were collected targeting the moist areas and stored in laboratory of Central Department of Biotechnology. For the further sample collection fern rare tree was targeted during collection of sample from Panchase area near to Pokhara due to its origin and saprophytic nature of Streptomyces on the basis of previous studies and assuming that novel *Streptomyces* can inhabitat over there.

3.3.2. Methods of collection

1. Scaple was used for the soil collection from the approximate depth of 5cm and stored in clean plastic bag.

2 .Samples were then stored in refrigerator.

3. The soil samples were further treated for the isolation of Streptomycetes from it and was further analysis was done for the confirmation of it.

3.4. Primary culture and Isolation pure *Streptomyces*

For isolation of *Streptomyces* from the different soils collected from different areas of Nepal. Following techniques where carried out:

Six sterile test-tube filled with 10 ml of sterile and autoclaved water were taken. 1gm of soil sample was diluted in first test-tube and further diluted in other five test tubes. Then the tubes were vortex and left for one minute and1ml from first test-tube was transferred to the second test-tube and continuously carried out to other test-tube until dilution was carried out till 10^{-6}.100ul were transferred from the labelled$10^{-4}$to specific media plates that is International Streptomyces Project 2 (ISP2) and International Streptomyces Project 4 (ISP4) (Shirling and Gottlieb,1966) with the antibiotic Nalidixic Acid.(50ug/ml)antifungal.(Appendix composition of ISP2 And ISP4).Then the plates were incubated at 28°c for 15 days under aseptic condition. But regular check was carried in each interval of 2-3 days to prevent contamination. Colonies with dry, powdery, non-mucoid and muddy smell were taken for pure culture from primary culture.

3.5. Gram staining of Putative *Streptomyces*

A clean and grease free slide was taken with a drop of distilled water. Then, sample of putative *Streptomyces* were transferred into slide with help of wooden stick. After that,

a smear was prepare and left for a minute to air dry and heat fixing was done. Drops of crystal violet was flooded over the smear for 60 seconds and washed with running water. Then after, iodine was flooded for 30 seconds and washed by running water. After that, decolourizer was used for destaining of the smear. At last, safranin was used for 20 seconds, washed with water and dried in air or with the help of blotting paper. Finally, microscopy was carried out for detail observation about *Streptomyces*.

3.6. Biochemical tests of putative *Streptomycetes* and Bacteria

3.6.1. Indole test

Some sterilized glass culture tubes were taken containing 5ml tryptophan broth. The tubes were inoculated with the culture under aseptically condition and incubated for the 24hours at 37°c.0.5ml of kovac's reagent was added to the broth.

3.6.2. Methyl Red-Voges-Proskauer (MR-VP) test

For methyl red test, prior to inoculation, medium was allowed to equilibrate to room temperature. Using organisms taken from an 18-24 hour pure culture, the medium was lightly inoculated. They were then incubated aerobically at 37°c for 24 hours. Following 24 hours of incubation, 1ml of the broth was aliquot to a clean test-tube. The remaining broths were incubated for 24 hours. Then 2 to 3 drops of methyl red indication was added to aliquot. And the red color was observed immediately.

Voges-Proskauer test, prior to inoculation, the medium was allowed to equilibrate to room temperature. Using organisms taken from an 18-24 hour pure culture, the medium was lightly inoculated. They were incubated aerobically at 37°c for 24 hours. Following 24 hours of incubation, 2ml of the broth was aliquot to a clean test tube. The remaining broth was re-incubated for an additional 24 hours.6drops of 5% alpha-naphthal was added, mixed well to aerate. Then 2 drops of 40% potassium hydroxide was added, and mixed well aerate. A pink-red color at the surface within 30 min is observed. The tube was shaken vigorously during the 30 min period.

3.6.3. Citrate utilization

The Simmons's citrate agar was boiled, autoclaved at 121°c under 15psi pressure for 15 minutes and cooled in slanted position(long slant, shallow butt).The inoculated medium will be a deep forest green due to the pH of the sample and the bromo thymol blue. A light inoculum picked from the center of a well-isolated colony was stabbed 37°c for up to 4-7 days. Observe a color change from green to blue along the slant in case of positive result.

3.6.4. Urease test

The urea agar was boiled, autoclaved at 121°c under 15 psi pressures for 15 minutes, and distributed 4 to 5ml per sterile tubes and tubes were slanted during cooling until solidified. The surface of a urea agar slant was streaked with a portion of a well–isolated colony or inoculated slant with 1 to 2 drops from an overnight brain-heart infusion broth

culture. The cap was left on loosely and incubated the tube at 35°c-37°c in ambient air for 48 hours to 7 days. In case of positive result the development of a pink color was examined for as long as 7 days.

3.6.5. Oxidase-Catalase test

3.6.5.1. Oxidase Test:

Strip of Watmann's No.1 filter paper was soaked in a freshly prepared 1% solution of tertramethyl-p-phenylene-diamine dihydrochloride.After draining for about 30 seconds, the strips were freeze dried and stored in a dark bottle tightly sealed with a screw cap. For use, a strip was removed, laid in a petri dish and moistened with distilled water. The colony to be tested was picked up with a (platinium) loop and smeared over the moist area. A positive reaction is indicated by an intense deep purple blue, appearing within 5-10 seconds, a "delayed positive" reaction by coloration in 10-60 seconds, and a negative reaction by absence of coloration or by coloration later than 60 seconds.

3.6.5.2. Catalase Test

A sterile wooden stick was taken to transfer a small amount of colony growth in the surface of a clean, dry glass slide. A drop of 3% H2O2 was poured in the glass slide. The evolution of oxygen bubbles was then observed.

3.6.6. Triple Sugar Iron Agar (TSIA) Test

The TSI agar was boiled, autoclaved at 121°c under 15 psi pressures for 15 minutes. The agar was cooled in slanted position (long slant, shallow butt).With a sterilized straight inoculation needle the top of a well-isolated colony was touched. The TSI Agar was inoculated by first stabbing through the center of the medium to the bottom of the tube and then streaking on the surface of the agar slant. The cap was left on loosely and incubated the tube at 35°c in ambient air for 18 to 24 hours.

3.7. Mass production of secondary metabolities

For the mass production, ISP2 broth were inoculated with pure putative Streptomyces for 7-15 days during these interval regular check was done to prevent it from contamination in shaking incubator for secondary metabolites production .Media was treated with ethyl acetate in ratio of 1:1 and separating funnel was used after keeping it for agitation for 30min.Then extract was concentrated by using rotavapour which was dissolved and preserved by use of methanol.

For high production of antimicrobial substances slight modification was done on media on basis of nitrogen and carbon. Indole and tannic acid was added in the media in the absence of glucose Putative Streptomyces were inoculated in the modified media for the production of the secondary metabolites and further analysis were carried out by Antibiotic Susceptible test (AST).

3.8 Extraction from Calendula

Dried sample of Calendula were collected and washed to remove all the dusts and extra particles from sample a dried at 28°c and weighed to be 0.1 gm. Then it was crushed and made paste was made, which was then dissolved in ethyl acetate in the ratio (1:1). Then, the rotavapour was used for extraction with temperature of 28◦c at 160rpm.Then weight was taken which was found to be 0.01 and was dissolved in 100% ethanol. Then, AST was carried out against MDR bacteria which showed zone of inhibition.

3.9. Antibiotic Susceptibility Test (AST)

3.9.1. Standardization of Inoculum

This was done as described by CLSI. A pure culture of identified Salmonella isolate (s) from a 24-hour plate culture was selected. Sterile wire loop was used to pick three colonies of each Salmonella serotype and emulsified in 5 ml of nutrient broth (NB) and then incubated for 24 hours at 37°C in aerobic condition. Next day, the tube containing the bacterial suspension was adjusted with extra inoculum or diluents, if necessary, until 0.5 McFarland standards were obtained.

3.9.2. Inoculation of test plates

After adjusting the turbidity of the inoculum suspension, 200µl of inoculum was mixed in culture tube containing 8 ml of soft agar (NB+0.75% agar), mixed gently when in liquid state (temp. around 60°C) and poured off to the dried surface of a 20 ml Mueller-Hinton agar plate in a 120 mm borosilicate Petri plate. The inoculated plates were air dried at 37°C to allow for any excess surface moisture to be absorbed before applying the antibacterial drug discs.

3.9.3 Application of discs to inoculated agar plates

All positive cultures of *Klebsiella* and *Pseudomonas* serovars were tested in vitro for susceptibility to different antibacterial drugs by agar diffusion technique as described by Kirby-Bauer and WHO (Biemer, 1973). This was carried out per WHO protocol. The susceptibility testing of *Klebsiella* and *Pseudomonas* isolates was carried out using Mueller-Hinton agar and was tested in vitro for susceptibility to seven different antibacterial drugs; Ampicillin (10mcg), Chloramphenicol (30mcg), Ciprofloxacin (5mcg), Nalidixic acid (30mcg), Gentamicin (10mcg), Amikacin (10 mcg) Ceftazidime (30 mcg)and Cotrimoxazole (25mcg).All the antibiotic discs were commercially produced by HiMedia.

The inoculated plates were air dried under aseptic condition to eliminate the liquid on the surface of the medium, sterile forceps was used to place the antibacterial discs on the inoculated plates. Within 30 minutes after applying the disc, the plates were inverted and incubated at 37°C for 18 hours. Measuring scale was then used on the underside of the plate to measure the diameter of each zone of inhibition in millimeter. Zone diameter for the sample was compared with NCCLS Published Interpretative.

Then, dried sample of Calendula were collected and washed to remove all the dusts and extra particles from sample and dried at 28°c and weight to be 0.1 gm. Then it was crushed and made paste, which was then dissolved in ethyl acetate at the ratio (1:1).

Then, rotavapour was used for extraction with temperature of 28°c at 160rpm.Then weight was taken which was found to be 0.01 and was dissolved in 100% ethanol. AST was carried out against MDR bacteria which showed slight zone of inhibition.

Moreover, soils from different areas of Nepal were collected from about 5cm below with help of spatula. Then soils were diluted and spread on plate International Streptomyces Project Medias (ISP2, ISP4) after sample was diluted at 10^{-6} which was incubated at 28°c for 15 days. The putative *Streptomyces* grown in the media was resteaked on the specific media and inoculate in broth media for secondary metabolites production. The extract extracted from these broths was tested against carbapenem resistant bacteria but did not show zone of inhibition.

So, on the basis of results shown by dry lab and with the concept of review, developed concept that phenolic groups can develop stress as well as can act as source of carbon and nitrogen sources and develop modified media. As we have reviewed that with help of stress Streptomycetes can produce high amount of antimicrobial products, media composition of modified media was calculated as:

Table 3.1: Modified media composition for Streptomyces

S.N	Macronutrients	Gram/Litre
a.	Di-potassium phosphate	0.5
b.	Magnesium sulfate	0.2
c.	Sodium chloride	0.1
d.	Calcium chloride	0.02
e.	Ferrous sulfate	0.1
f.	Ammonium sulfate	2.0
g.	Potassium chloride	0.1
h.	Indole/Tannic acid	0.025M

S.N	Micronutrients	Gram/Litre
a.	Copper sulfate penta hydrate	0.4
b.	Zinc sulfate	0.12
c.	Phosphorous acid	1.4
d.	Sodium molybdate	1.0
e.	Manganese (ii) sulfate mono hydrate	1.5

Antimicrobial test was carried out by 30 strains of *Streptomyces* which were inoculating in modified media in sterile condition and incubated at 28°c in shaker at 150rpmm for 3 days. Regularly culture of checked to observe presence of contamination and growth of bacteria on the culture. The antimicrobial test was carried out with extract extracted in modified media. For the antimicrobial test the crude extracts were used, tests were carried out in MHA (Muller Hinton Agar) where carpet culture was done of carbapenem resistance bacteria. Among 30 strains of Streptomyces extracted only 2 strains showed the positive result.

3.10. Molecular Diagnostic Test

3.10.1. Genomic DNA isolation

A. Preparation of cells

Klebsiella, *Pseudomonas* and *Streptomyces* were revived and streaked on specific agar plate and incubated overnight at 37°c and at 25°c respectively. Then, single isolated colony was inoculated in 2ml of LB medium and incubated at 37°c and 25°c respectively for 12 hours. From this overnight culture 1.5ml was transferred to sterilized Eppendorf tube and centrifuged at 5,000rpm for 5minutes at 4°c.Immediately, supernatant was discarded by aspiration and remaining overnight culture (0.5ml) was also added to same tube containing the cell pellet and centrifuged again at 5,000 rpm for 5minutes at 4°c.Then, supernatant was removed as much as possible without disturbing the cell pellet.

B. Cell lysis

The cell pellet was re-suspended in 450ul of TE1 buffer by gentle pipetting .The solution was split into two fresh sterilized eppendrof tubes by transferring 225ul of above suspension to each tube. To each tube 180ul of lysozyme (1mg/ml) was added. Both tubes were incubated at 37°c for 30 minutes gently mixing the solution by inverting the tube and incubated at 37°c for 45 minutes or until the solution was added in both tubes and incubated at 37°c for 45minutes until the solution is clear due to the cell lysis, with gentle inversion in between the ice incubation period. Equal volume of chilled phenol (450ul) was added and mixed well. The mixture was centrifuged at 13,000 rpm for 10 minutes. The upper aqueous layer containing DNA was transferred to fresh sterilized eppendorf tube without carry-over of lower organic phase. Again, equal volume of chilled phenol:chloroform:isoamyl alcohol(25:24:1)was added to above aqueous solution, mixed well and centrifuged at 13,000 rpm for 2 minutes and the aqueous phase was collected in a fresh Eppendrof tube.

C. Genomic DNA recovery

To the aqueous solution (450ul)containing genomic DNA,100ul of 3M chilled sodium acetate(pH5.2) and double volume of 95% ethanol (1,100ul)was added and incubated at -20'c for 30 minutes. Then the mixture was centrifuged at 13,000rpm for 20minutes at 4'c.The supernatant was poured off and pellet was washed with 250ul of 70% ethanol without disturbing the pellet. Then the solution was centrifuged at 13,000 rpm for 10 minutes at 4°c.After draining the supernatant, remaining ethanol was removed by keeping the tube open in room temperature for 5-10 minutes. Care was taken not over dry the DNA pellet. The genomic DNA was resuspended in 100ul of autoclaved MilliQ water or TE buffer (pH 8.0) and stored at 20'c until use.

D. Quantification of template DNA

1ul of the isolated genomics DNA was taken and loaded in the nano drop.

3.10.2. Polymerase Chain Reaction (PCR)

Chromosomal DNA extracted from *Klebsiella*, *Pseudomonas* and *Streptomyces* were carried out by using it as a template DNA for PCR amplification under given PCR condition of universal 16S primer BLa NDM conditions is:

Table 3.2: Condition for Polymerase Chain Reaction

Conditions	Temperature	Time	
Initial Denaturation	95°c	3min	
Denaturation	95°c	1min	29cycle
Annealing	50°c	31min	
Extension	73°c	1min	
Final extension	72°c	5min	
Hold	4°c	Infinity	

3.10.3. Phylogenetic Tree

Samples were send for sequencing process after PCR was carried out, sequence obtained were further analyzed by BLAST program. On the basis of similarity shown by isolated samples phylogenetic tree was constructed by the use of Clustal W by further analysis.

CHAPTER-4

RESULT AND DISCUSSION

On the basis of WHO, classification *Mycobacterium tuberculosis* is not included under any priority but it is one of the most immerging causes of tuberculosis. It is one of the difficult diseases to cure and the antimicrobial resistance emerging in these bacteria is compounding its management. As it is transmitted through the areoles research on this organism Biosafety level 3 with Class II biological safety cabinet, the present research could not focus on this pathogens but the envisaged antimicrobial efficiency of new molecule has been taken in consideration that the mechanism could be replicated in *M.tuberculosis*,also.Thus,the focus was in WHO prioritized pathogens available.

The desperate need of new molecule to manage infections by the WHO prioritized pathogens have ignited concerted efforts by different scholars around the globe and Central Department of Biotechnology, Tribhuvan University was isolated. The project focused on screening of soil dwelling strains that could potentially make antimicrobials against Carbapenem resistant *Klebsiella* and *Pseudomonas* isolated from clinical samples obtained from Tribhuvan University Teaching Hospital (Nutan Thakur and Krisha Sthapit, 2017).

4.1. Characterization and revival of Bacteria

The pathogens that were cryo-preserved as glycerol stocks of *Klebsiella* and *Pseudomonas* were retrieved in Luria-Bertani Agar (Figure 4.1) to check and confirm their originality. For other subsequent experiments either Luria-Bertani broth or Luria-Bertani Agar were used.

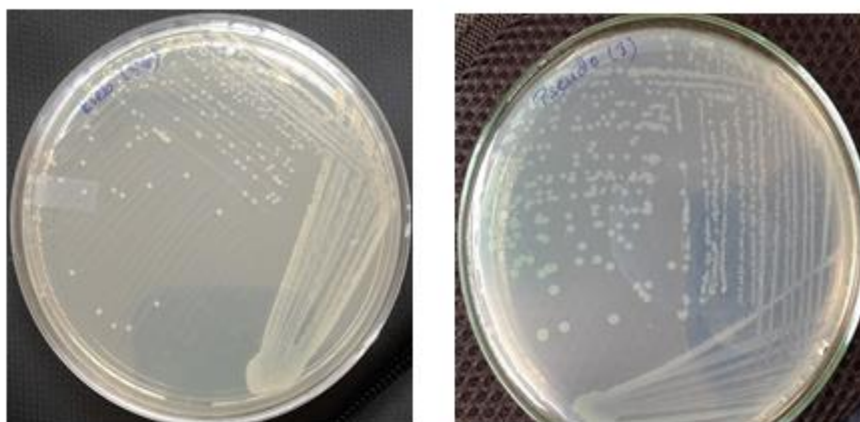


Figure 4.1: Isolation of *Klebsiella* and *Pseudomona*s from cyro-stock

4.1.1. Biochemical Tests

The biochemical test and gram staining is considered as the preliminary test for the confirmation and identification of bacteria that can also distinguish their families on the basis of Bergey's Manual.These tests were carried out to confirm that the isolates were the respective pathogens *Klebsiella* and *Pseudomonas.* As illustrated in Tables 4.1 and 4.2 the biochemical test are in concordance to that are reported for respective pathogens.

Table4.1: Biochemical tests for identification of *Klebsiella* and *Pseudomonas*

S.N	Biochemical tests	Results	
		Klebsiella	*Pseudomonas*
1.	Indole	Positive	Negative
2.	Methyl Red	Negative	Negative
3.	Vokes Proskauer	Negative	Negative
4.	Citrate	Positive	Positive
5.	Catalase	Positive	Positive
6.	Oxidative -Fermentative	Fermentative	Oxidative
7.	Capsule	Capsulated	Non-Capsulated
8.	H2S	Negative	Negative
9.	Gas	Positive	Positive
10.	Gram staining	Negative	Negative

4.1.2. Antibiotic Susceptibility Test (AST)

The retrived strains were tested for their antibiotics sensitivity towards different antibiotics. Those strains that showed sensitivity towards the antibiotics were not taken for further research and those showing resistance to three or more antibiotics and carbapenem were(Figure 4.2) taken for further research.

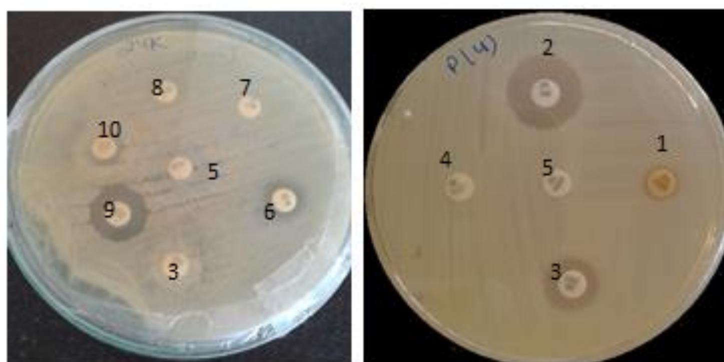


Figure 4.2: Antibiotics susceptibility tests for *Klebsiella* and *Pseudomonas* isolated.

1.MRP(10):Meropenem ,2.PI(100):Pipemidic acid,3.CAZ(30):Ceftazidime ,4.CTX(30):Cefotaxime ,5.GEN(10):Gentamicin ,6.E(15):Erythromycin 7.MET(5):Metronidazole,8.NA(30):Nalidixic acid, 9.CL(25):Cephalexin, 10.IPM(10):Imipenem

The preliminary test such as Biochemical and Antibiotics Susceptible test were carried out for the reconfirmation and to check the presence carbapenem resistance genes in bacteria.

There are various essentials genes which helps in virulence activity of bacteria.Among them, some genes found in *Eschericia coli* are as follow:

4.2: Lists of essential genes in *Eschericia coli*

Genes	Activities
bcsB	Cellulose biosynthesis
dnaA	Chromosomal replication initiator protein DnaA
infC	Factor 3 for the initation of protein translation
dnaX	DNA polymerase III/DNA elongation factor III.
rplE	Ribosomal protein E of the large ribosome subunit
alsK	D-allose kinase
serS	Seryl-tRNA synthetase(SerRS)
trmD	SAM-dependent tRNA m(1)G37 methyltransferase
racR	Rac prophage repressor
chpS	ChpS antitoxin;toxin in ChpB

4.2. Computational screening for Antibacterial potential.

4.2.1. Drug target protein identification

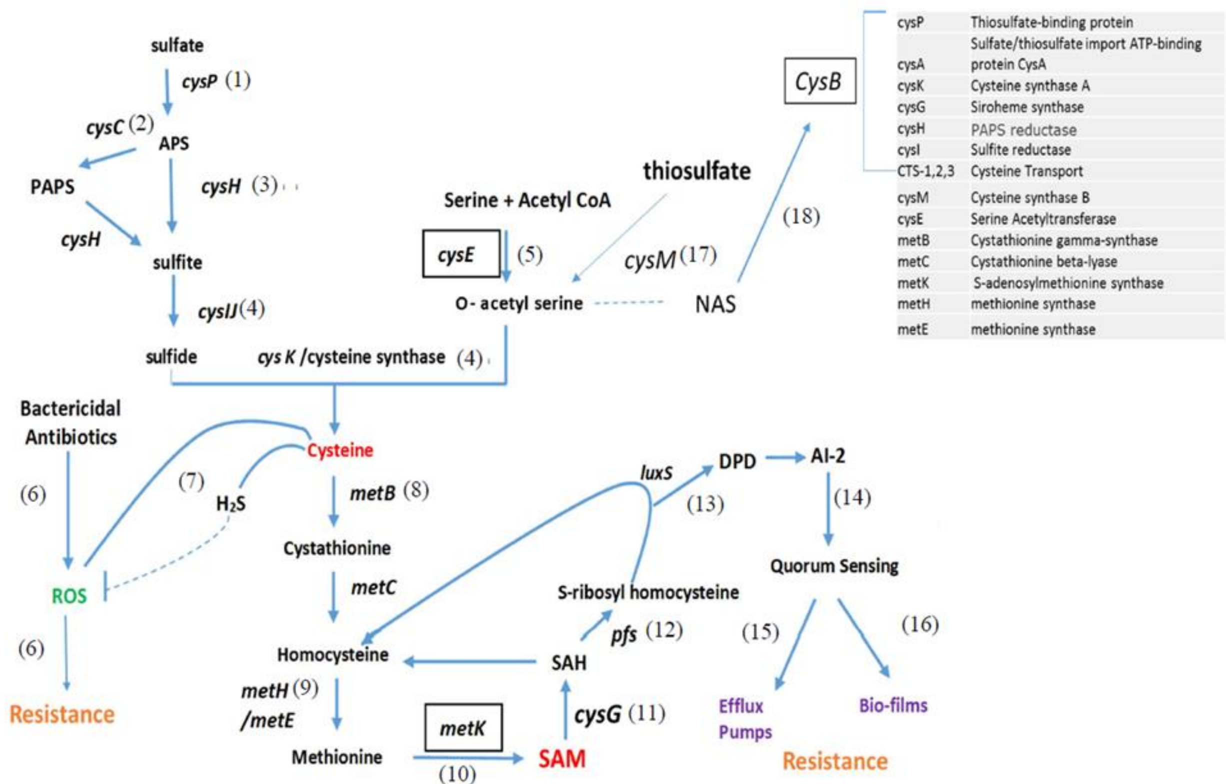


Figure4.3: Schematic diagram of bacterial antibiotics resistance mechanism involving different thiol compounds as illustrated through sulfur assimilation pathway in bacteria. 1,. (Mansilla and de Mendoza, 2000); 2. (Loughlin, 1975); 3.(Krone et. al., 1991) ; 4.(Pinto et. al., 2007) 5.(Kredich, 1971).(Zatyka et. al., 1992) 6.(Becerra et. al., 2013)(Michael a. Kohanski et. al., 2007) 7.(Turnbull and Surette, 2010; Shatalin et. al., 2011) 8.(Qureshi et. al., 1975)9. (Kaplan and Flavin, 1966).10. (Hobson and Smith, 1973)(Wei and Newman, 2002) 11.(Stroupe et. al., 2003)(Fazzio and Roth, 1996) 12.(Belval et. al., 2006)13.(Chen et. al.,

2002;De Keersmaecker et. al., 2005) 14.(S. T. Miller et. al., 2004) 15.(Rahmati et. al., 2002)(Sawada et. al., 2004) 16. (Huang et. al., 2008;Hogardt et. al., 2004) 17. (Nakamura et. al., 1983) 18. (Kredich and Tomkins, 1966).

One of the enzymes involved in sulfur assimilation(Figure4.3)is Met –K that was found to be critical and essential for growth of,*Eschericia coli* in earlier works(Bipin Rimal and group thesis , 2016) inhibition of Met-K causes decrease in genomic methylation(Collier, Justine.,2016) and cell division(Z.W.EI-Hajj et al.,2013).Since this is essential protein for converting methionine to *S-adenosylmethionine*(SAM) (Wüthrich, D et al.,2018) and as SAM is involved in several methylation process in bacteria(names are, Wrong et al.,1996). Furthermore, *S-Adenosylhistidine* (SAH) is formed with combination of cysG protein which and SAH is involved in inducing quorum sensing(Miller MB1, Bassler BL,2001) and develop resistance against antibiotics(Chen,F et al.,2013). This also helps in recycling of the cysteine groups in bacteria (Kunjapur, A et al., 2016)

One of the enzymes that use SAM as substrate for methylation is bacterial ,tRNA (guanine-N(1)-)-methyltransferase (TrmD)(Zhang, Y et al.,2017) that methylates $tRNA^{Pro}$ at G^{37} position(Gamper, H et al.,2015) and protein is taken as target protein due to its binding property towards SAM for methylation in bacteria .In addition, Trm5 gene is present in human which has similar function but are different from each other by its tertiary folds. Due to this unique fold drug designed against TrmD protein may not affect Trm5 protein of eukaryotes. Thus , this protein was taken as a potential drug target to act against prokaryotes without affecting Eukaryotes .In both TrmD and Trm5, key element is SAM that is essential for both eukaryotes and prokaryotes for methylation and is critical for ,bacterial growth and replication.

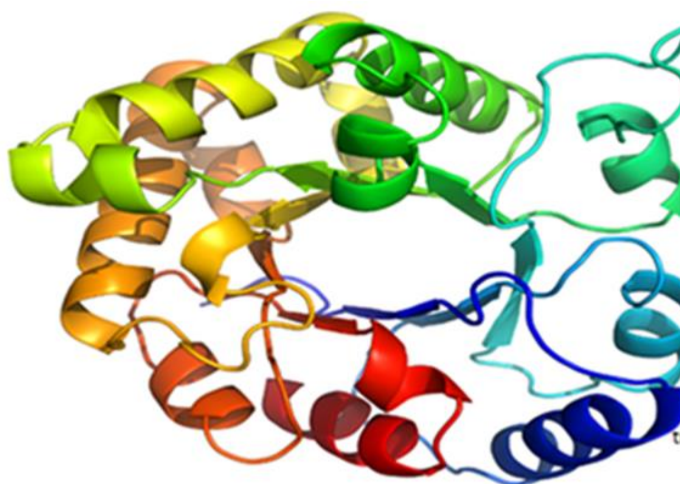


Figure 4.4: Structure of TrmD of *Escherichia coli*

(http://www.rcsb.org/pdb)

4.2.2 Drug target ligand identification.

Some compounds which are used against different bacterial infections are heterocyclic compounds which are: furans, hydrazides, pyrimidine, thiazepines, pyrazolines, chalocones, imidazoles, etc.

Among them, imidazole is found to be most preferred one having five membered ring with 2 nitrogen atom present at position 1 and 3 of the ring constituting an important pharmacophore (Kumar M, et al.,2017) which exhibits the scaffold property and can act as the building block of biomolecules such as histidine, histamine and natural products i.e., pilocarpine alkaloid (Pilocarpus jobarandi) . It only does not act as a antimicrobial but can also act as anti-inflammatory, analgesic, anti-ulcerative, histamine H3 antagonist, antioxidant, farnesyltransferase and geranylgeranyl transferase-I inhibitor, antitumoral, antiparasitic, antiprotozoal, and antidiabetic activities(Derivatives, IT S,2016) as well as Some imidazole derivatives such as Cimetidine, Etomidate, Ketoconazole, Metronidazole, Ornidazole, Azomycin, Oxiconazole, and Clonidine also used as drug therapy.

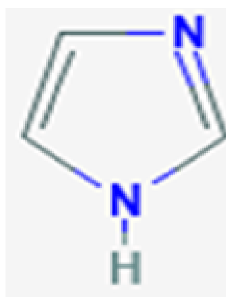


Figure4.5: Structure of Imidazole
(https://pubchem.ncbi.nlm.nih.gov/compound/imidazole#section=2D-Structure)

Imidazole acts differently through different mechanisms. The nitroimidazoles enter in the cell by passive diffusion (Mazuryk, O et al.,2015) forming nitro radical(Orozco, E.,2017) causing breakdown of DNA strand and cell death .It also reacts with the flavohemoglobin(Bahadur, Vet al.,2015) and inhibit it's NO dioxygenase (NOD) function(Gardner, P et al. ,1998) inhibiting the metabolism of NO and finally leading to bacterial cell death(Vallyathan, V et al.,1997) . It also inhibits the metabolism of NO groups which enhance cell death(Murphy, M,1999).

Another function of imidazole is,-inhibition of enoyl acyl carrier protein reductase (FabI) as, -an enzyme involved in the synthesis of bacterial fatty acids is a novel target for antibacterial activity. Imidazole binds to NOD in presence of flavoHb-FE(III) intermediate and cause inhibition of it. It can also bind without the O2 but with help of CO for which the binding affinity is increased in formation of high-affinity flavoHb-Fe(II) CO complex . The reduction of NADH cannot effect the mechanism but reduction of FAD and heme only cause the turnover of NOD(Helmick, R,et al.,2005) The combination of imidazole forming mono-substituted and di-substituted can act as antimicrobial agents(Rani et al.,2013).

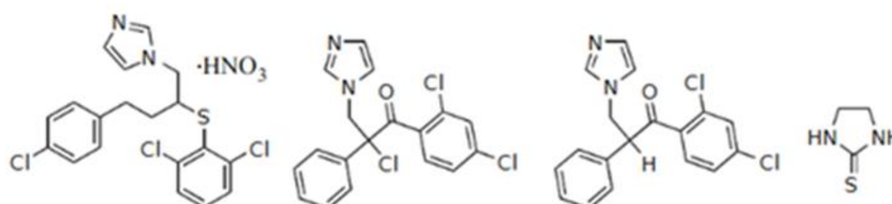


Figure 4.6: Chemical structure of antimicrobial Imidazole (Rani,et al.,2013)

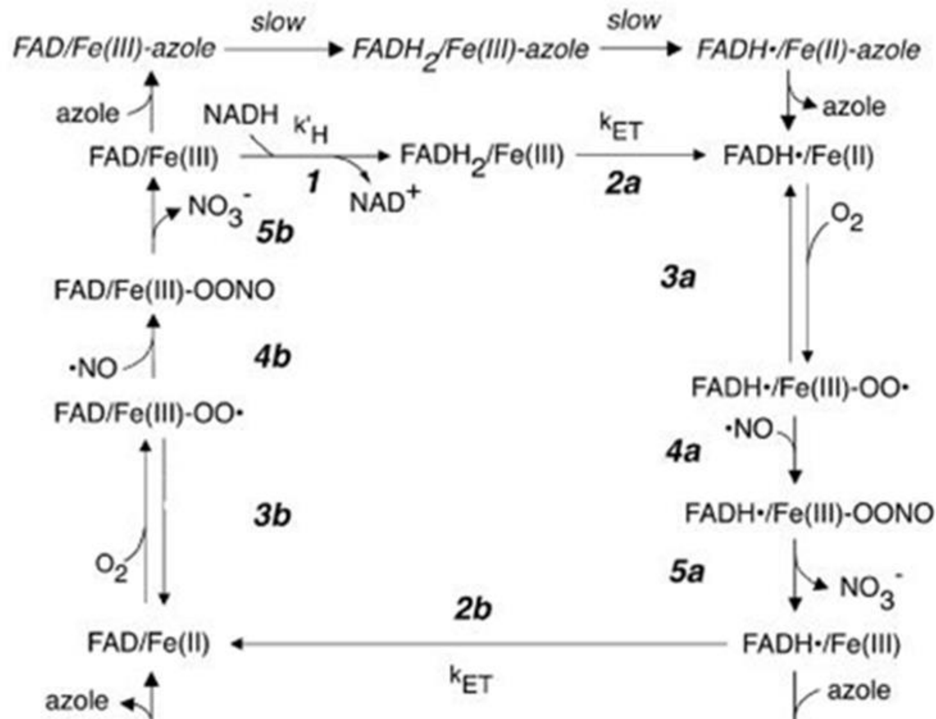


Figure 4.7: Mechanism for imidazole for NOD inhibition. (Helmick R, et al.,2005)

Similarly, addition of one N group in imidazole can form pyrrole, which is basic in nature (https://www.quora.com/What-is-the-difference-between-the-indole-ring-and-imidazole-ring) activity shown by imidazole could be demonstrated by pyrroles, too. And Indoles are derivatives of pyrrole so, it can be predicted that indole compounds could also exhibit similar activities as substituted imidazoles.

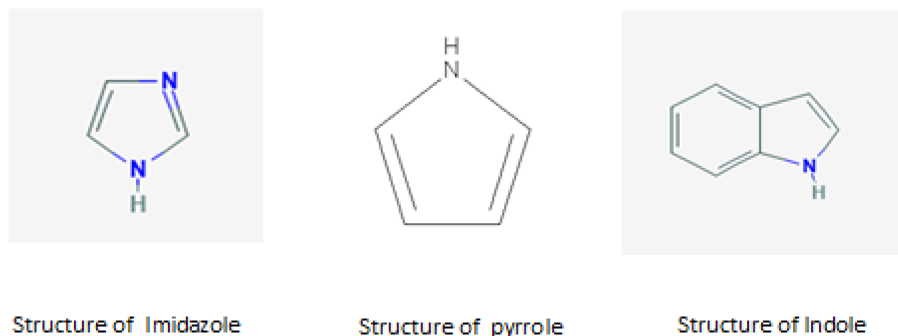


Figure4.8: Structural comparision between Imidazole, pyrrole and Indole

For bacteria growth activation of several enzymes are necessary and is carried out by the activator commonly called kinases that transfer phosphate groups from high-energy to low-energy. It utilizes ATP to transfer phosphate and convert it into ADP (Grangeasse, C et al,2012).So, it plays important part in cell signaling, protein regulation, cellular transport etc. It has been reported that indole and its derivatives as well as imidazole act as inhibitors against the kinases especially (GSK-3). The structural similarity between the purine molecule and indoles, mostly at the pyrrole ring, indicates that it could compete with ATP binding pocket.

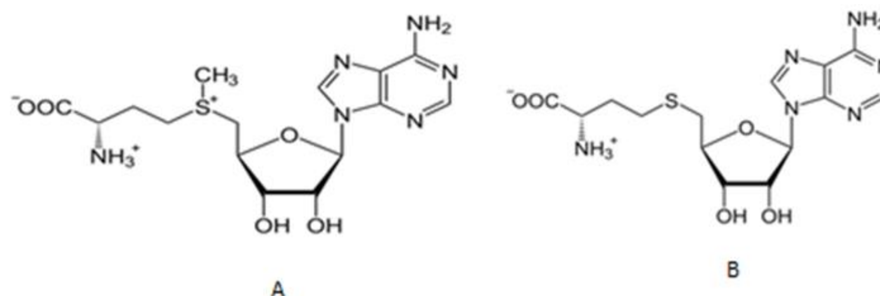


Figure 4.9: Structures of SAM and SAH

A: S-AdenosylMethionine. B: S-Adenosyl-L-homocysteine.

During making of different indirubins as potential kinase inhibitors (Wu et al.,2005) some indoles were cytotoxic to E. coli HME5tnaA<>cat expressing human cytochrome P4502A6 double mutant (Table 4.3). Because of structural similarity and potential kinase inhibitor it was presumed that some indoles could potentially with compete with SAM and/or SAH in their respective sites in those enzymes that use SAM as the substrate and give SAH as the product. One of such enzymes TrmD of prokaryotes could be a target without causing any effect to human but causing effect on bacterial cells. Thus, indoles and its derivatives were taken as ligands.

Table 4.3: List of Indoles and Indole derivatives.(Wu.Z, Aryal ,et.al.,2005)

Cytotoxic at high concentrations
5-Bromo-1H -indole
6-chloro-1H-indole
4-Bromo-1H-indole
6-Bromo-1H-indole
7-Chloro-1H-indole
5-Chloro-1H-indole
4-Chloro-1H-indole
5-Iodo-1H-indole
7-Bromo-1H-indole

The crystal structure of TrmD was retrieved from https://www.rcsb.org/ and different indole derivatives from ZINC database and was docked with help of Discovery studio. The structure of TrmD binding with amino acids was observed using Autodock vina that helped in locating the SAM binding site in TrmD (Figure 4.9).

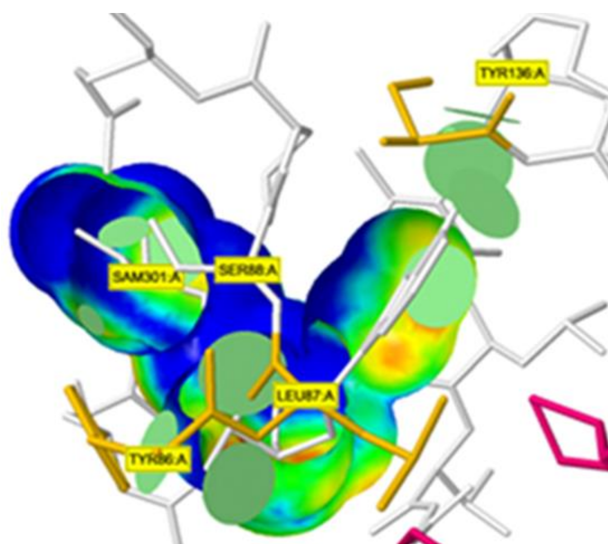


Figure 4.10: Structure of TrmD with different amino acids bound to SAM

The original structure of SAM and SAH found to be ring structures and compared with structures of indole and its derivative for prediction whether it matches with each other or not on basis of structures.

TrmD protein was docked with different indoles and its derivatives as ligands in binding site of SAM and SAH.. Then binding energy was compared with their original ligands, SAM and SAH (Table 4.4 and Table 4.5, respectively) with the help of computational tool called pyrx. It helped in the identification of the potent indole and its derivatives as competitive ligands against SAM and SAH.

Table 4.4: Docking results of TrmD with SAM, indoles and its derivatives.

S.N	Ligands	Target	Binding energy
1.	Zinc_3638431(1H-benz(f)indole-2,3-dione)	1uak	-9.1
2.	Zinc_5159022(3-metylbut-2-enyl)indoline-2,3-dione	1uak	-8
3.	Zinc_2047514(isatin)	1uak	-7.6
4.	Zinc_4228231(S-Adenosyl-1-methionine)	1uak	-6.9
5.	Zinc_154242(6-Methylsulfonyl-1-oxiran _2_ ylmethoxy-1H-indole	1uak	-6.6
6.	Zinc_154243(6-Metylsulfonyl-1-oxiran_2_yl methoxy-1H-indole)	1uak	-6.5
7.	Zinc_ 13462972(2R)-2-methyl-1-nitroso-indoline	1uak	-6.5
8.	Zinc_13462974(2S)-2-methyl-1-nitroso-indoline	1uak	-6.4
9.	Zinc_ 14516984(1-Azaindene)	1uak	
10.	Zinc_ 95864419(indole)	1uak	-6.3
11.	Zinc_ 95864420 (indole)	1uak	-6.1
12.	Zinc_ 175204(2,8-dimethyl-2,3,4,5-tetrahydro-1H -pido(4,3-b)indole	1uak	-5.9

For binding energy calculation SAM molecule that is the native ligand was removed from the binding site of the protein and other indole derivatives were docked at the binding pocket of the SAM. The software calculates the binding energy and the binding energy of 1H-benz (f) indole-2, 3-dione was found to be -9.1 kcal/mol compared to that of SAM that was found to be -6.9kCal/mol.

Thus, indole derivative named 1H-benz (f) indole-2, 3-dione could be taken as competitive ligand of SAM in TrmD based on Gibbs energy and presumed that it could which can inhibit methylation of $tRNA^{pro}$ in resulting +1 frame shift during protein translation and hindering the function of some of critical enzymes that are prone to this mutation and this could lead to cytoxicity for bacteria.

Table 4.5: Docking results of TrmD gene of SAH, indoles and its derivatives

S.N	Ligands	Target	Binding energy
1.	Zinc_3638431(1H-benz(f)indole-2,3-dione)	1ual	-8.9
2.	Zinc_5159022(6-(3-methylbut-2-enyl)indoline-2,3-dione)	1ual	-8.4
3.	Zinc _2047514(isatin)	1ual	-7.6
4.	Zinc_13462974(2S-2-methyl-1-nitroso-indoline)	1ual	-6.7
5.	Zinc_13462972(blahdione)	1ual	-6.4
6.	Zinc_14516984(1-Azaindene)	1ual	-6.4
7.	Zinc_95864419(indole)	1ual	-6.3
8.	Zinc_95864420(indole)	1ual	-6.1
9.	Zinc_4228232(S-Adenosylhomocysteine)	1ual	-5.8
10.	Zinc_175204(2,8-dimethyl-2,3,4,5-tetrahydro-1H-pyido(4.3-b)indole)	1ual	-5.4
11.	Zinc_154243(6-Methylsulfonyl-1-oxiran_2_ylmethoxy-1H-indole)	1ual	-4.8
12.	Zinc_154242(6-Methylsulfonyl-1-oxiran_2_ylmethoxy-1H-indole)	1ual	-4.5

The 1ual derivative of TrmD containing the S-adenosyl homocysteine (SAH) was docked with different indole and its indole derivatives such as isatin, indirubin and other derivatives at binding site of SAH. The binding energy of 1H-benz(f)indole-2,3-dione was found to be -8.9 kcal/mol compared to , the binding energy of the SAH that was found to be -5.8 kcal/mol. Thus, those compounds having better binding energy than SAM or SAH at respective binding sites indicate that it could prevent substrate binding and product release in inhibiting TrmD function. This molecule could be further explored as potential drug candidate lead.

The ligands which are docked in TrmD are:

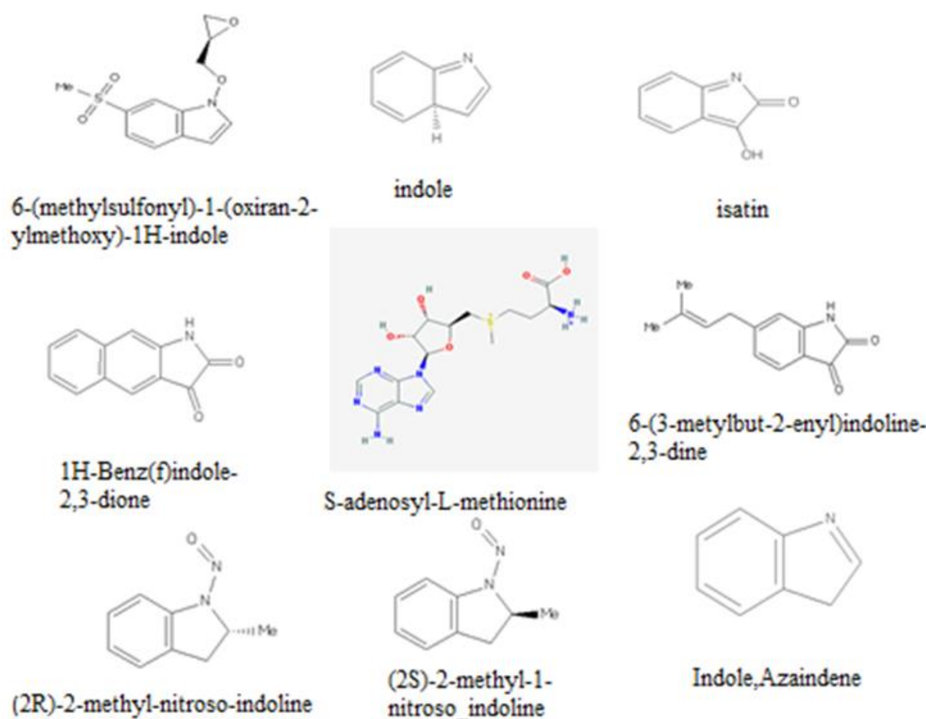


Figure4.11: Structure of indole derivatives docked in TrmD instead SAM and SAH

TrmD protein helps in methylation of DNA with presence of SAM. Methyl group which is obtained from SAM is converted to SAH by the utilization of ATP so it can be a good target to inhibit growth, structural components such as phosphotidyl choline and transfer information between bacteria. Binding energy was calculated by different computational tools with different indoles and derivatives of indole .Among them, indole and its derivatives which has high binding energy is considered to be competitive ligands for blockage of all activities of SAM and SAH in bacteria.

Bacteria named *Eschericia coli* found in the gut of human produce indole to form a niche environment to be protected the bacteria ,by biofilm formation, plasmid stability, drug resistance and virulence (Lee,2010).Furthermore it also helps in prevention of biofilm formation of different bacteria (Di Martino et al., 2003; Domka et al., 2006; Lee et al., 2007b). Moreover, indole is highly produced in presence of antibiotics and temperature (50◦c) to protect themselves from other bacteria (Han et al., 2011). Thus, in treating indole positive bacteria the including indole derivatives as drug could potentially sense indole moiety of the derivative and deregulate expression of tryptophanase gene(tnaA) (Mueller, R.et al.,2009)in making indole from tryptophan by being confused and make bacteria susceptible to antibiotics administered.

4.3 Phytochemical analysis

Indoles and indirubin derivatives are colored compounds, as Calendula flower is used as coloring compound in some food items such as butter and cheese, was of interest because it is edible and the colors of the flower that is golden could have some compounds that could exhibit antimicrobial potential. This was hypothesized because Calendula has been used for various kinds of health related problems such as cuts,burns and skin irritation . In addition, it has also been used as anti-inflammatory, antitumor agent, healing wounds, antiviral,antigen toxic, treating acne, controlling bleeding and soothing irritated tissue ,treating radiation tissue as well as traditionally it was used as for abdominal cramps and constipation.

Chemically, Calendula contains flavonol glycosides, triterpene oligoglycosides, oleanane – type glycisidase, saponins and sesquiterpene glucoside .Flavonol glycosides can pro-drug due to its weak activity. The free radical scavenging activity of flavonol may justify ethnomedicinal value so itand can be used for mitigating the etiology of many inflammatory diseases .(Taiwo et al.,2014).

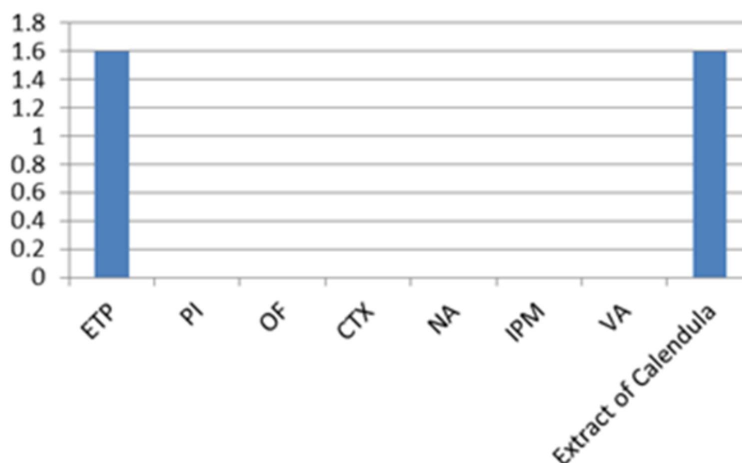


Figure.4.12: Zone of inhibition between antibiotics and extract of calendula against *Klebsiella* (isolate number 3) carbapenem resistance bacteria .X axis:Antibiotics.,Y axis:Zone of inhibition in centimeter

ETP: Ertapenem (Resistance), PI:Penicillium, OF: Ofloxacin, CTX: Cefotaxime, NA: Nalidixic Acid, IPM: Imipenem, VA: Vancomycin.

Multi-drug resistant *Klebsiella* sps. that is sensitive to Ertapenem was sensitive to Calendula flower extract. The zone of inhibition of Ertapenem and Calendula extract were similar size of 1.6 cm. Different antibiotics as well as extract were tested against multidrug resistance bacteria. Zone of inhibition shown by Ertapenem and extract of Calendula was found to be similar that is 1.6 cm representing effectiveness of calendula and ETP are found to be similar.

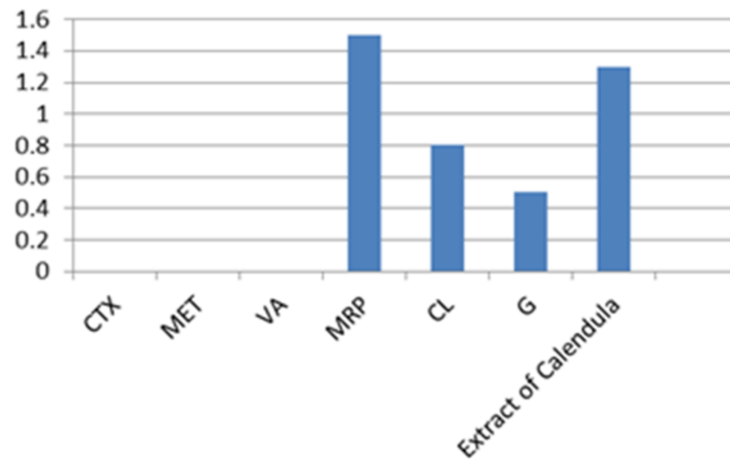


Figure.4.13: Zone of inhibition (cm)between of respective antibiotics and extract of calendula against carbapenem resistance bacteria *Klebsiella* (isolate 24). X axis:Antibiotics.,Y axis:Zone of inhibition in centimeter.

CTX: Cefotaxime, MET: Metronidazole, VA: Vancomycin, MRP: Meropenem (Sensitive), Cl: chloramphenicolChloramphenicol,Acid,6. G: Gentamicin

Pseudomonas sps when tested some antibiotics exhibited some zone of inhibition but they were relatively small so bacteria were presumed to be resistant to these drugs. Moreover, extract of Calendula flower showed zone of inhibition to this multidrug resistance bacteria. These findings indicate that Calendula flower extract could be further explored to identify active constituent that has antimicrobial potential to multi-drug resistant bacteria.

4.4. Isolation, Characterization and Analysis of Streptomyces from soil sample in specific media

Search for antimicrobials is urgently needed due to emergence of MDR pathogens (van Duin, D,2016). One of the areas of interest could be Streptomycetes sps. since it has been reported that still their potential to make secondary metabolites and antimicrobials is still under explored probably by 90% (https://www.ncbi.nlm.nih.gov/pubmed/19844637/) Thus, Ssoils from different parts of Nepal were collected, .Bbut present study focused on the rhizospheric soil of Fern Tree, most preferred site was the fern tree site considered it to beone of most prehistorical plant , from the time of dinosaurs period, still surviving in its own natural habitat indicated of its intricate interaction with rhizospheric microbiome including endophytes some of which are known to be Plant Growth Promoting Rhizosphere (Islam, S et al.,2016) along with the relation with some microbes that could have supported in suppressing pathogenicity. As mentioned above the potential of Streptomyces sps. to produce the antimicrobial and its saprophytic nature (de Lima Prooópio, R,et al.,2012) it was predicted that in the damp area with decaying plant materials(Bonanomi, G et al.,2006) would harbor these organisms and the most growth of Fern Tree at the site of flowing spring waters (observation made during site observation) it was presumed that this species could be found in identified sites around rhizospheric parts.

Hence, soils were collected from moist area around rhizospheric region of Tree Fern removing top soil and soil bored around 5-10 cm for screening. Serial diluted soil samples were subjected to pasteurization to kill any vegetative cells because Streptomyces sps are

spore formers (Yague P et al.,2013)whose spore is resistant to pasteurization(Gopal,N et al.,2015) International Streptomyces Project Medias (ISP2, ISP4) were used for the primary isolation because it contains the simple carbon sources like glucose or starch, respectively , sucrose and maltose which can be easily utilize by other bacteria. In ISP2 other spore forming organisms that can utilize would also survive but in ISP4 only those spore forming organisms that have breakdown starch would survive.

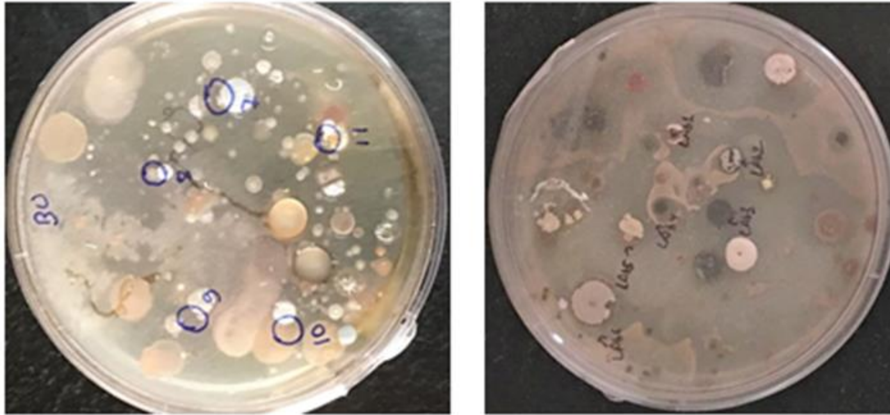


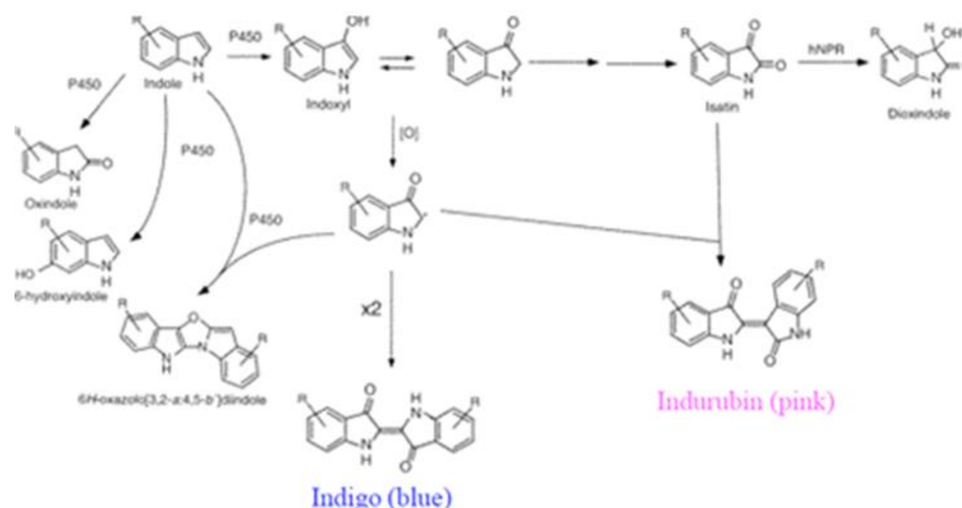
Figure 4.14: Primary screening of *Streptomyces* from collected soil.

Due to the presence of simple metabolizable sugars and other nutrients several organisms grow so putative *Streptomyces* sps. have to be meticulously looked for cottony type, surface and other morphological character whether it has substrate mycelia or not and along with that it has aerial hyphae, and has also muddy odor or not. Initial screening in separating from other organisms is cottony color (Islam, M, 2014) and comparatively slow grower than others . Then, putative Streptomyces sps. were further streaked in ISP2 and ISP4 media and their above mentioned characteristics are observed as depicted in Table 4.6.

Table4.6: Characterization of extracted putative Streptomyces from soil

S.N	Soils	Sample codes	Media	No of days	Growth and colonies	Aerial mycelia	Substrate mycelia
I.	Sample6 (a)	R11	ISP4	15	Single/Small/Smooth	Yes	Invaded
		R12	ISP4	15	Single/Medium/Smooth	Yes	Invaded
		R13	ISP4	15	Single/Medium/Smooth	Yes	Invaded
		R16	ISP4	15	Single/Small/Smooth	Yes	Invaded
II.	Panchase lake	R18	ISP4	15	Single/Small/Smooth	Yes	Invaded/brown
		20	ISP2	15	Single/Metallic yellow	White	Invaded
		R26	ISP4	15	Small colony	Greenish	Invaded
		R27	ISP4	15	Single/Small	Yes	Invaded
		R28	ISP4	15	Single/larger	Powdered	Invaded
		R29	ISP4	15	Single/Medium	White powdered	Invaded
		R30	ISP4	15	Single/Medium	Yes	Invaded
		R31	ISP4	15	Single/Medium	Light brown	Non- invaded
		R32	ISP4	15	Single/Medium	Dusty brown	Invaded
		R33	ISP4	15	Single/small	Yes	Invaded
III.	Bhadauray	R34	ISP4	15	Single/large/bulging	White	Invaded
		R35	ISP4	15	Single/large	White	Invaded
		R36	ISP4	15	Single/large	Brown	Invaded
		R37	ISP4	15	Single/medium	White	Invaded
		R38	ISP4	15	Single/medium	Brown	Invaded
		R39	ISP4	15	Single/medium	Brown	Invaded
		R40	ISP4	15	Single/medium	White	Invaded
		R41	ISP4	15	Single/medium	White	Invaded
		R42	ISP4	15	Single/medium	White	Invaded
IV.	Kothiyaghat	S	ISP2	15	Single/medium	Brown with white centered	Invaded/brownish
		O	ISP2	15	Single/medium	Purple	Invaded
		R1	ISP4	15	Single/small/bulging	White	Invaded
		R2	ISP4	15	Tiny colonies	Grey	Invaded/Reddish
		R3	ISP4	15	Tiny colonies	White	Yellowish red
		VI(R)	ISP4	15	Small colony	Small purple	Yellowish
		R9	ISP4	15	Small colony	White	Yellowish

After analyzing by naked eyes putative Streptomyces from primary culture it was re-streaked in ISP2 media which help in isolation of pure culture for further analysis and mass production for antimicrobial test.



4.15: Pathway of cytochrome P450 2A6 mediated metabolism of indole to colored metabolites indigo and indirubin. (Gillam, E.,2000)

Human cytochrome P450 2A5 in particular has been found to metabolize indole to colored compound indigo and indirubin , which help to convert indole into indigo, indurubin and other products (Figure 4.16). It is also found that there is a presence of cytochrome P450 or CYPome in *Streptomyces* (Chun, Y et al.,2007) and has been suggested that they could be exploited for drug discovery (https://www.ncbi.nlm.nih.gov/pubmed/16863466). In addition,some Streptomycetes sps. could metabolize indole derivatives to give antimicrobial compounds because of their CYPome (https:// www. ncbi. nlm. nih. gov/ pmc/ articles/PMC5585785/).Furthermore, the CYPome of (https:// www.ncbi .nlm.nih. gov/ pubmed/ 16863466) similar to human which help to convert indole into indigo,indurubin and other products .So Thus, screening for Streptomyces having antibacterial, antifungal and antiparasitics properties those isolates showing colorful colonies and/or culture medium identification as a confirmation colorful colonies were more preferred (Figure 4.15) to be Streptomyces showing antibacterial, antifungal and antiparasitics property.

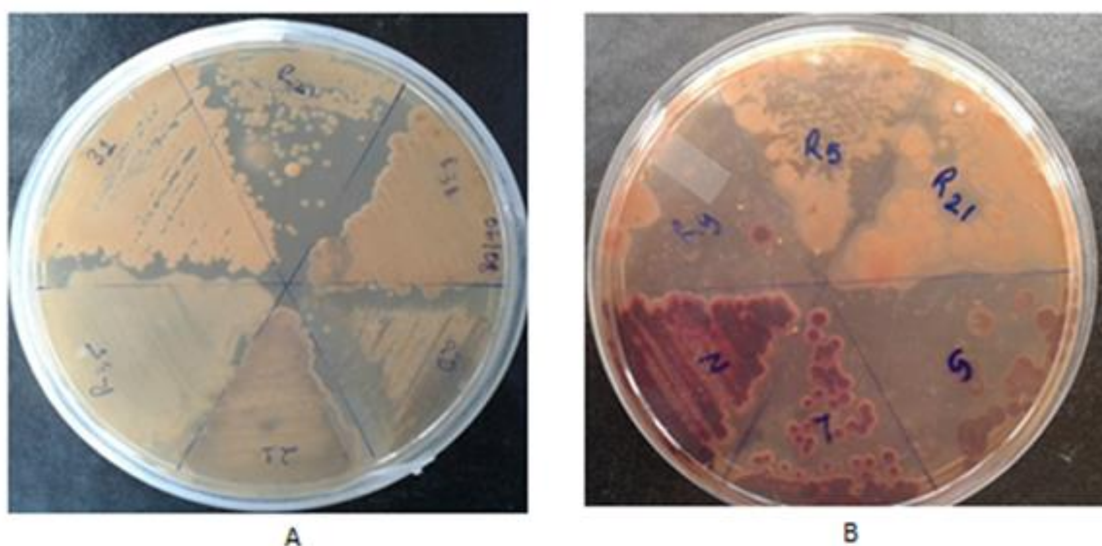


Figure4.16: Different colorful Streptomyces isolated from the different soils samples.

After selection of putative Streptomyces of interest Gram staining process was carried out and side by side microscopy was done to observe colony characteristics related to the putative Streptomyces. Gram staining was done and they were found to be Gram positive (Figure 4.16). They were of various size and morphology with their own respective signature color (Table 4.7).

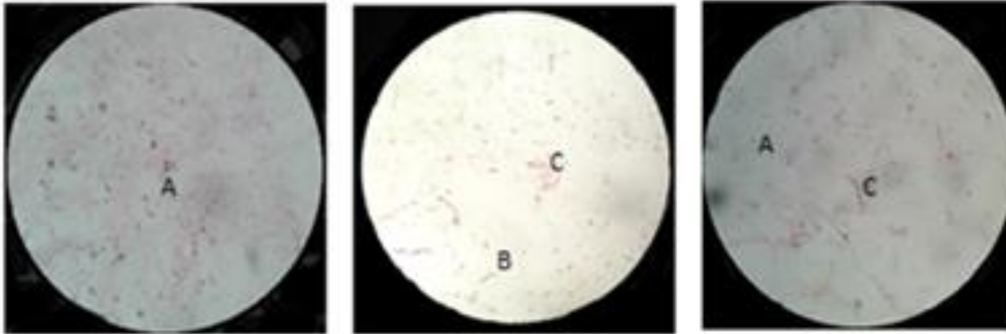


Figure4.17: Gram staining of putative of Streptomyces

A:Cocci bacteria ,B:Cocci bacteria in chain,C:String,

Table4.7: Gram Staining Size, morphology and respective color of putative *Streptomyces*.

S.N.	Samples	Shape and color
a.	O	Cocci,Purple
b	R38	Cocci,Purple
c.	R41	Cocci,Purple
d	1	Cocci,Purple
e.	Sample 4	Cocci,Purple
f.	R3	Purple,Rod
g	S	Purple,Rod with string
h	6	Cocci,Positive with string
i.	R50	Chain cocci ,positive with string
j.	R11	Small cocci,Positive chain

Biochemical analysis was carried out for further confirmation of putative Streptomyces the data obtained was as follow:

Table 4.8: Biochemical test of putative *Streptomyces* S

S.N	Biochemical Tests	Results
1.	Indole	Positive
2.	Methyl Red(MR)	Positive
3.	Vokes Proskauer(VP)	Positive
4.	Citrate	Positive(Blue)
5.	Oxidative	Positive(Yellowish)
6.	Fermentative	Positive(Yellowish)
7.	Urease	Positive(Pinkish)
8.	Triple Sugar Iron	Positive(Yellowish)
9.	Gas	Positive
10.	H2S	Positive
11.	Motility	Positive
12.	Catalase	Positive
13.	Oxidase	Positive

Table4.9: Biochemical test of putative *Streptomyces* R38

S.N	Biochemical Tests	Results
1.	Indole	Positive
2.	Methyl Red(MR)	Positive
3.	Vokes Proskauer(VP)	Positive
4.	Citrate	Positive
5.	Oxidative	Positive
6.	Fermentative	Positive
7.	Urease	Positive
8.	Triple Sugar Iron	Positive
9.	Gas	Positive
10.	H2S	Positive
11.	Motility	Positive
12.	Catalase	Positive
13.	Oxidase	Positive

During the biochemical test, all the tests showed a positive reaction which was similar to Streptomyces so by predicting it is a putative Streptomycetes further analysis was carried out.

4.5. Antioxidant potential of isolated strains

Since, *Streptomyces* sps. make different secondary metabolites (Stulberg, E,,2016) including antioxidant and antimicrobials. Recently, it has been established that apart from the respective gene targets of the respective antibiotics they have additional mechanism of killing through generation of reactive oxygen species (ROS) (Van Acker, H., 2017) that destroy macromolecules like proteins, lipids and nucleic acids. However, MDR pathogens

exhibit additional bactericidal antibiotics drug resistance through mitigating reactive oxygen species (ROS) produced by different bactericidal antibiotics in use (Bhardwaj, A, 2014). This is mostly performed by production of H_2S from cysteine, electron sink in glutathione (Winterbourn, C et al., 1993), reductive TCA cycle and expression of different enzymes(Bostwick, D et al.,2000) . Thus, the discovery of new antibiotics could have been compromised during screening if the extract had antioxidants that could have quenched the effect of antimicrobial present in the extract if it had killing effect through ROS (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3791156/). Thus, it is prudent to check for antioxidant potential of the extracts before searching for antimicrobial potential.

Those isolates that showed positive results in morphological characteristics, microscopy, and biochemical tests were incubated in ISP2 medium and the cell free extracts were examined for their antioxidant potential using 1, 1-Diphenyl-2-picrylhydrazyl (DPPH). DPPH is considered as radical and as being efficient radical (OKAWA, M et al., 2001)trap it also acts as a strong inhibitor of radical-mediated polymerization(Kedare, S et al.,2011) . Thus, compounds that trap the electron from it is considered as antioxidant. The DPPH assay is robust and convenient where the reaction mixture Color changes from blue (oxidized DPPH) to yellowish color (reduced) as indication of antioxidant property of the test compound (Figure 4.17).

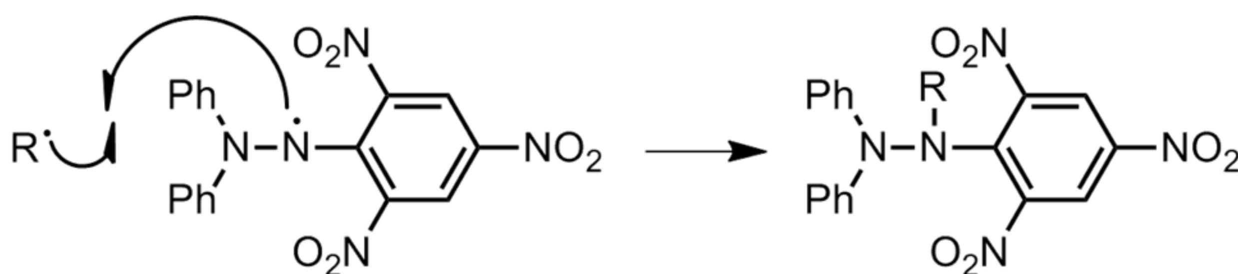


Figure 4.18: Schematic chemical reaction of DPPH assay

Rate of reduction from the sample depends upon chemical reaction with DPPH and that acts as an indicator of antioxidant potential due to the absorption at 520nm. Originally the reaction mixture is deep violet in color before addition of antioxidants and becomes pale yellow or colorless when neutralized that can be quantified by measuring at this wave length that can be then further explained as quercetin (Zhang, M et al.,2011)(Sak, K.,2014) equivalent. Moreover, this result can be easily seen by visualization and if qualitative investigation is only required but not the quantitative then color change could be taken as antioxidant. The antioxidant potential of some of the isolates is illustrated in Table 4.8. This is only qualitative data where + indicates color change and – indicates no color change, hence without any antioxidant activity.

Table4.10: Antioxidant test of putative *Streptomyces*

S.N	Samples	Results
a)	R5	+
b)	R9	-
c)	R22	-
d)	R37	-
e)	R38	+
f)	R42	+
g)	T	-
h)	1	+
i)	17	+
j)	19	-
k)	31	+
l)	Sample4	-
m)	S	+

4.6. Antimicrobial potential of isolates

The different isolates (40) from different rhizospheric soils of Cyathea gigantean(Tree Fern) that were presumed to be putative *Streptomyces* were cultured in ISP2 medium for screening of antimicrobial potential activity. The cell free culture medium was extracted with ethyl acetate and concentrated in rotary evaporator which was finally dissolved in ethanol. In antimicrobial susceptibility assay (AST) in plates none of the extracts show positive results.

However, among those 40 samples only 30 samples that exhibited colored colonies were further pursued for antimicrobial potentials since indigo and indirubins(Gillam et.al.,2000) are colored compounds and have been reported to be antimicrobials in plant and microbial extracts((Krivogorsky et al., 2008; Al-Dhabi et al., 2012; Fogaça et al., 2017) . In addition, the hydrophobic indole nucleus is known to increase permeability of bacterial membrane (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5962266/) and that could be one of the mechanisms of bactericidal activity.

With this hypothesis the culture medium was modified. Since *Streptomyces* sps. are known saprophytes(de Lima Procópio,R2012), in competitive niche environment of the rhizosphere (Bauer, M et al.,2018)these slow growers (Flowers, T. H.Williams, S. T.,1977)would find difficult to compete for easily mrtabolizable hexose sugars that are preferred reduced carbon source for many organisms with other fast growers (Kim, J et al.,2010) and even to pentose that are regulated by carbon catabolite repression (CCR) for simultaneous metabolism with hexoses because these fast growers exhibit diauxic growth when different forms of reduced sugars are present (Editors, AccessScience,2015). Thus, to compete with these fast growers relying on decaying matters the Streptomycetes must be able to use lignin degradation products and should have developed ability to grow in lignin degradation products' molecules, often termed as cometabolomics (https: //www .ncbi .nlm. nih.go v/pmc/articles/PMC3923840; https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5812493/). Some of the Streptomycetes sps are indole positive(Hossain, M et al.,2014) They probably produce secondary metabolites as antimicrobials (https://www.ncbi.nlm.nih .gov/pmc

/articles/PMC3487804/) to compete in niche environment with other fast growing organisms. In addition, *Streptomycetes* sps. are known to produce metabolite that act as antimicrobials and they also produce indole derivatives as antimicrobial agent (Igarashi Y. et al.,2004). In addition, peptoid with multiple indole moieties which are more hydrophobic have higher membrane permeability (https://www.ncbi.nlm.nih.gov/pmc/articles/ PMC5962266 /) indicating that indole itself could act as antimicrobial agents.

Taking this into account the culture medium was modified. The sugar or starch in ISP2 and ISP4 were replaced with lignin degradation product tannic acid (Figure 4.20(1)). In addition, indole (Figure4.20(5)) was also used as carbon source for growth of the isolated organisms. In addition, the indole moiety or pyrimidine type structure was thought that it could compete with the SAM at TrmD active site and could help in killing the pathogens. Out of 40 isolates, two isolates gave antimicrobial potential upon co-culturing with indole and tannic acid gave zone of inhibition to MDR *Klebsiella* sps. and *Pseudomonas* sps. (Figure 4.20).

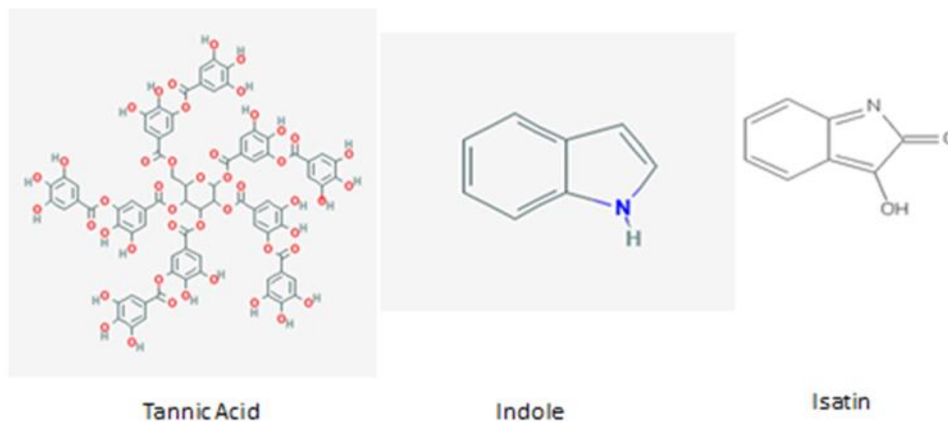


Figure 4.19: Structure of cyclic compounds used in Streptomycetes in dry lab.

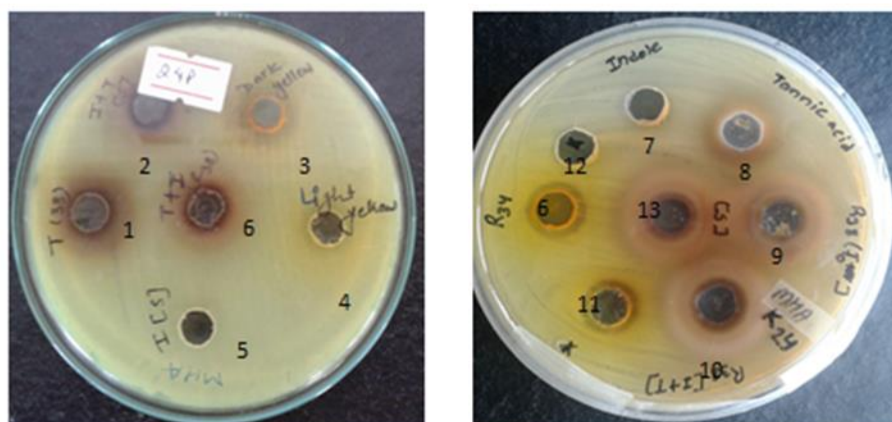


Figure4.20: Antibiotics susceptibility test on *Pseudomonas* (isolate 24) and *Klebsiella* (isolate 24) with different extracts of *Streptomyces*.

1:Sample R38 with Tannic acid,2:Sample S with indole and tannic acid, 3.Candellula(Dark), 4.Candellula(light),5.Sample S with indole, 6.R34, 7.Indole(control), 8.Tannic(control),

9.Sample R38 with tannic acid 10.Sample R38 with indole and tannicacid, 11.candellula, 12.negative control, 13.sample S with tannic acid

Plotting the zone of inhibition in bar graph indole or tannic acid did not have potent antimicrobial effects. Moreover, tannic acid when used as reduced carbon source one of the isolates, R38, showed zone of inhibition. This effect was pronounced when both tannic acid and indole were used in growth medium (Figure 4.21, 4.22). This indicated that indole and tannic acid metabolites that could have been fused together could have given antimicrobial compound. Since, isatin and indirubin are reported to possess some antimicrobial potential (Fogaca .V M et al.,2014)it could be presumed that compound could be of similar nature with additional benzene type ring attached to indolo moiety. This is the first report of development of antimicrobial using indole and tannic acid as the substrate as far as of our knowledge. The antimicrobial potential was better than calendula extracts (Figure 4.25;4.26). Both isolates had similar activity.

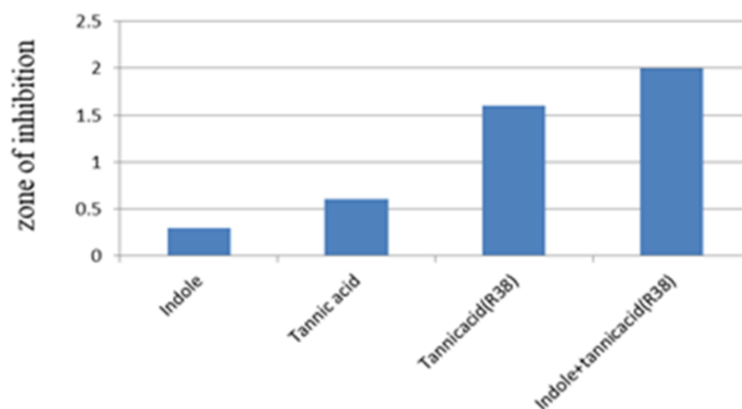


Figure 4.21: Bar chart of zone of inhibition of *Klebsiella (*isolate 24)by *streptomyces* isolate R38

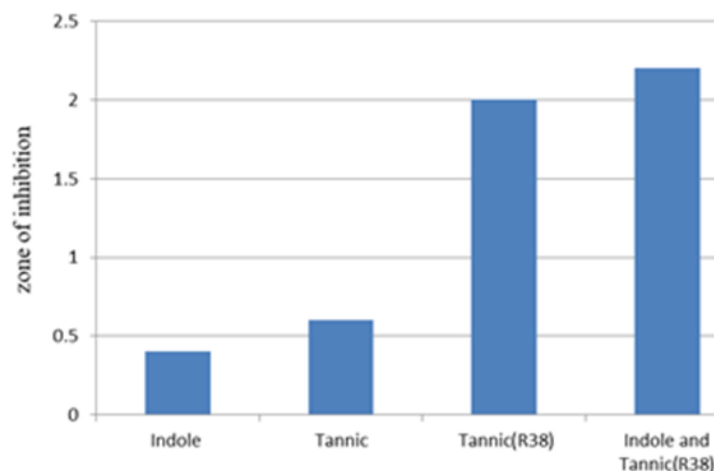


Figure 4.22: Bar chart of zone of inhibition of *Pseudomonas* (isolate24)by *streptomyces* isolate R38

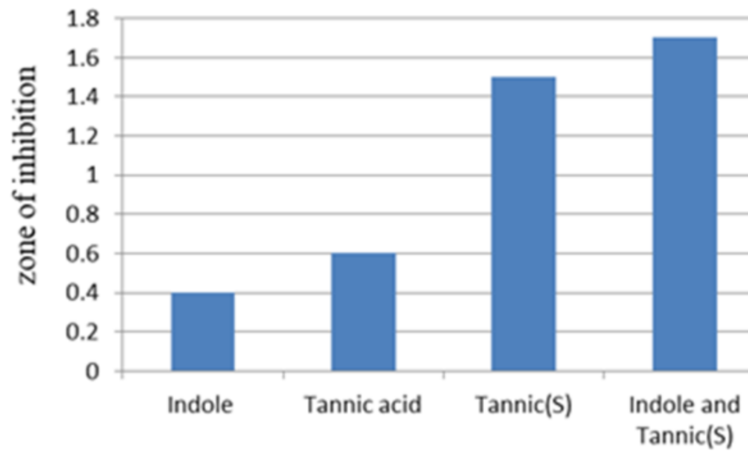


Figure 4.23: Bar chart of zone of inhibition of Pseudomonas (isolate24)by Streptomyces isolate S

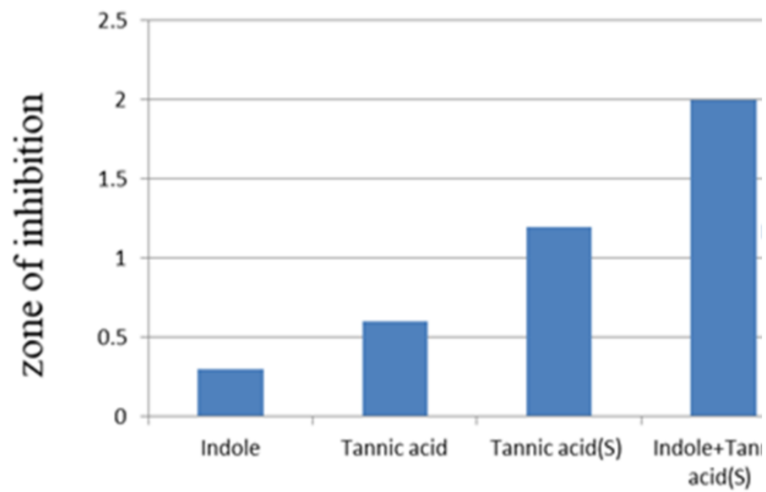


Figure 4.24: Bar chart of zone of inhibition of *Klebsiella* (isolate24) by isolate *streptomyces* S

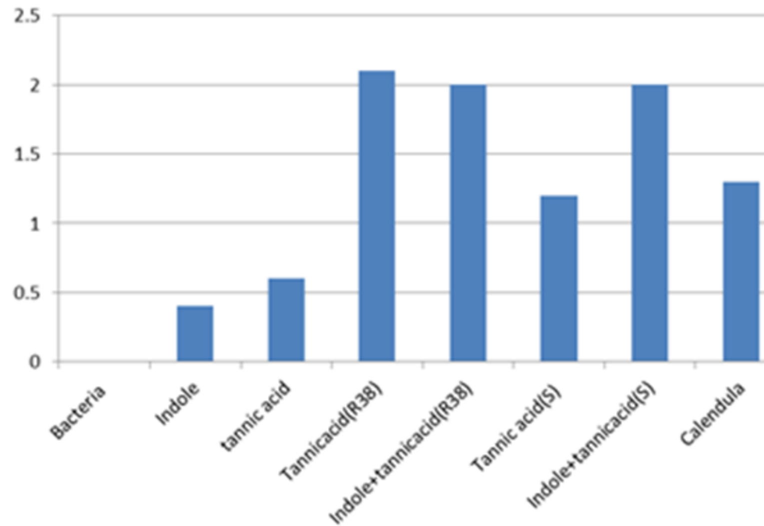


Figure 4.25: Comparative bar chart of zone of inhibition by the extracts of *Streptomyces* and flower for *Klebsiella(isolate* 24).Y-Axis: zone of inhibition.

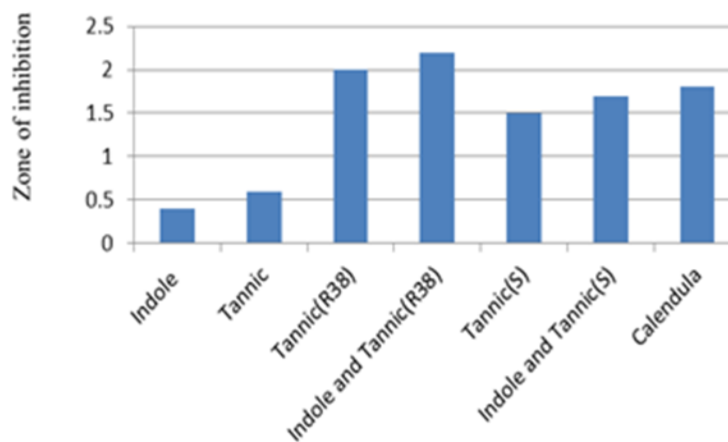


Figure 4.26: Comparative bar chart of the zone of inhibition by the extracts of *Streptomyces* for *Pseudomonas*(isolate 24)

Furthermore, minimal inhibitory concentration of the crude extract revealed that as low as 50 µl/ml was enough to exhibit the antimicrobial potential (Figure 4.27; 4.28). Thus, it can be presumed that the antimicrobial produced could be highly efficient if it could be extracted and purified. Thus, purification of the active constituent is recommended.

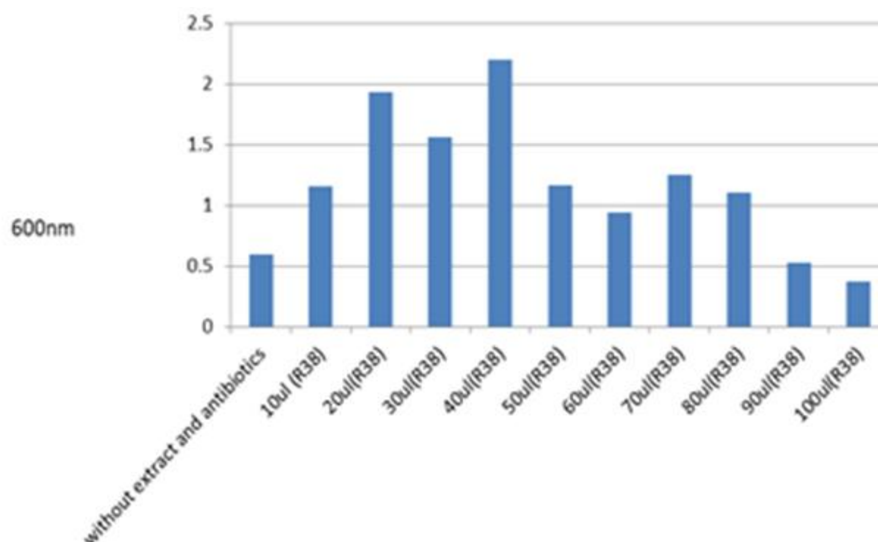


Figure4.27: The bar chart which represents the minimal inhibitory concentration by using extract of R38 with combination of indole and Tannic acid at different concentration against *Klebsiella(isolate 24)*

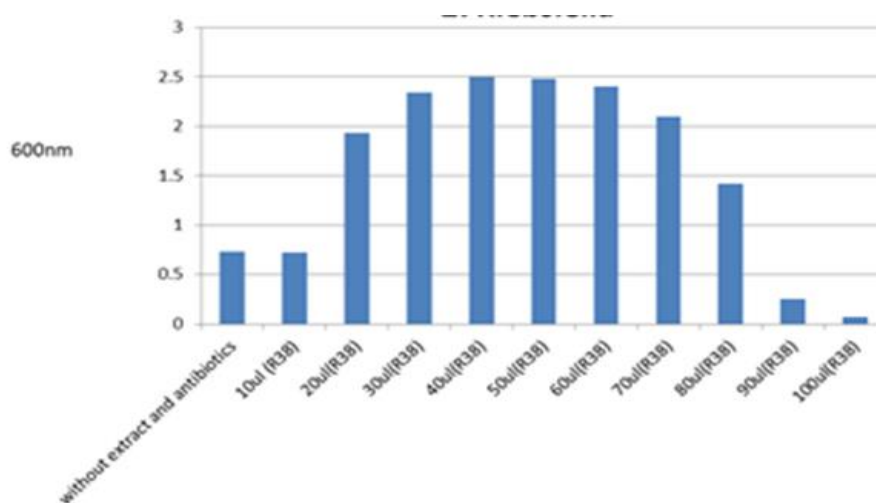


Figure4.28: The bar chart which represents the minimal inhibitory concentration by using extract of R38 with combination of indole and Tannic acid at different concentration against *Klebsiella (isolate 27)*

4.7. Molecular Analysis

Morphological and biochemical test was carried out for identification of family Actinomycetes that indicated that the isolates were *Streptomycetes* sps. This lead for molecular characterization and analyse whether the sample is bacteria or not 16s rRNA gene amplification was devised using universal primer used to amplify bacterial 16s rRNA gene. Genomic DNA was extracted and analyzed with nanometer and was found to be approximately 1.75ng. After that, molecular analysis of selected isolates was done by Polymerase Chain Reaction (PCR), by 16s universal primers. The PCR product was then subjected to gel electrophoresis and was visualized under UV light after intercalating ethydime bromide. For interpretation, 1Kb ladder was loaded in the gel.

Reverse primer sequence (R): CGGTTACCTTGTTACGACTT

Forward primer sequence (F): AGAGTTTGATCMTGGCTTCAG

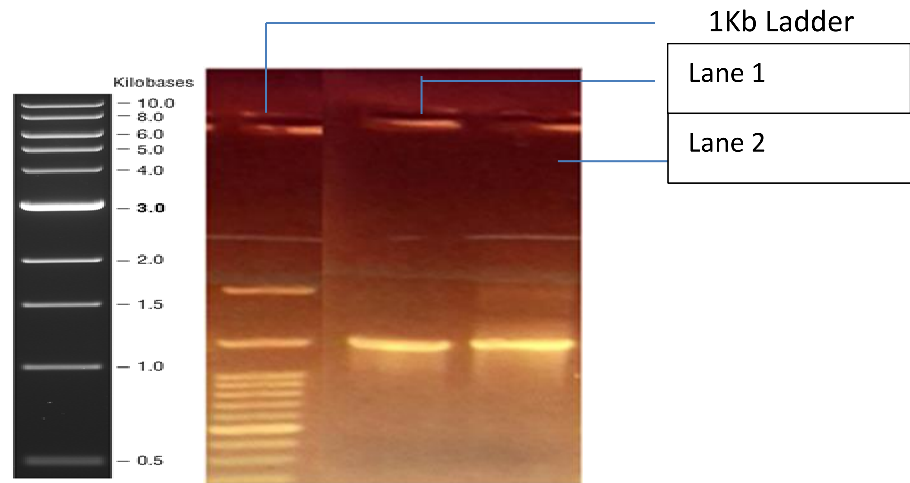


Figure 4.29: PCR products of the putative *Streptomyces*

The band corresponding to 1,500 bp of PCR product (Figure 4.18) confirmed that the isolates were bacteria. **Lane1** represents the sample isolate 'R38' and similarly **Lane 2** represents isolate 'S' that have shown antimicrobial potential.

Furthermore, PCR amplification of genomic DNA using StrepB 5'-ACAAGCCCTGGAAACGGGGT-3' (forward), StrepE 5'-CA CACCAGGAATTCCGATCT-3'(reverse) and StrepF 5'-ACGTGTGCAGCCCAAGACA-3' (reverse) (Rintala et al. 2001;Suutari et al. 2001) that amplifies gene, respectively, gave PCR product of 520bp and 1070 bp amplicon.

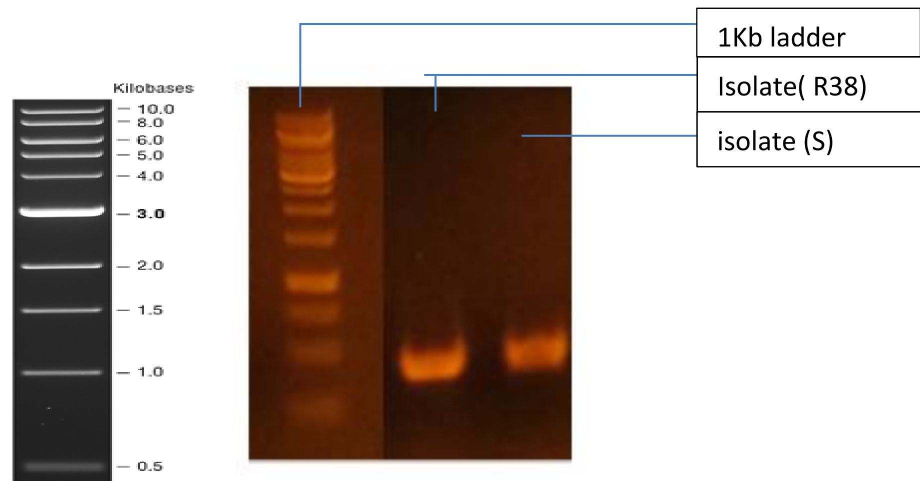


Figure 4.30: PCR product of *Streptomyces* by specific primers

It indicated that the isolates are *Streptomycetes sps*. because these genes are specific to this species(*S. ambofaciens*)(Pernodet et al. 1989) .

Isolates that represented *Streptomyces* sps. were then subjected for antimicrobial production. Since in ISP media antimicrobials were not produced, it was presumed that modifying the media could be an alternative strategy due to carbon catabolite repression

(CCR)(Bruckner,R.,TitgemeyerF.,2002) so reduced carbon sources have been used to produce different antibiotics(Sanchez et al.,2010).Those strains that had shown antimicrobial production against MDR *Klebsiella* sps. and *Pseudomonas* sps. in media consists of stress inducing molecules indole-3-butyric acid and lignin degradation product tannic acid were prioritized as potential organism to further pursue the research. These isolates had showed that they were *Streptomycetes* sps. PCR amplification of specific gene it was thought to sequence the 16s rRNA genomic sequence to construct phylogenic tree.

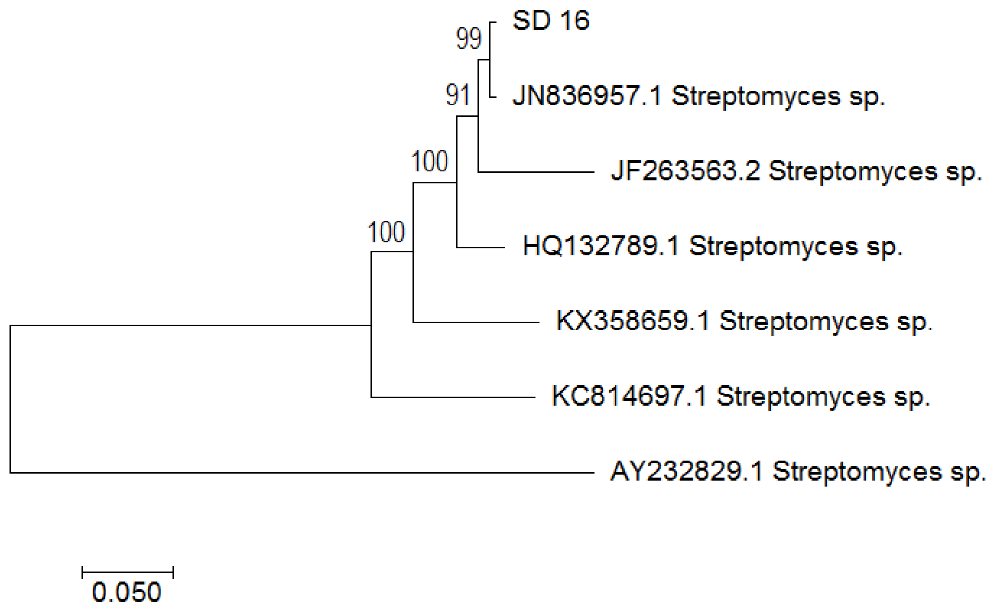


Figure 4.31: phylogenetic tree of *streptomyces* isolate S

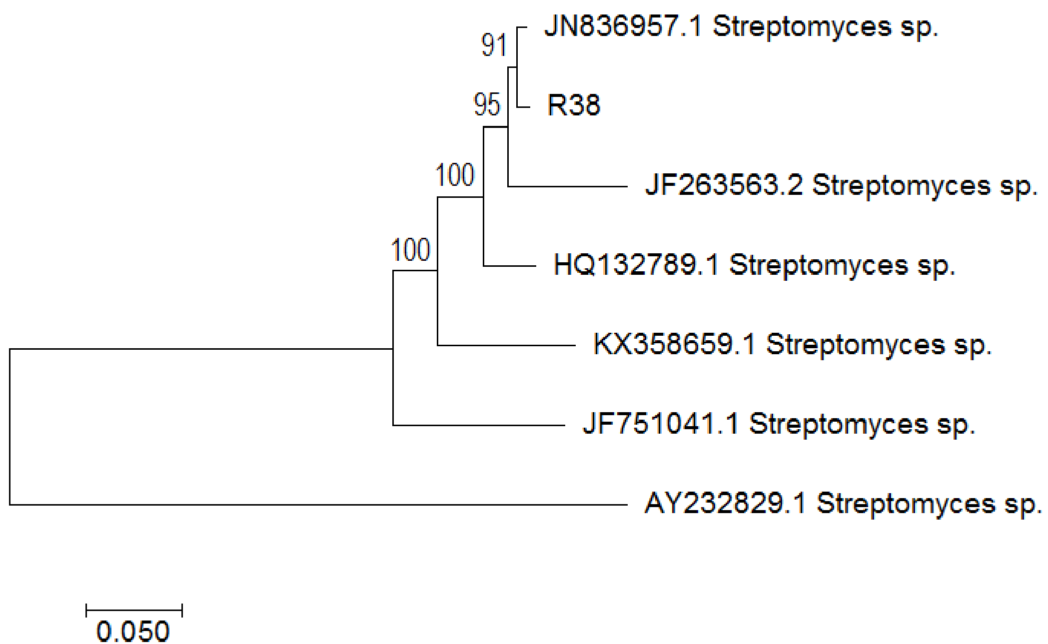


Figure 4.32: phylogenetic tree of *streptomyces* isolate R38.

Phylogenetic tree was constructed on basis of similarity between the different *Streptomyces* by BLAST.Generally, species generated separate clusters in the tree with bootstrap value (>50%) were considered to be discriminated successfully.

The average bootstrap value of both isolates was found to be slightly different from each other. In context of isolate *Streptomyces* R38, it found to be 96.5% similarly isolate *Streptomyces* S is found to be 97.5%.By the help of phylogenetic tree it is found that both isolates were similar with Streptomyces sp. AQB.SKKU 20 16S ribosomal RNA gene, partial sequence but identity of them were found to be different with each other.

The identity were found to be slightly different with each other which was found to be 91% in context of R38 whereas it is found to be 99% similarity with Streptomyces sp. AQB.SKKU 20 16S ribosomal RNA gene, partial sequence.

Table 4.11: Isolate S 16s rRNA genomic DNA partial sequence alignment

Description	Max score	Total score	Query score	E value	Identity
Streptomyces sp. AQB SKKU20 16S ribosomal RNA gene, partial sequence	2479	2479	95%	0.0	99%
Streptomyces sp. MSU 2261 16S ribosomal RNA gene, partial sequence	1554	1554	60%	0.0	98%
Streptomyces sp. N56(2010)16S ribosomal RNA gene, partial sequence	2073	2073	90%	0.0	95%
Streptomyces sp. NEAE-123 16S ribosomal RNA gene, partial sequence	1764	1764	85%	0.0	93%

Table 4.12: Isolate R38 16s rRNA genomic DNA sequence partial alignment

Description	Max score	Total score	Query score	E value	Identity
Streptomyces sp. N56(2010)16S ribosomal RNA gene, partial sequence	2435	2435	100%	0.0	100%
Streptomyces sp. AQB SKKU 20 16S ribosomal RNA gene, partial sequence	2119	2119	100%	0.0	96%
Streptomyces sp NEAE-123 16S ribosomal RNA gene, partial sequence	1434	1434	100%	0.0	89%
Streptomyces sp. USCO36 16S ribosomal RNA gene ,partial sequence	1576	1576	100%	0.0	88%
Streptomyces sp. 2011 16S ribosomal RNA gene, partial sequence	1293	1293	99%	0.0	87%

The partial sequence results obtained (Figure 8.5.1; 8.5.2, Appendix 8.5) were *Streptomyces* sps. and both the organisms above 80% similarity with different Streptomyces sps. Since, some of *Streptomyces* sps. can metabolize indole derivatives to give antimicrobial compounds because of their CYPome (https:// www.ncbi. nlm.nih.gov/ pmc/articles /PMC 5585785/). It can be confirmed that the isolated strains were *Streptomyces* sps**.**

CHAPTER-6

CONCLUSION

Multi drug resistance bacteria are being immerging problem which should be control by doing scientific research against them. As we know that, in our environment there are many bacteria which can affect our health can cause health problems. Some get resistance due to the misuse of antibiotics which should be controlled. Also, there are different bacteria which can help in treatment of such kind of problems by producing antimicrobial compound. The bacteria are selected on the basis of their metabolic pathway, their product, environmental condition where they are found.

Streptomyces are taken as the bacteria of interest because it has been used for different kinds of health related problems. Being our country a diverse one so in concept of new Streptomyces, soils of different areas were collected. The soils collected were from moist site where large amount of bacteria are found. Mostly preferred site was fern tree which is considered to be the oldest one.

Dilution was carried out to remove the extra particles and decrease the concentration of bacteria present in soil. The diluted solution that is 10^{-6} was spread in autoclaved media that is ISP2 and ISP4 which is specific media for isolation of Streptomyces. They use to form a colorful pigment which gave the indication that there was presence of indigo type of substance. As well as, it was also seen that the presence of simple sugar in the media caused contamination in the media. So, putative Streptomyces isolated from primary culture were re-streaked in the pure culture media for the further analysis and mass production.

So, computational technique was used for analysis of indole and their derivative for the confirmation and analysis of result seen without using the sophisticated and expensive lab equipment and compounds. In this technique, the protein named TrmD was taken as target protein due to its importance in methylation of DNA in presence of SAM and SAH. The binding energy of different ligands such as indole and indole derivatives (indurubin,isatin) were compared with all the docked with different binding energy.

Furthermore, to inhibit the contamination of bacteria in media containing Streptomyces modified media was prepared which contain the phenolic compounds which provide Carbon, Nitrogen as a source and also provide stress to produce antimicrobial product. Then, AST were carried out to observe the zone of inhibition of Carbapenem resistance bacteria as well as the Calendula was used as comparative molecule for observing zone of inhibition. As a result, zone of inhibition was seen in different bacteria from extract of

selected Streptomyces which was comparatively larger than zone of inhibition shown by Calendula.

Hence, phenolic compounds could act as antimicrobials and can also create the stress to bacteria which can produce secondary metabolites and can be easily utilized by other bacteria to convert them in useful products. The products produced by bacteria can be used against the emerging drug resistance bacteria by inhibiting their DNA methylation, transcription, transfer of genes etc.

Recommendations

Molecules used for the production of high amount of secondary metabolites may also increase potential of other bacteria by crating stress as well as it can also act as antimicrobial molecules. Also, molecules which are produced by *Streptomyces* should also be further analysed and tested in animal which can lead to potent drug design.

CHAPTER-5

SUMMARY

In this investigation, potent molecule against multidrug resistance bacteria (MDR), the emerging problem of world was targeted. For identification of specific molecules computational technique was used through which TrmD was taken as target molecule. In which SAM is essential for bacteria for methylation was replaced by different indoles and its derivatives also its B.E was calculated. Indole and its derivatives were preferred as ligand on basis of review of Wu et.al., (2005) in which different indoles where used against bacteria in which 5mM was found to be toxic. As well as, human cytochrome P450 2A6 was found to convert indoles in indirurubin causing inhibition of kinase enzyme named Glycogen Synthase kinase GSK-3β and found to be more potent against bacteria, without effecting human due to presence of Trm5. It is found that same cytochrome was found in Streptomyces so different areas of Nepal mainly targeting moist area due to its saprophytic nature. During selection main priority was given to color producing *Streptomyces* predicting it to be secondary metabolites and able to compete SAM and SAH for inhibition activity. As well as it was predicted that it can degrade indole and tannic acid for their metabolic process by creating stress on their bacterial components enhancing their secondary metabolites production. During computational analysis, different indole and derivatives were used among them, it was found that [1H-benz(f)indole-2,3-dione] was found to have less binding energy by replacing SAM and SAH in TrmD. So, including them in modified media, *Streptomyces* were grown and their antimicrobial property was analyzed by testing them against Carbapenem resistance bacteria. Zone of inhibition shown by isolated Streptomyces were confirmed by comparative study. For comparative analysis of zone of inhibition extract of flower named Calendula was used which is known for treatment of various kinds of diseases.

For the further confirmation of isolated bacteria as *Streptomyces* sequencing process was carried out. Whereas Antibiotics susceptibility result shown by extract help to conclude that indoles and its derivatives can be a molecule to act against bacteria.

CHAPTER 7

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8. APPENDIX

Appendix 8.1.Characterization and revival of Bacteria

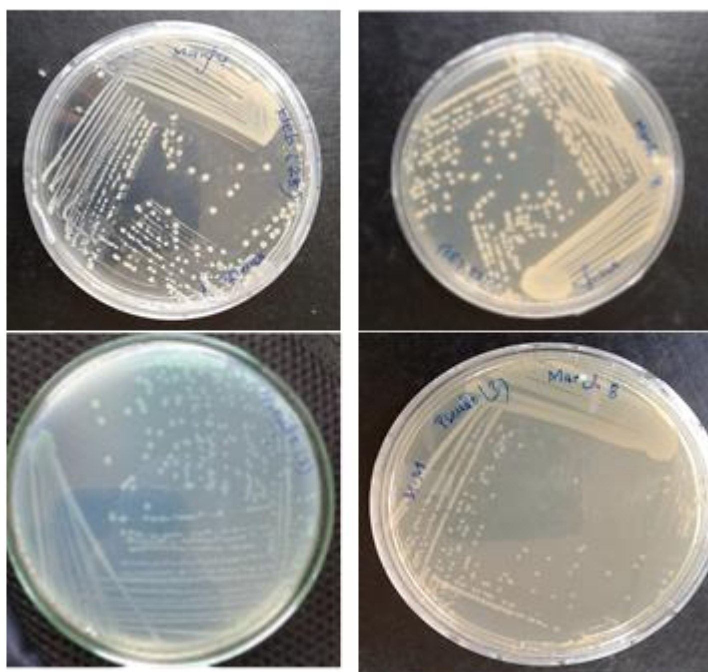

Figure 8.1 Isolation of *Klebsiella* and *Pseudomona*s from cyro-stock

Appendix 8.1.1.Biochemical test

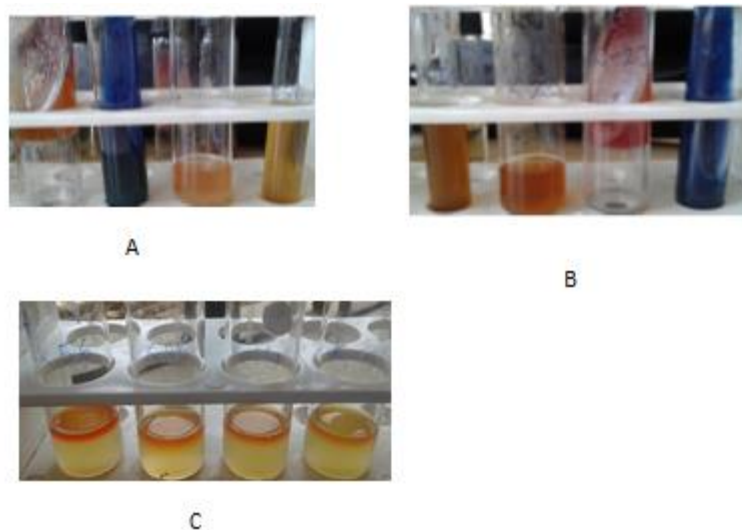

Figure 8.2:Biochemical test of bacteria and *Streptomyces*

A: TSI,MR-VP,citrate of bacteria;B TSI,MR-VP,citrate of *Streptomyces;* C:urease test

Appendix 8.1.2: Antimicrobial test

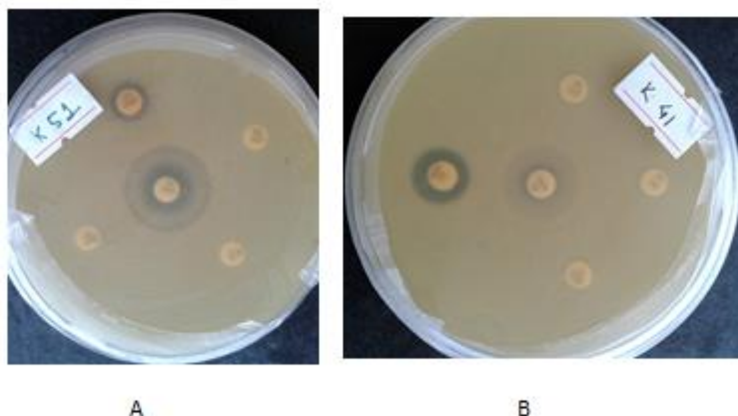

Figure 8.3: Antibiotics susceptibility tests for *Klebsiella* and *Pseudomonas* isolated

Appendix Table 8.1: List of database used for analysis

GenBank	http://www.ncbi.nlm.nih.gov/Genbank/	General nucleotide sequence database
UniProt	http://www.ebi.ac.uk/uniprot/	Universal Protein Resource gathering protein sequences and annotations from SwissProt (manually reviewed), trEMBL (computer annotated), and PIR
Pubchem	http://pubchem.ncbi.nlm.nih.gov/	Database on small molecules
PubMed	http://www.pubmed.org/	Database on biomedical literature
Protein data bank	http://www.rcsb.org/pdb/	Database on protein

Appendix Table 8.2:List of different ligands with its binding energy in TrmD replacing SAH

S.N	Ligands	Target	Binding energy
i.	zinc_4228232(S-Adenosylhomocysteine)	1p9p	-7
ii.	zinc_18825333(couroupitineB)	1p9p	-6.3
iii.	zinc_3844872(BLAHdione)	1p9p	-6.3
iv.	zinc_3217621[2-(p-tolyl)benzoi-acid-[2-(3-acetylanilino)-2-keto-ethyl]-ester	1p9p	-6.2
v.	zinc_84397755(indirubin)	1p9p	-5.9
vi.	zinc_32915988(Indurubin)	1p9p	-5.8
vii.	zinc_3641599[1H-benz(g)indole-2,3-dione]	1p9p	-5.5
viii.	zinc_5159022[6-(3-metylbut-2-enyl)indoline-2,3-dione]	1p9p	-5.5
ix.	zinc_3640842[1H,2H,3H-benzo(e)indole-1,2-dione]	1p9p	-5.3
x.	zinc_3638431[1H-benz(f)indole-2,3-dione]	1p9p	-5
xi.	zinc_2047514(isatin)	1p9p	-4.9
xii.	zinc_154243{6-(methylsulfonyl)-1-(oxiran-2-ylmethoxy)-1H-indole	1p9p	-4.9
xiii.	zinc_175204(2,8-dimethyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole	1p9p	-4.8
xiv.	zinc_154242{6-(methylsulfonyl)-1-(oxiran-2-ylmethoxy)-1H-indole	1p9p	-4.8
xv.	zinc_13462972{(2R)-2-methyl-1-nitroso-indoline}	1p9p	-4.6
xvi.	zinc_13462974{(2S)-2-methyl-1-nitroso-indoline}	1p9p	-4.3
xvii.	zinc_95864419(Indole)	1p9p	-4
xviii.	zinc_95864420(Indole)	1p9p	-3.9
xix.	zinc_967454(indoline)	1p9p	-3.7
xx.	zinc_14516984(indole)	1p9p	-3.7

Appendix Table 8.3: List of different ligands with its binding energy in TrmD replacing SAM

S.N.	Ligands	Target	Binding Energy
I.	zinc_5159022[6-(3-methylbut-2-enyl)indoline-2,3-dione]	4yvg	-8.1
II.	zinc_4228231(S-Adenosyl-L-methionine)	4yvg	-7.7
III.	zinc_175204(2,8-dimethyl-2,3,4,5-tetrahydro-1H-pyridol[4,3-b]indole	4yvg	-7
IV.	zinc_13462974{(2S)-2-methyl-1-nitroso-indoline}	4yvg	-6.7
V.	zinc_13462972(blahdione)	4yvg	-6.5
VI.	zinc_154242[6-(methylsulfonyl)-1-(oxiran-2-ylmetoxy)-1H-indole]	4yvg	-6.5
VII.	zinc_154243[6-(methylsulfonyl)-1-(oxiran-2-ylmetoxy)-1H-indole]8	4yvg	-6.5
VIII.	zinc_14516984(indole)	4yvg	-6.4
IX.	zinc_95864419(indole)	4yvg	-6.3
X.	zinc_3638431[1H-benz(f)indole-2,3-dione]	4yvg	-6.1
XI.	zinc_95864420(indole)	4yvg	-6
XII.	zinc_2047514(isatin)	4yvg	-5.7

Appendix 8.4: colorful isolates in plates of *streptomyces*

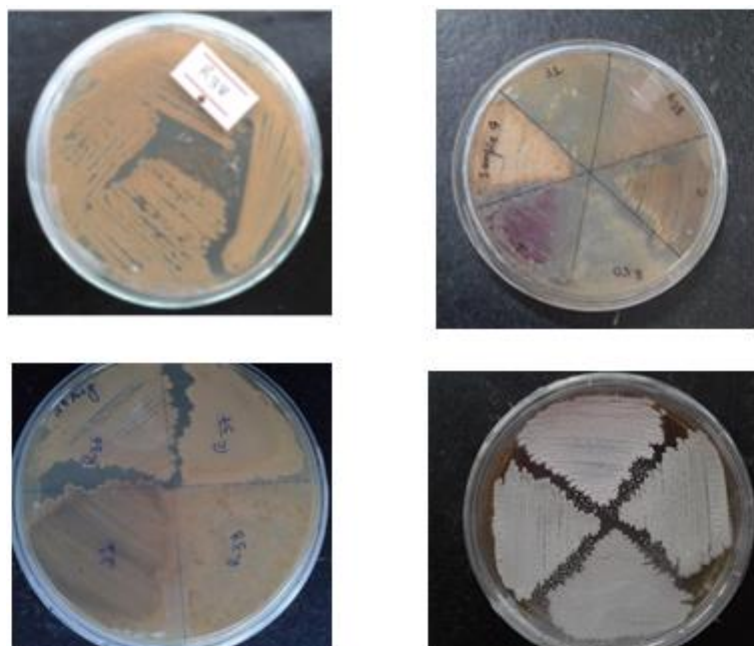

Figure8.4: Different colorful Streptomyces isolated from the different soils

Appendix Table 8.5Media composition of ISP2 and ISP4

ISP2 growth media

Ingredients	Gms / Litre
Peptic digest of animal tissue	5.000
Yeast extract	3.000
Malt extract	3.000
Dextrose	10.000
Agar	20.000
Final pH (at 25°C)	6.2±0.2

ISP4 growth media

Ingredients	Gms / Litre
Starch, soluble	10.000
Dipotassium phosphate	1.000
Magnesium sulphate. Heptahydrate	1.000
Sodium chloride	1.000
Ammonium sulphate	2.000
Calcium carbonate	2.000
Ferrous sulphate, heptahydrate	0.001
Manganous chloride, 7H2O	0.001
Zinc sulphate, 7H2O	0.001
Agar	20.000
Final pH (at 25°C)	7.2±0.2

Appendix 8.6 Gram staining

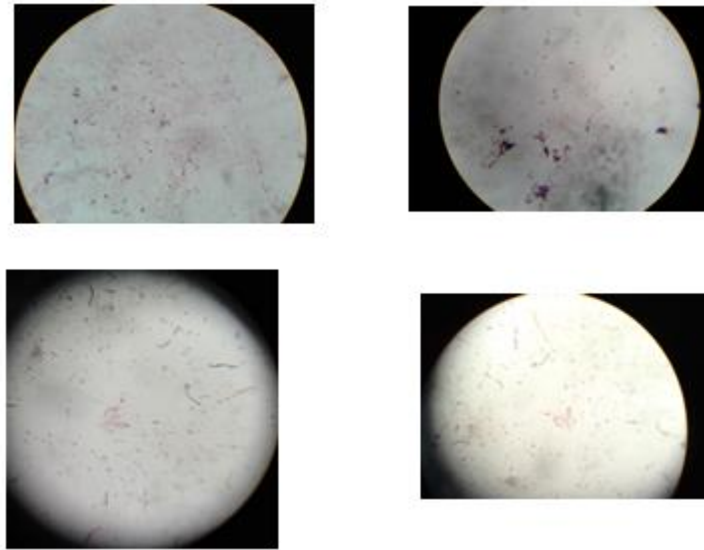

Figure 8.6: Gram staining of Streptomyces

Appendix 8.7: Antioxidation test

Figure 8.7: Antioxidation test of putative Streptomyces

Appendix 8.8 Alignment sequences of putative Streptomyces (R38 and S)

Appendix8.8.1: Alignment sequence of R38 by blast

Streptomyces sp. strain XZ-18 16S ribosomal RNA gene, partial sequence(94%)

>MF059155.1:3-696 Streptomyces sp. strain XZ-18 16S ribosomal RNA gene, partial sequence

TCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAA
CTAGTTGTTGGGGATTCATTTCCTTAGTAACGTAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGT
CGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATTCGAT
GCAACGCGAAAAACCTTACCTACCCTTGACATGGTCGGAATCCCGAAGAGATTTGGGAGTGCTCGAAAG
AGAACCGGCGCACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
ACGAGCGCAACCCTTGTCCTTAGTTGCTACGCAAGAGCACTCTAAGGAGACTGCCGGTGACAAACCGGA
GGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGGGTAGGGCTTCACACGTCATACAATGGTCG

GAACAGAGGGTTGCCAACCCGCGAGGGGGAGCTAATCCCAGAAAACCGATCGTAGTCCGGATTGCACTC
TGCAACTCGAGTGCATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCC
GGGTCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTTTACCAGAAGTGGCTAGTCTAACCGCAAG
G

Streptomyces sp. NEAE-123 16S ribosomal RNA gene, partial sequence(89%)

>JF263563.2:12-1240 Streptomyces sp. NEAE-123 16S ribosomal RNA gene, partial sequence

ATGCAGTCGAACGGCAGCACGGACTTCGGTCTGGTGGCGAGTGGCGAACGGGTGAGTAATGTATCGGA
ACGTGCCTAGTAGCGGGGGATAGCTACGCGAAAGCGTAGCTAATACCGCATACGCCCTACGGGGGAAA
GCAGGGGATCGTAAGACCTTGCACTATTAGAGCGGCCGATGTCGGATTAGCTAGTTGGTGGGGTAAGGG
CTCACCAAGGGAACAATCCGTATCTGGTTTGAGAGGACGACCAGCCACTCTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGCAGCAGTGGGGAATTTTGGACAATGGGGGAGACCCTGATCCCCCGTCCCGCGTG
TGCCATGAAGGCCTTCGGATTGTAAAGCACTTTTGGCAGGAAAGAAACGGCCTGGGTTAATACCCCGTG
AAACTGACGGTACCTGCAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTG
CAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTCGGAAAGAAAGATGTGAAATCCC
AGAGCTTAACTTTGGAACTGCATTTTTAACTACCGAGCTAGAGTGTGTCAGAGGGAGGTGGAATTCCGCG
TGTAGCAGTGAAATGCGTAGATATGCGGAGGAACACCGATGGCGAAGGCAATCTCCTGGAATTGTACTG
ACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTACACGATG
TCAACTAGGTGTTGGGGACCTTCGTCTCTTAGTAACGCAGCTAACGCGTGAAGTTGACCGCCTGGGGAGT
ACGGTCGCGGGATTAAAACTCAAAGGAATTTGACGGAGACCCGCACAGCGGGAGGATGATGGTGGATT
ATTCGATGCACGCGAAAACCCTTACCTACCCCGTACATGTAAGGAATTAGAAAGAGATTGGGAAGTGCTG
GCAAGAGAACCCGCACACAGGGGATGCATGTCCGTCGTCAGCTTGGGTCGTGGGAGGTTGGGTTAAATC
CCGCAACGCGACCTCCCCCATTCATTACTTCCTTCGGAAGGGCACTCTAAGGAGACTGCCGGTGAC
AAACCGGAGGAAGGTGGGGAGGACGTCAAGTCCTCATGGCCCTTATGGCTTGGGCTTCACACG
TCATCCAATGGTTGGGACAGAGGGTCGCCAACCCGCGAGGGGGAGCCAATCCCAGAAACCCTA
TC

Streptomyces sp. N56(2010) 16S ribosomal RNA gene, partial sequence(100%)

>HQ132789.1 Streptomyces sp. N56(2010) 16S ribosomal RNA gene, partial sequence

GAACGTGCCCAGTAGCGGGGGATAACTACTCGAAAGAGTGGCTAATACCGCATACGCCCTACGGGGGAA
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CAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGGGAAACCCTGATCCAGCCATCCCGC
GTGTAGTGATGAAGGCCTTCGGGTTGTAAAGTACTTTTGGCAGAAAAGAAAAGGTATCCCCTAATACGG
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GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGTGTAGGCGGTTCGGAAAGAAAGATGTGAA
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CCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAAGGCAGCCCCCTGGGATAA
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AATGGTCGGGACAGAGGGTCGCCAACCCGCGAGGGGGAGCCAATCTCAGAAACCCGATCGTAGTCCGG
ATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGGATCAGAATGTCGCGGTGA

ATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTTCACCAGAAGTAGGTAGCC
TAACCG

Streptomyces sp. AQB.SKKU 20 16S ribosomal RNA gene, partial sequence(96%)

>JN836957.1:61-1378 Streptomyces sp. AQB.SKKU 20 16S ribosomal RNA gene, partial sequence

GAACGTGCCCAGTAGCGGGGGATAACTACGCGAAAGCGTAGCTAATACCGCATACGCCCTACGGGGGA
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GGCTCACCAAGGCGACGATCCGTAGCTGGTTTGAGAGGACGACCAGCCACACTGGGACTGAGACACGG
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TACAATGGTCGGGACAGAGGGTCGCCAACCCGCGAGGGGGAGCCAATCCCAGAAACCCGATCGTAGTCC
GGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGTCGCGGT
GAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTTTACCAGAAGTAGTTAG
CCTAACCG

Streptomyces sp. MSU 2261 16S ribosomal RNA gene, partial sequence (94%)
>AY232829.1:1-881 Streptomyces sp. MSU 2261 16S ribosomal RNA gene, partial sequence
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TAGTTATCCCCCGCTACTAGGCACGTTCCGATACATTACTCACCCGTTCGCCACTCGCCACCAGACCGAA
GTCCGTGCTGCCGTTCGACTTGCATGTGTAAGGCATCCCGC

Streptomyces sp. strain RK-307 16S ribosomal RNA gene, partial sequence(92%)
>MH244357.1:608-1262 Streptomyces sp. strain RK-307 16S ribosomal RNA gene, partial sequence
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TCGCAAGATTAATACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATTTGA
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GTACAGAGGGTTGCCAACCCGCGAGGGGGAGCCAATCCCAGAAAACGCATCGTAGTCCGGATCGTAGTC
TGCAACTCGACTACGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCC
GGGTCTTGTACACACCGCCCGTCACACCAT

Appendix 8.8.2: Alignment sequence of S by blast

Streptomyces sp. AQB.SKKU 20 16S ribosomal RNA gene, partial sequence(99%)
>JN836957.1 Streptomyces sp. AQB.SKKU 20 16S ribosomal RNA gene, partial sequence
TCGAACGGCAGCACGGACTTCGGTCTGGTGGCGAGTGGCGAACGGGTGAGTAATGTATCGGAACGTGC
CCAGTAGCGGGGGATAACTACGCGAAAGCGTAGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGG
GATCGCAAGACCTTGCACTATTGGAGCGGCCGATATCGGATTAGCTAGTTGGTGGGGTAACGGCTCACC
AAGGCGACGATCCGTAGCTGGTTTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTC
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CGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTCGGAAAGAAAGATGTGAAATCCCAGAG
CTTAACTTTGGAACTGCATTTTTAACTACCGGGCTAGAGTGTGTCAGAGGGAGGTGGAATTCCGCGTGTA
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GTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATTCG
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GAGAACCGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC
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GGGACAGAGGGTCGCCAACCCGCGAGGGGGAGCCAATCCCAGAAACCCGATCGTAGTCCGGATCGCAG
TCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGTCGCGGTGAATACGTTC
CCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTTTACCAGAAGTAGTTAGCCTAACCGCA
AGGGGGGC

Streptomyces sp. N56(2010) 16S ribosomal RNA gene, partial sequence(95%)
>HQ132789.1 Streptomyces sp. N56(2010) 16S ribosomal RNA gene, partial sequence
GAACGTGCCCAGTAGCGGGGGATAACTACTCGAAAGAGTGGCTAATACCGCATACGCCCTACGGGGGAA
AGGGGGGGATCGCAAGACCTCTCACTATTGGAGCGGCCGATATCGGATTAGCTAGTTGGTGGGGTAAAG
GCTCACCAAGGCGACGATCCGTAGCTGGTTTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCC
CAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGGGAAACCCTGATCCAGCCATCCCGC
GTGTAGTGATGAAGGCCTTCGGGTTGTAAAGTACTTTTGGCAGAAAAGAAAAGGTATCCCCTAATACGG
GATACTGCTGACGGTATCTGCAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG
GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGTGTAGGCGGTTCGGAAAGAAAGATGTGAA
ATCCCAGGGCTCAACCTTGGAACTGCATTTTTAACTGCCGAGCTAGAGTATGTCAGAGGGGGGTGGAATT
CCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAAGGCAGCCCCCTGGGATAA
TACTGACGCTCATACACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAA
CGATGTCAACTAGCTGTTGGGGCCGTTAGGCCTTAGTAGCGCAGCTAACGCGTGAAGTTGACCGCCTGG
GGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGG
ATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGTCTGGAAAGCCGAAGAGATTTGGCCG
TGCTCGCAAGAGAACCGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTT
AAGTCCCGCAACGAGCGCAACCCTTGTCATTAGTTGCTACGCAAGAGCACTCTAATGAGACTGCCGGTGA
AAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGGGTAGGGCTTCACACGTCATAC

AATGGTCGGGACAGAGGGTCGCCAACCCGCGAGGGGGAGCCAATCTCAGAAACCCGATCGTAGTCCGG
ATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGGATCAGAATGTCGCGGTGA
ATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTTCACCAGAAGTAGGTAGCC
TAACCG

Streptomyces sp. NEAE-123 16S ribosomal RNA gene, partial sequence
>JF263563.2:7-1240 Streptomyces sp. NEAE-123 16S ribosomal RNA gene, partial sequence(93%)
TAACAATGCAGTCGAACGGCAGCACGGACTTCGGTCTGGTGGCGAGTGGCGAACGGGTGAGTAATGTAT
CGGAACGTGCCTAGTAGCGGGGGATAGCTACGCGAAAGCGTAGCTAATACCGCATACGCCCTACGGGG
GAAAGCAGGGGATCGTAAGACCTTGCACTATTAGAGCGGCCGATGTCGGATTAGCTAGTTGGTGGGGTA
AGGGCTCACCAAGGGAACAATCCGTATCTGGTTTGAGAGGACGACCAGCCACTCTGGGACTGAGACACG
GCCCAGACTCCTACGGGAGCAGCAGTGGGGAATTTTGGACAATGGGGGAGACCCTGATCCCCCGTCCCG
CGTGTGCCATGAAGGCCTTCGGATTGTAAAGCACTTTTGGCAGGAAAGAAACGGCCTGGGTTAATACCCC
GTGAAACTGACGGTACCTGCAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
GTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTCGGAAAGAAAGATGTGAAA
TCCCAGATTAACTTTGGAACTGCATTTTTAACTACCGAGCTAGAGTGTGTCAGAGGGAGGTGGAATTCCG
CGTGTAGCAGTGAAATGCGTAGATATGCGGAGGAACACCGATGGCGAAGGCAATCTCCTGGAATTGTAC
TGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTACACGA
TGTCAACTAGGTGTTGGGGACCTTCGTCTCTTAGTAACGCAGCTAACGCGTGAAGTTGACCGCCTGGGGA
GTACGGTCGCGGGATTAAAACTCAAAGGAATTTGACGGAGACCCGCACAGCGGGAGGATGATGGTGGA
TTATTCGATGCACGCGAAAACCCTTACCTACCCCGTACATGTAAGGAATTAGAAAGAGATTGGGAAGTGC
TGGCAAGAGAACCCGCACACAGGGGATGCATGTCCGTCGTCAGCTTGGGTCGTGGGAGGTTGGGTTAAA
TCCCGCAACGCGACCTCCCCCATTCATTACTTCCTTCGGAAGGGCACTCTAAGGAGACTGCCGGTGACAA
ACCGGAGGAAGGTGGGGAGGACGTCAAGTCCTCATGGCCCTTATGGCTTGGGCTTCACACGTCATCCAA
TGGTTGGGACAGAGGGTCGCCAACCCGCGAGGGGGAGCCAATCCCAGAAACCCTATC

Streptomyces sp. MSU 2261 16S ribosomal RNA gene, partial sequence
>AY232829.1 Streptomyces sp. MSU 2261 16S ribosomal RNA gene, partial sequence (98%)
CCGTCAATTCCTTTGAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGTCAACTTCACGCGTTAGCTGCG
CTACCAAGGCCCGAAGGCCCCAACAGCTAGTTGACATCGTTTAGGGCGTGGACTACCAGGGTATCTAATC
CTGTTTGCTCCCCACGCTTTCGTGCATGAGCGTCAGTGTTATCCCAGGAGGCTGCCTTCGCCATCGGTGT
TCCTCCGCATATCTACGCATTTCACTGCTACACGCGGAATTCCACCTCCCTCTGACACACTCTAGCTCGG
TAGTTAAAAATGCAGTTCCAAAGTTAAGCTCTGGGATTTCACATCTTTCTTTCCGAACCGCCTGCGCACG
CTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGC
CGGTGCTTATTCTGCAGGTACCGTCAGTTTCACAGGGTATTAACCCGTGACGTTTCTTTCCTGCCAAAAG
TGCTTTACAACCCGAAGGCCTTCATCGCACACGCGGGATGGCTGGATCAGGGTTTCCCCCATTGTCCAAA
ATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGGTCGTCCTCTCA
AACCAGCTACGGATCGTCGCCTTGGTGAGCCGTTACCCCACCAACTAGCTAATCCGATATCGGCCGCTCT
AATAGTGCAAGGTCTTGCGATCCCCTGCTTTCCCCCGTAGGGCGTATGCGGTATTAGCTACGCTTTCGCG
TAGTTATCCCCCGCTACTAGGCACGTTCCGATACATTACTCACCCGTTCGCCACTCGCCACCAGACCGAA
GTCCGTGCTGCCGTTCGACTTGCATGTGTAAGGCATCCCGCTAGCGTTCAATCTGAGCCATGATCAACTC
TAATCG

Streptomyces sp. strain XZ-18 16S ribosomal RNA gene, partial sequence
>MF059155.1:1-696 Streptomyces sp. strain XZ-18 16S ribosomal RNA gene, partial sequence (94%)
GCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTC
AACTAGTTGTTGGGGATTCATTTCCTTAGTAACGTAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACG
GTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATTCG

ATGCAACGCGAAAAACCTTACCTACCCTTGACATGGTCGGAATCCCGAAGAGATTTGGGAGTGCTCGAAA
GAGAACCGGCGCACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC
AACGAGCGCAACCCTTGTCCTTAGTTGCTACGCAAGAGCACTCTAAGGAGACTGCCGGTGACAAACCGG
AGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGGGTAGGGCTTCACACGTCATACAATGGTC
GGAACAGAGGGTTGCCAACCCGCGAGGGGGAGCTAATCCCAGAAAACCGATCGTAGTCCGGATTGCACT
CTGCAACTCGAGTGCATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCC
CGGGTCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTTTACCAGAAGTGGCTAGTCTAACCGCAA
GG

Appendix 8.6.Antimicrobial test by extract of *Streptomyces*

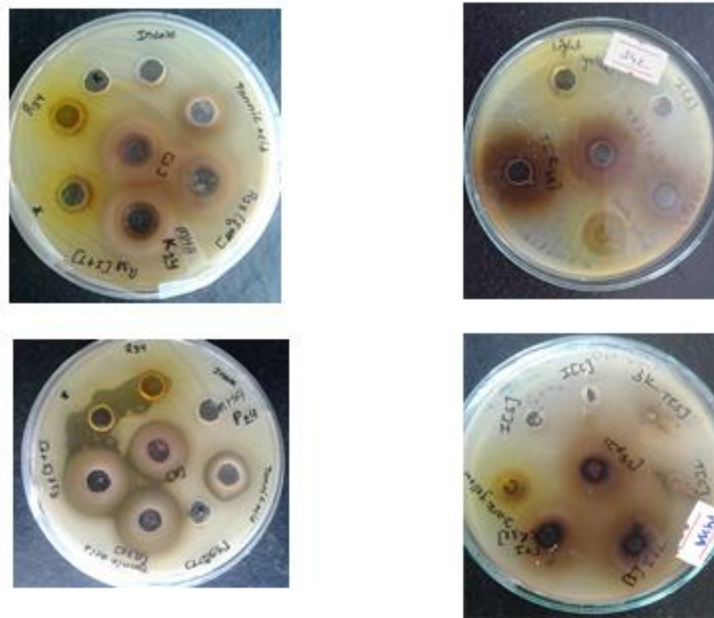

Figure 8.6: Tests of extracts in different bacteria

Appendix 8.7. Comparative Antimicrobial test by extract of *Streptomyces* and antibiotics discs

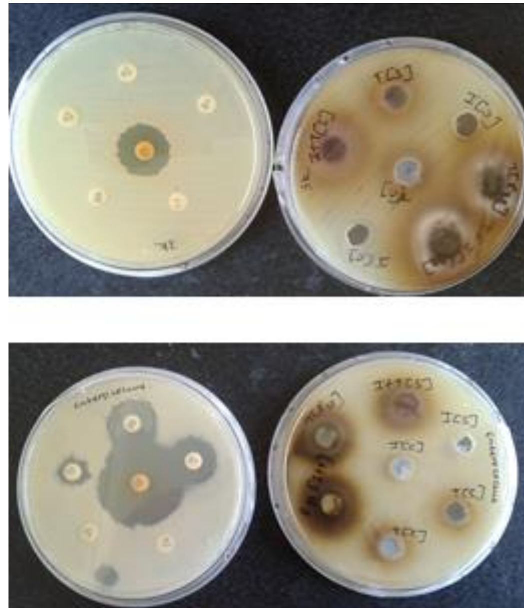

Figure 8.7: Comparative tests of extracts in different bacteria

Appendix 8.8 Zone of inhibition in bar

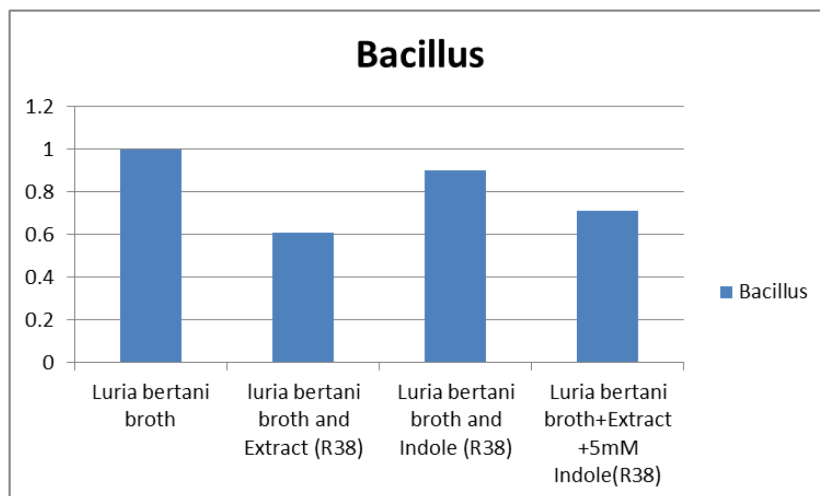

Figure: 1

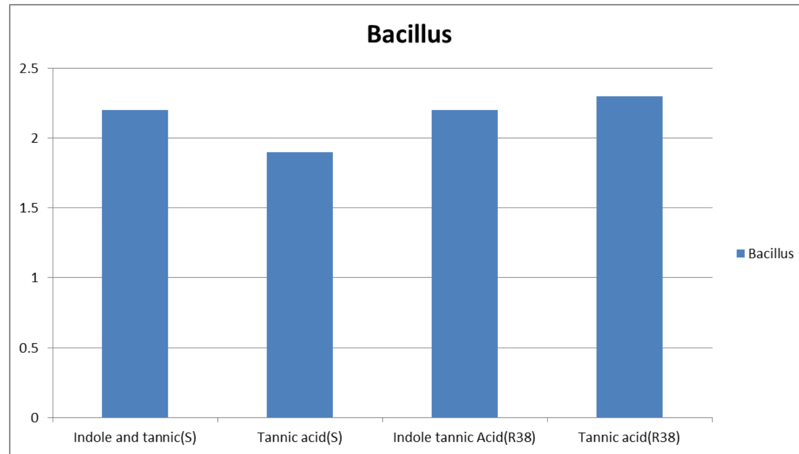

Figure :2

Figure8.8: Zone of inhibition in bar chart of *Bacillus*

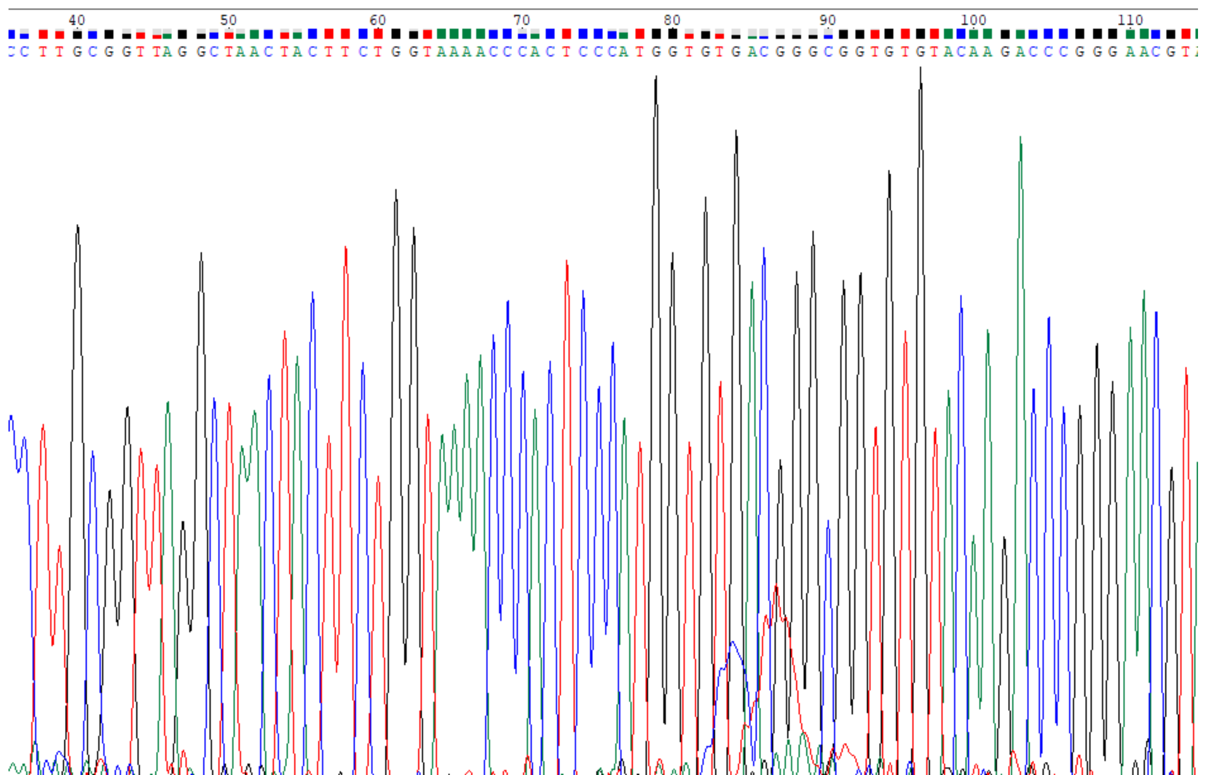

Figure 4.31:16S Reverse chromatography of S

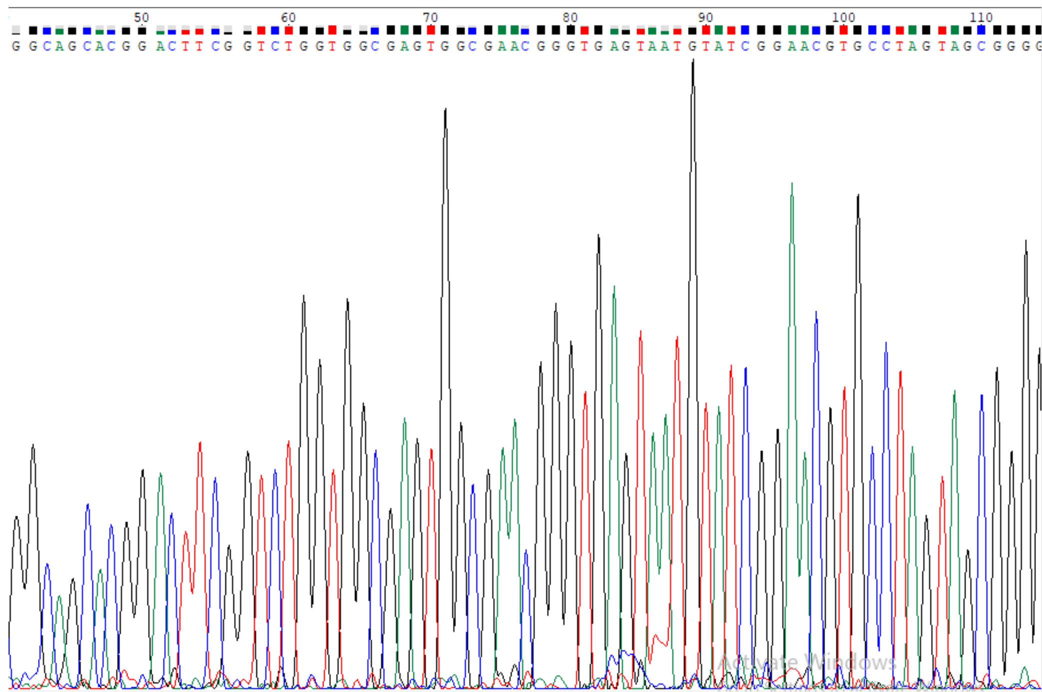

Figure4.32: 16S Forward chromatography of S

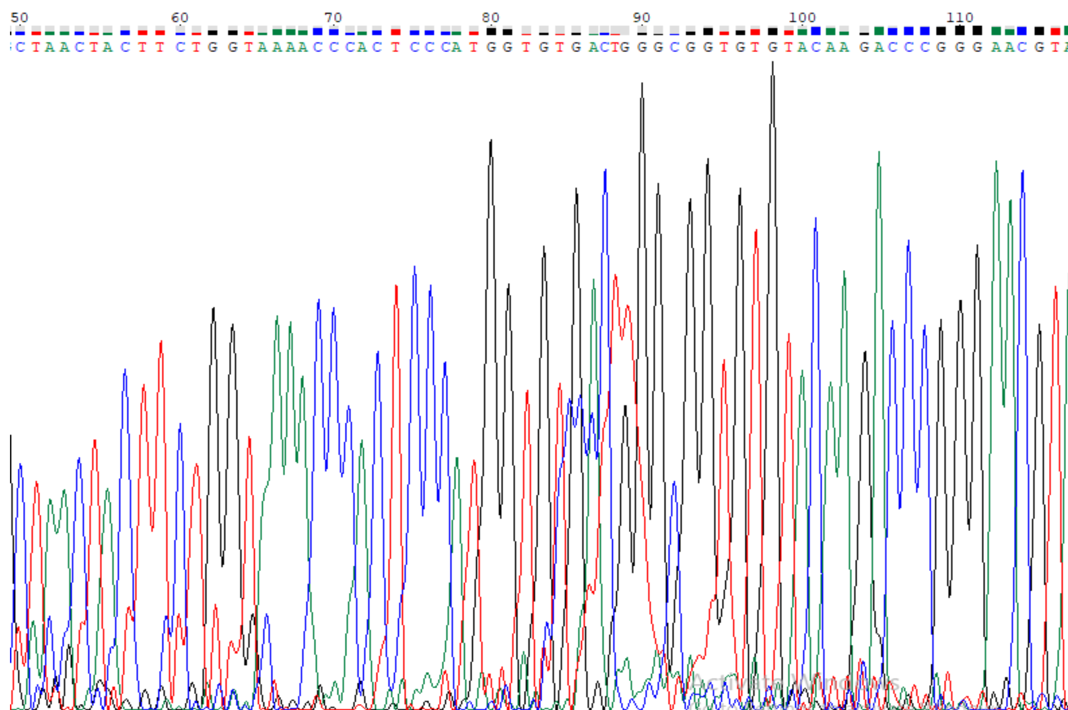

Figure 4.33:16S Reverse chromatography of R38

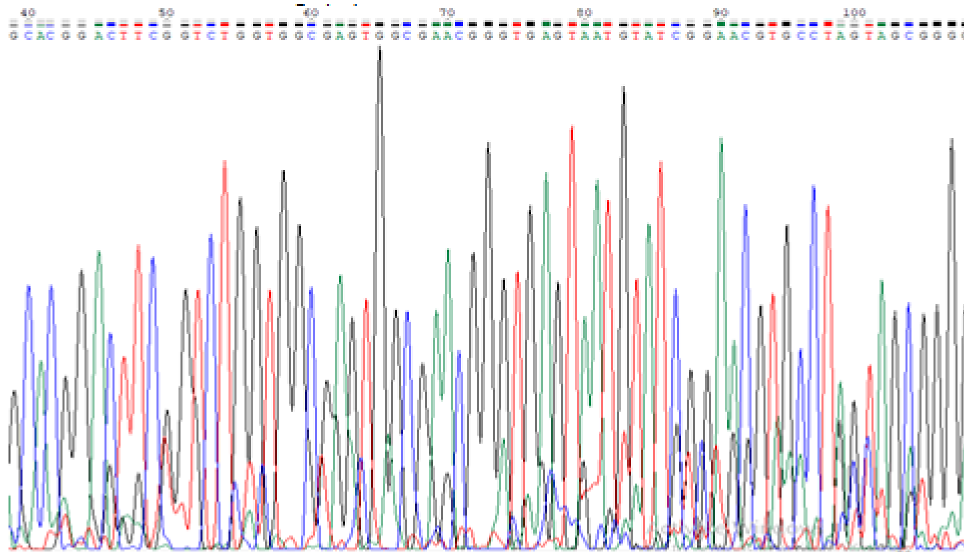

Figure 4.34:16S forward chromatography of R38