IN SILICO MEDIATED TWEAKING OF *Streptomyces* FOR ANTIMICROBIAL PRODUCTION: CLONING FOR RIBOSWITCH MEDIATED INHIBITION

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By
Sita Ghimire
Registration No.: 5-2-33-204-2011
Supervisors
Senior Scientist Dr. Pramod Aryal
Prof. Dr. Rajani Malla

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LIST OF ABBREVIATIONS

Ala	Alanine
ACT	Actinorhodin
ADME/Tox	Absorption, Distribution, Metabolism, Excretion, Toxicity
AfsK	Threonine Kinase
AHLs	Acylhomoserine Lactones
AMR	Anti Microbial Resistance
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
Bp	base pair
CCR	Carbon Catabolite Repression
CDBT	Central Department Of Biotechnology
CDC	Centres for Disease Control and Prevention
CLSI	Clinical Laboratory Standards Institute
Cm	Centimeters
DMSO	Dimethyl sulfoxide
DNA	Deoxyribo Nucleic Acid
DNTI	Drugs from Nature Targeting Inflammation
ECDC	European Centre for Disease Control
EUCAST	European Committee on Antimicrobial Susceptibility Testing
Glk	glucose kinase
HGT	Horizontal Gene Transfer
HTS	High Throughput Screening
IDT	Integrated DNA technology
LB	Luria Bertani broth
MDR-TB	Multidrug Resistance Tuberculosis
Ml	Microlitres
mM	milimolar
mRNA	messenger Ribonucleic Acid
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NPs	Natural products
ORF	Open Reading Frame
PBP	Penicillin Binding Protein
PDR	Pandrug-Resistant
PKS	Polyketide Synthase
RCSB	Research Collaboratory for Structural Bioinformatics
SCA	Starch Caseine Agar
STE	Sodium Tris EDTA
TAE	Tris Acetate Ethylene Diamne Tetra Acetic Acid
UTR	Un -Translated Region
WHO	World Health Organization
XDR	Extensively Drug-Resistant

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ABSTRACT

The potential worldwide disastrous health calamities rendered by multidrug resistant pathogens, recognized by World Health Organization seek an effective therapeutics, demands noble antibiotics to be developed. Pharmaceuticals companies and researchers have to be dedicated to discover new potent antibiotics against the last line antibiotic resistant bugs. Moreover, the new antibiotic candidate should have noble mechanism against the pathogens. The Computer Aided Drug Discovery (CADD) along with combinatorial chemistry was taken into account to identify putative drug candidates against the essential Uroporphyrinogen III methyltransferase (CobA) protein of *Salmonella* sps. Virtual screening of 1,788 ligands screened on the basis of ADME/Toxicity testing identified top three lead drug candidates. The ligand library was generated from kinase inhibitors that gave N-[3-[4-[4-(trifluoromethyl) benzoyl] piperazine-1-carbonyl] phenyl] pyridine-3-carboxamide and 1-(4-phenylpiperazin-1-yl)-2-[4-[2-(trifluoromethyl) benzoyl] piperazin-1-yl] ethanone and one indole derivative which gave (1R,4R)-4-(1H-indol-3-ylmethyl) -1-methyl-2,4-dihydro-1H-pyrazino [5,4-b] quinazoline-3,6-dione as the putative noble drug candidates. These compounds gave clear pictures that tweaking of saprophytic *Streptomycetes* sps. with some stress modulating compounds could help in developing antibiotics. Hence, antibiotics producer *Streptomyces* sps were screened and isolated from soils of various geographical areas of Nepal and subjected for the production of secondary metabolites by addition of different lignin degradation natural products or different carbon sources to the modified media. Out of total isolated *Streptomyces* strains, four strains named as PA2, PA3, MA1 and KA10 were found to produce compound(s) that inhibited the carbapenem and colistin resistant biofilm producing *Salmonella* sps. Upon further expansion to carbon catabolite repression mediated antibiotic production, the crude extracts of standard *Streptomyces* strain, *S. coelicolor* and isolated PA3 strain showed good inhibitory effect against *Salmonella* sps. and methicillin resistant *Staphylococcus aureus* in robust resazurin assay when secondary metabolites were produced with addition of 5 mM coumarin and two amino acids, valine and tryptophan. Furthermore, pectin and cellulose as the sole reduced carbon sources were found to induce development of good inhibitors against those two pathogens. The results indicate that these substrates could potentially be used to develop putative drugs against CobA protein of pathogens. Thus, the prospect of riboswitch mediated translation inhibition of essential CobA protein as the mechanism of action of produced antibiotics by *Streptomyces* was designed by gene promoter cloning where effect on inhibition of translation could be assayed by β-galactosidase *LacZ* reporter system when subsequently cloned under this promoter that would assist in to explore whether the *cob*A gene product mRNA could have *S*-adenosyl methionine (SAM) riboswitch at its 5'-UTR region as it uses SAM for methylation but is still unknown for this to gene.

Keywords: Carbon catabolite repression, CADD, essential gene, Multidrug resistance, resazurin, riboswitch, *Streptomyces*

1 INTRODUCTION

1.1 Background

The gradual discovery of antibiotics till 2000 were taken as the milestone in medical field to decrease the fatality from simple communicable infections. In addition, antibiotics have also been used as probiotic and as prophylactics to diminish the secondary infections since past years. However, the rapid emergence of resistance appears to have resulted from selective pressure for survival of the pathogens (Tello *et.al.,* 2012). Such resistances have emerged due to inappropriate antimicrobial consumption, misuse of antimicrobials in livestocks, mutations in bacteria (Woodford and Ellington, 2007) and horizontal gene transfer (Burmeister, 2015). The emergence of the last resort antibiotic, carbapenem and/ or colistin resistant pathogenic strains (Caniaux *et. al.*, 2017) is of great concern.

A set of synchronized plans and strategies for the improvement of proper use of antimicrobial medications is referred to as antibiotic stewardship. It aims in enhancing health outcomes of patient by reducing resistance mechanism of pathogens. Centers for Disease Control and Prevention in 2013 launched the program to track the causes of infections and analyzing it to verify whether it is pathogenic or not and is that of human origin found in the people and community. It is also conducting the regular programs to aware about the antibiotics proper usages, diseases transmission routes and technique to be safe from various food borne pathogenic diseases, too. All at aiming for minimal antibiotics uses to aware the people for judicious and rational uses of the antibiotics.

WHO has launched the program named as Global Antimicrobial Resistance Surveillance System (GLASS) in October 2015 to establish a worldwide standardized approach in order to move forward by analyzing and sharing the datas on antimicrobial resistance. After two years, it published the global list of Critical and High priority carbapenem and colistin resistant pathogens against which new antimicrobials with noble drug target is in urgent need (February 27, 2017). Since 2000, only two noble classes of antibiotics, the oxazolidinones (Barbachyn and Ford, 2003) and cyclic lipopeptides (Kern, 2006) have entered into the market but that is yet is not enough to combat the resistance problem.

Thus, the alarming increased rate of antimicrobial resistance demands the noble families of antibiotics against bacteria with noble mechanism of inhibition. One of the areas could be computer aided drug discovery (CADD) approach (Das, 2017) of selecting some molecules through *in silico* approach. High throughput computational screening method could be employed to identify and draw the noble lead compounds to narrow down the drug candidates with more specific mode of action against resistant strains. In addition, repurposing of drugs (Oprea and Mestres, 2012) have been a new tools to look for the molecule that has been used in treatment of one cause or had been developed for one purpose that could be used in for another. CADD could be helpful in this, especially with

molecular docking (Das, 2017) of ligands present in the database to identify as probable lead candidate against the protein of interest that is essential to the survival of the pathogens.

Moreover, the strategy to develop new antibiotics should not shy from exploration of natural products as well as soil bacteria itself (Cragg and Newman, 2013). *Streptomyces* spp. are soil dwelling antibiotic producing bacteria from which world's two third of antibiotics are produced (Hasani *et. al.*, 2014). The uplifted geographical variation of Nepal could harbor different *Streptomyces* sps. that could be of the marine or terrestrial habitats that could provide potentially new microorganisms and their secondary metabolites to address the global problem of communicable diseases, their etiology and resistance carried by these pathogens.

In the present work both CADD and *in vitro* approaches have been used to identify putative molecule that could potentially be developed as antibiotics. Based on the CADD results the culture conditions were modified to culture known *Streptomyces* sps. or the isolated from the Nepalese soil to develop antibacterial compound. The new strategy of combination of both *in silico* and *in vitro* approaches lead towards the discovery of new potential antimicrobial drugs. Based on CADD data, the mechanism of action of newly developed drugs could be presumed to have the putative potential to exhibit off riboswitch structure as analogous compound to bind at the SAM riboswitch. In addition, the gene that have been selected to study riboswitch have not been yet reported to be having such structure at the 5'-end of the mRNA. The constructs have been designed in such a way that the mechanism of killing could be probably assayed by off riboswitch mechanism through reporter assay by cloning the promoter region and the 5'-UTR region of the essential genes of MDR pathogens under whose regulation *lac*Z gene to be cloned to assay β-galactosidase activity.

1.2 Current Studies

The global emergence of AMR that has diminished the effectiveness of existing antibiotics treatments and is creating problems to control the infectious pathogens. This has become a major concern in clinical study as well as for researchers. It is reported that synergistic administration of intravenous colistin, meropenem and ertapenem aids in the treatment of *Klebsiella pneumoniae* carbapenemases (KPC) producers *K. pneumoniae* infections in elderly patient (Caniaux *et. al.*, 2017). But, this strategy could not provide complete solution to combat the resistance problem. Hence, rapid development of new potential classes of antibiotics and new treatment strategy are required.

With this prospect, different pharmaceutical companies are trying to develop and discover the new antibiotics against MDR pathogens and are at research and development pipeline. The recent news saying "A first-in-human study with a new class of antisense oligonucleotide therapeutics showed the ability to target the RNA-silencing drug to the liver, resulting in improved potency and safety at therapeutic doses" is

exciting news that some dramatic approaches have been used (https://www.news-medical.net/news/20190128/New-class-of-antisense-oligonucleotide-therapeutics-tested-in-humans.aspx).

In January 30, 2019, "The 15-valent pneumococcal conjugate vaccine V114 has received Breakthrough Therapy Designation from the U.S. Food and Drug Administration (FDA) for the prevention of invasive pneumococcal disease (IPD) caused by the vaccine serotypes in pediatric patients 6 weeks to 18 years of age. V114 is also under development for the prevention of IPD in adults. Both indications are currently being studied in Phase 3 clinical trials" (https://www.drugs.com/clinical_trials.html). Similarly, in February 2019, Food and Drug Administration (FDA) approved the drug Egaten (triclabendazole)againstfascioliasis (liver fluke infestation) (https://www.centerwatch.com/drug-information/fda-approved-drugs/).

Researchers are applying various strategy to launch the new antibiotics into the market. Since, conventional techniques take about fifteen years to develop a single antibiotic*, in silico* approaches have been recognized as efficient and cost effective method to develop and screen the drug target.

CADD is time effective dry laboratory approach to discover various therapeutics agents. It has been successfully used in development of angiotensin-converting enzyme (ACE) inhibitor captopril (approved in 1981) as an antihypertensive drug (Talele *et. al.*, 2010) and noble transforming growth factor (TGF)- β1 receptor kinase inhibitors. This high throughput screening method can be applied to identify the novel drug target in clinical medicine and medicinal biochemistry too. In addition, natural products also have been explored (Newman and Cragg, 2012). Among them, soil dwelling *Streptomyces* can be used by feeding various substrates in their growth media such that they can act as positive inducer for antibiotics production.

Thus, the present study includes both CADD and isolation and screening of antimicrobial producing *Streptomyces* strains to support the wet laboratory experiment for the development of new leads against MDR pathogens. The putative mechanism of killing could be studied after promoter cloning of the essential genes of MDR pathogens whose regulation can be studied by β galactosidase reporter assay. The riboswitch mechanism could also be elucidated, if present.

1.3 Hypothesis

1.3.1 Null Hypothesis

Noble antibiotics could not be produced against MDR strains by the isolated *Streptomyces.*

1.3.2 Alternative Hypothesis

Noble antibiotics could be produced against MDR strains by the isolated *Streptomyces.*

1.4 Objectives

1.4.1 General Objective

- Development of antibiotics against WHO prioritized MDR pathogens based on CADD aided compound feeding to isolated *Streptomyces* sps.

1.4.2 Specific Objectives

- To perform molecular docking against the drug target.
- To screen Streptomycetes strains isolated from different soil samples of Nepal.
- To perform Antimicrobial assay of crude extracts from putative Streptomycetes.
- To characterize the antimicrobial positive *Streptomyces* at the molecular level.
- To perform promoter cloning of the essential gene of *Pseudomonas aeruginosa.*
- To perform β galactosidase assay by using reporter gene.

1.5 Rationale

The emerging serious resistance among the MDR pathogens demands new antibiotics to reduce the economic and health burden caused by these pathogens. The present study focuses on the identification of noble potential antibiotics against WHO prioritized pathogens with specific mechanism of inhibition of the drug target.

1.6 Scope of the Study

The study focuses to identify potential lead molecules among the Indole derivatives and kinase inhibitors against MDR pathogens. Also, robust screening of antimicrobial assays such as resazurin assay have been performed. In addition to this, promoter cloning of essential gene of pathogen have been performed to understand the unknown riboswitch mechanism of *cob*A gene.

2 LITERATURE REVIEW

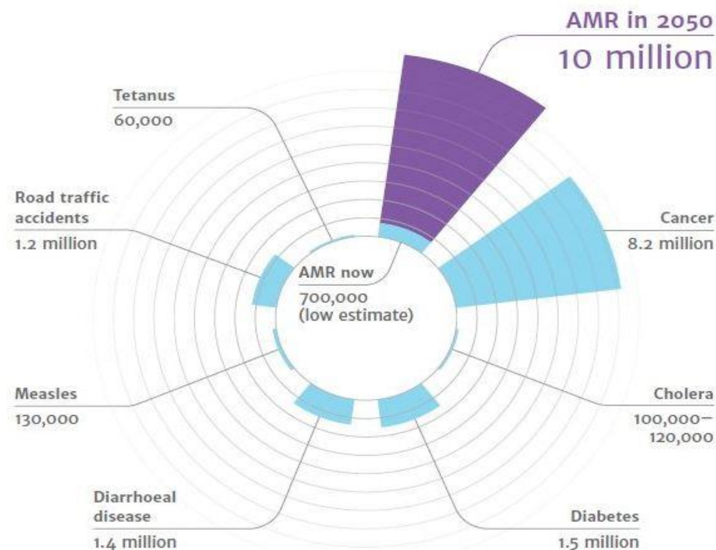
2.1 Literature review of antimicrobial resistance

The increasing emergence of antimicrobial resistance among the most threatening bacterial pathogens is currently recognized as a major global pandemic health threat affecting humans worldwide (Munita *et.al.,* 2016).

According to World Health Organization (WHO),(http://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance) antimicrobial resistance (AMR) is the mechanism by which the microorganisms exhibit resistance against at least three existing antimicrobial drugs (antibiotics, antifungals and antivirals). Thus, management of these microorganisms sometimes referred as superbugs, further increases the cost of health care. It threatens the effective prevention and treatment of increasing range of infections that are caused by bacteria, parasites, viruses and fungi. Therefore, WHO in 2019 has named antibiotic resistance as top five most important public health threats of the 21st century indicating that the world is heading towards a post-antibiotic era, the era where antibiotics would be ineffective and new antibiotics are not found and developed.

The remarkable and continuous increasing number of bacterial as well as fungal pathogens resistance to the existing drugs discovered till date is alarming. The intrinsic and extrinsic mutations and different parameters also govern for increasing resistance to the pathogens. According to Centers for Disease Control and Prevention (CDC), more than two million people are agonized with multi drug resistant pathogens in the U.S. Among them, at least 23,000 are dying as a result of that infections caused by these pathogens (CDC, 2013).

The new resistance mechanisms against various pathogens are emerging (Ferri *et.al.,* 2015) in every corner of the world in these days. Among them, MDR-Tuberculosis (TB) was one that possessed a great threat for health security in 2017. WHO makes an estimate of about 558,000 new cases of MDR-TB having resistance against rifampicin (first line drug), hence accounts for nearly 50 % of the global TB cases (WHO). It was found that 8.5% of MDR-TB were extensively drug–resistance TB (XDR-TB) (resistant to isoniazid, rifampicin plus any fluoroquinolone) in 2017 (CDC, 2017). In addition, according to surveillance report of European Centre for Disease Control (ECDC, 2018), about 33,000 people die each year due to multidrug resistant *Klebsiella pneumoniae*, *Escherichia coli, Pseudomonas aeruginosa* and others. It also shows that 39% of the disease burden is caused by carbapenem and colistin resistant bacteria which are regarded as last line of antibiotics (ECDC report).



Source: The Review on Antimicrobial Resistance, Jim O'Neill (2016)

Figure 1: Deaths attributable to AMR every year compared to other major causes of death

Moreover, extensively drug-resistant (XDR) and pandrug-resistant (PDR) pathogens have also been well defined and categorized by CDC and ECDC to describe resistance patterns deviated from the Clinical Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Magiorakos *et.al.,* 2012). They report that XDR pathogens are non susceptible to at least one antimicrobial agent in all but of two or fewer categories where as PDR pathogens are non-susceptible to all agents in all antimicrobial categories indicating alarming nature of resistance.

WHO declared that by 2050, around 10 million people will be affected from antimicrobial resistance that will be more than that of death due to cancer (Jim O'Neill, 2016). This might be because of exponential growth of new strains of multidrug resistant organisms worldwide. Hence, it is globally presumed that this condition will be a catastrophic threat.

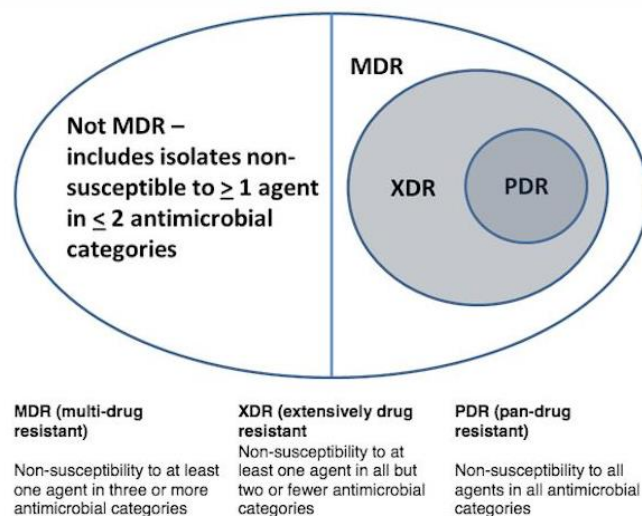


Figure 2: Relationship of PDR, XDR and MDR to each other (Magiorakos *et.al.,* 2012)

World wide spread of multidrug resistance 'pandemic bacterial strains' and the displacement of naturally existing competitors tend to succeed regularly. Beyond this, various factors such as misuse of antibiotics in agriculture, animals and environment (Prestinaci *et.al.,* 2015), self prescription by patients, insufficient doses and use of antibiotics for viral diseases also promote for spreading this threat. Similarly, resistance also emerges instantly after noble drugs had been launched into the clinical market (Nübel, 2016). Therefore, to address this issue, WHO (February 27, 2017) has characterized the resistant bacteria into three priorities on the basis of need of new antibacterial drugs to treat the serious infections caused by them.

WHO priority pathogens list for Research and Design of new antibiotics

Priority 1: Critical

- *Acinetobacter baumannii*, carbapenem-resistant
- *Enterobacteriaceae*, carbapenem-resistant, ESBL-producing
- *Pseudomonas aeruginosa*, carbapenem-resistant

Priority 2: High

- *Enterococcus faecium*, vancomycin-resistant
- *Staphylococcus aureus*, methicillin-resistant, vancomycin-intermediate and resistant
- *Helicobacter pylori*, clarithromycin-resistant
- *Campylobacter* spp., fluoroquinolone-resistant
- *Salmonellae*, fluoroquinolone-resistant
- *Neisseria gonorrhoeae*, cephalosporin-resistant, fluoroquinolone-resistant

Priority 3: Medium

- *Streptococcus pneumoniae*, penicillin-non-susceptible
- *Haemophilus influenzae*, ampicillin-resistant
- *Shigella* spp., fluoroquinolone-resistant

Source: WHO (February 27, 2017)

2.2 Mechanism of Antibiotics Resistance

There are various reasons for overcoming by the pathogens to the existing antibiotics. Though antibiotics are needed for the treatment of complex medical approaches such as organ transplantation, surgical approaches (Munita and Arias, 2016) for management of infections, but various reasons cause them to be ineffective. According to them, mechanism of antimicrobial resistance can be categorized into two main categories.

2.2.1 Genetic basis of antimicrobial resistance

The epigenetic and phenotypic variation (Nishikawa and Kinjo, 2014) of bacteria allows them to respond to the presence of antibiotic molecules that might be risk for their existence. They might produce resistance by sharing the same environmental niche with antimicrobial producing organisms. Thus, have evolved evolutionary resistance mechanisms intrinsically to endure the effect of the harmful antibiotic molecules.

Bacteria use two major genetic approaches to adapt to the antibiotic rich environment; **i)** mutations in gene(s) associated with the target gene against which mechanism of action of the antibiotics occurs, and **ii)** possession of resistance coding foreign DNA through horizontal gene transfer (HGT) from the same niche.

Mutations in gene(s)

The development of mutations in certain genes of bacteria alter the activity and target site (Lambert, 2005) of the drug such that they are able to survive in the presence of antibiotic molecules. The resistance through genes mutations allows them predominate the niche over the sensitive bacterial population.

Mutations of the bacterial gene amend the antibiotic action via one of the following mechanisms: i) modifications of the antimicrobial target to decrease the affinity for the drug, ii) Declination in the drug uptake rate, iii) Stimulation of efflux mechanisms against harmful molecule, iv) Changes in metabolic pathways by modulating the regulatory network.

Horizontal Gene Transfer (HGT)

The process of acquiring the foreign unrelated DNA sequences by bacteria and insert them into their genome is called Horizontal Gene Transfer. Since these genes are not inherited from the parent DNA molecule, also known as, "Lateral Gene Transfer" (Syvanen, 1985). It increases the evidence of transfer and driving of antibiotic resistant genes among bacteria in the same ecological niche (Harris *et. al.*, 2010).

Horizontal gene transfer occurs through three primary mechanisms mainly transformation, conjugation and transduction (Gyles and Boerlin, 2013).

The process of transformation involves acquisition of a whole or a piece of foreign DNA from their niche and incorporating it to the genomic material of the recipient cell. It was first observed by the British bacteriologist Fredrick Griffith (in 1928), and discovered the mechanisms that avirulent strain of *Streptococcus pneumoniae* could be infectious after being contacted to heat-killed virulent strains. Further, this process was identified in 1944 (Avery *et.al.*, 1944). It permits the organism to take up extracellular naked DNA, when a cell is in a state of competence (Chen and Dubnau, 2004). The presence of specific membrane proteins, components of the type IV pilus (T4P) and type II secretion systems (T2SSs) help in the import of foreign DNA into the cytosol (Russel, 1998; Sandkvist, 2001).

Conjugation was first discovered by Tatum and Lederberg (1947) during recombination of *Escherichia coli*. In general, important resistance genetic information is transferred via chromosome but mobile genetic elements (MGEs) such as F-plasmids and transposons also act as vehicles for development of antimicrobial resistance (Manson *et. al.*, 2010). In conjugation the direct cell contact between two species by bridge formation (Gyles and Boerlin, 2013) occurs and is mostly predominant in the gastrointestinal tract of human beings under the treatment after infections.

In addition to conjugation, some of the resistant markers are transferred through transduction. The genetic materials are transferred from one bacterium to another via bacteriophages (bacterial viruses). After the viral infection to bacteria, virus traps the replication machinery of host and replicates its DNA or RNA. During this, occassionally, the phage might pick up short segments of host DNA thus allowing host genes to be circulated to another bacterium in another round of infection (Gyles and Boerlin, 2013). The discovery of transduction was traced back in 1952 when two scientists Joshua Lederberg and Norton Zinderwere studying the recombination in *Salmonella* Typhimurium.

Besides these primary mechanisms, other factors for dissemination of resistance genes are governed by other systems too. The Integrons (site-specific recombination system) recruit open reading frames by forming mobile gene cassettes (Hall and Collis, 1995) and provide efficient mechanism for incorporation of new resistant genes into new bacterial chromosomes. This robust strategy of genetic recombination also ensures their expression in the hosts including antimicrobial resistance trait.

2.2.2 Mechanistic basis of antimicrobial resistance

Bacteria have developed advanced mechanisms against different drugs to avoid suppressing and killing by antibiotics. Resistance can also be achieved by modification of various essential biochemical pathways. The mechanistic emergence of antimicrobial resistance occurs through the following processes.

2.2.2.1 Modifications of the Antibiotic Molecule

2.2.2.1.1 Chemical or enzymatic alterations of the antibiotic molecules

The production of certain enzymes by the bacteria bring chemical changes to antimicrobial compounds. In some cases, the protein synthesis inhibition occurs at ribosome level (Wilson, 2014) of the bacteria. The modification of enzymes involved in different biological pathways like acetylation, phosphorylation and adenylation causes steric hindrance to antimicrobial compounds that diminishes the potency of the antibiotics towards its target. One classic example involves chemical modification of chloramphenicol (inhibits protein synthesis by binding with 50S ribosomal subunits) by CATs (chloramphenicol acetyltransferases) enzyme by group transfer in both Gram negative and Gram positive bacteria (Shaw, 1983). Type A chloramphenicol modification confers high level of resistance where as Type B results low level of chloramphenicol resistance (Schwarz *et. al.,* 2004)

2.2.2.1.2 Chemical modification of the antibiotic molecule

One of the defense strategy of bacteria against antimicrobial compound is to produce various enzymes for the chemical modification of antibiotics. The enzyme β- lactamases produced by various gram negative *Enterobacteriaceae* destroy the amide bond of the β-lactam ring of β-lactam antibiotics (Munita and Arias, 2016) by hydrolysis thus rendering the antibiotics less effective or ineffective.

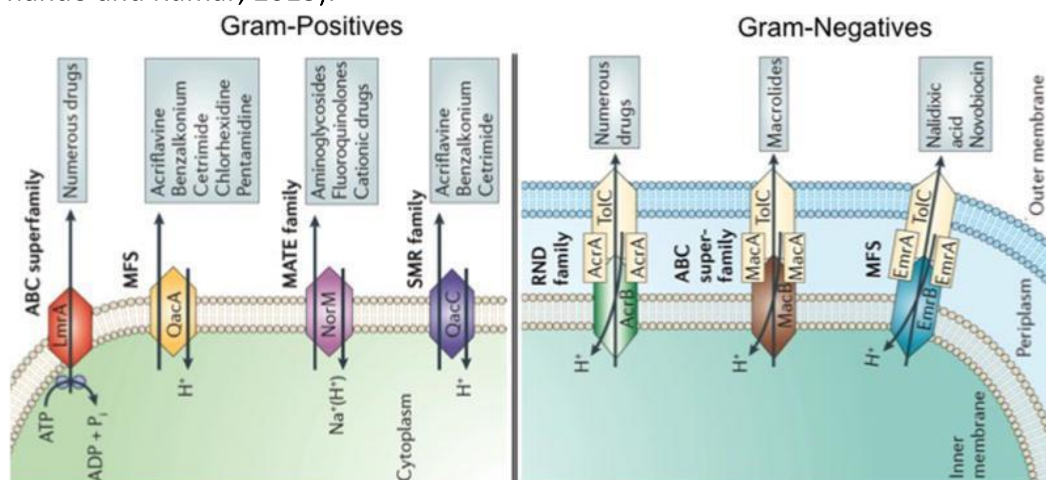
2.2.2.1.3 Decreased Antibiotic uptake and Efflux

2.2.2.1.3.1 Decreased permeability

Hydrophilic antibiotics (β-lactam, tetracyclins and some fluoroquinolones) require porins to cross the membrane barrier (Pagès *et. al.*, 2008) where as hydrophobic antibiotics (macrolides, rifamycins, aminoglycosides) use the transport system via the outer membrane bilayer (Delcour, 2009). So, these processes are predominantly affected by alteration in the outer membrane of bacteria. Hence, the development of the resistance system by hindering the antibiotic from reaching their target by lowering the rate of the uptake of the antimicrobial molecule in making the bacteria more resistance (Miller *et. al.*, 2014).

2.2.2.1.3.2 Efflux pumps

Active efflux pump is a mechanism which is responsible for flushing out of antibiotics and other toxic compounds from the bacterial cells. It can bestow the bacteria for antibiotic resistance at high level. The chromosomal and plasmids mediated efflux pump (Machuca *et.al.,* 2014) contributes to both natural and acquired resistance by allowing the resistance for simple and fast spread of efflux associated genes between unrelated species also. There are five major families of efflux transporters. They are the major facilitator superfamily (MFS) (Pao *et. al.*, 1998), the small multidrug resistance (SMR) family (Chung and Saier, 2001), the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily (Choi, 2005), the multidrug and toxic compound extrusion (MATE) family (Kuroda and Tsuchiya, 2009) and the resistance-nodulation-division (RND) family (Fernando and Kumar, 2013).



(Munita and Arias, 2016)

Figure 3: Representation of five different types of efflux pumps in Gram-positive and Gram negative bacteria

2.2.3 Changes in target sites

Another approach to avoid the antimicrobial activity by bacteria is development of different mechanism by interefering with their target sites. Most common strategy includes target protection and modification of the target sites.

2.2.3.1 Target protection

The reserved stratagem of antibiotic binding sites by bacterial enzymes and proteins prevents antimicrobial compounds from binding to the required sites thereby drawing off its activity. One of the target protection mechanism includes tetracycline resistance determinants TetM and TetO, both of which compete with tetracycline to bind for the same ribosomal space and binding site as well (Donhofer *et. al.*, 2012; Li *et. al.*, 2013).

2.2.3.2 Modification of the target sites

The resistance mechanism makes almost all of the antibiotics ineffective for the treatment of infectious diseases by modifying the target sites through i) enzymatic alterations of binding site of antibiotics, ii) mutations in the target sites encoding genes and iii) substitution of the original target. Some bacteria can evolve new drug targets having similar biochemical functions and pathways as the original but are unfamiliar to the existing antibiotics. The acquisition of an exogenous Penicillin Binding Protein (PBP2a) by Methicillin resistant *Staphylococcus aureus* in addition to PBP is the most relevant clinical cases (Stapleton and Taylor, 2007). Similarly, macrolide resistance due to methylation of the ribosome activated by an enzyme that codes the **erm** genes (***e***rythromycin ***r***ibosomal ***m***ethylation) is the result of modification of the target sites (Fyfe *et. al.,* 2016). Due to dimethylation of an adenine residue, binding of antibiotic to its required target is weakened and bacteria become more resistance against antibiotics.

2.3 Other mechanisms of resistance

2.3.1 Quorum Sensing

The physiological adaptation of microorganisms depends upon the changes in the surrounding environment. Such adaptation mechanisms involve the ability of cell to cell communication, called Quorum Sensing (QS), in a microbial population (Waters and Bassler, 2005). After required threshold of population is reached, it enables bacteria to coordinate expression of various associated behaviors such as, swarming motility, virulence factors, antibiotics production, drug resistance, and biofilm formation. The mechanism of quorum sensing is similar in both Gram positive and Gram negative proteobacteria.

It is also essential for regulation of intra and inter-bacterial genes and for maintaining micro as well as macrocolonies (Waters and Bassler, 2005) attached together by extracellular polymeric substances (EPS) for biofilm formation. The use of wide range of auto-inducers (act as signaling molecules) such as Acylhomoserine lactones (AHLs), fatty acids, oligopeptides contribute for microbial density to be attached in the same microbial community (McDougald *et. al.,* 2007). This increases the probability of establishing the infections and spreading them by maximizing the pathogens survival. The bacteria sense the quorum in microbial community and signal transduction cascade is activated resulting in the production of associated virulence factors and triggers biofilm formation (Castillo-Juárez *et. al.*, 2015).

Table 1: Examples of Bacterial Quorum Sensing Systems and their Controlled Social Traits (Li and Tian, 2012)

Microorganism	Major Signal Molecules	Regulatory System	Group –Derived Benefits
Bacillus subtlis	ComX CSF (PhrC) Phr A,-E, -F, -K, -H	ComP/ComA Rap proteins	Competence, sporulation, biofilm formation, antibiotic production
Myxococcus Xanthus	A-signal C-signal	SasSRN	Fruiting body formation or Sporulation
Pseudomonas Aeruginosa	3O-C12-HSL C4-HSL	LasI/LasR RhlI/RhlR OscR (orphan	Structured biofilm formation, virulence factors
Staphylococcus Aureus	AIP-I, AIP-II, AIP-II, AIP-IV	AgrC/AgrA	Biofilm formation, virulence Factors
Streptococcus Pneumonia	CSPs	ComD/ComE	Competence, fratricide, biofilm formation, virulence
Vibrio harveyi	HAI-1, CAI-1 AI-2	LuxLM/LuxN LuxP/LuxQ	Bioluminescence emission, Symbiosis

QS regulates the biofilm associated EPS matrix and efflux pump genes for dessimination of multidrug resistance against different antibiotics molecules. For example, when cell population of *Pseudomonas aeruginosa* is reached upto critically high, QS mechanism is activated, through which auto inducer C4–HSL is generated. Thus, inducing the expression of mexAB-orpM operon or inactivating MexR repressor for enhanced transcription of MexAB-OrpM efflux pump (Subhadra *et.al.,* 2016).

2.3.2 Biofilm Formation

Bacteria also develop unique mechanism to escape from the antibiotics such that they can develop resistance towards them. Biofilms are closely associated well organized multicellular microbial communities (Hall and Mah, 2017) that either adhere to biotic or abiotic sessile surfaces by producing EPS matrix. The EPS is made up of macromolecules such as carbohydrates, nucleic acids and proteins. They enable bacteria to defend against their adverse environment, thus assuming increased microbial resistance to antimicrobials (Flemming *et.al.*, 2016).

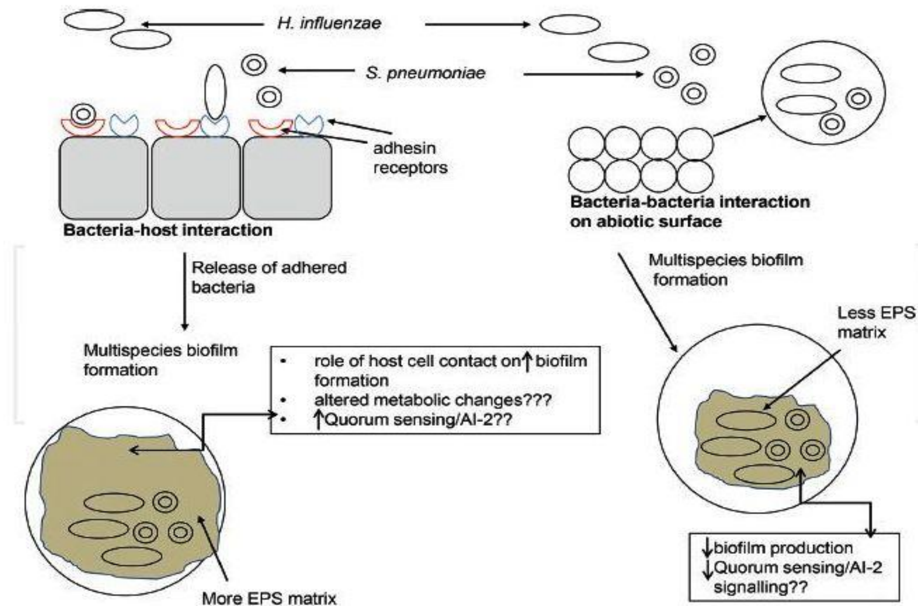


Figure 4: Mechanisms involved in biofilm formation when different species are in close proximity (More biofilm is produced by Mixed bacterial species. The figure shows that increased biofilm production by *H. influenzae-S. pneumoniae* in a multispecies environment when in contact with human epithelial cells (left panel). Theright panel shows a decreased production of biofilm when grown on anabiotic surface (tissue-culture polystyreneplate). The potential role of host cell contact, possibility of any altered metabolic changes or increased quorum sensing(QS)/autoinducer-2 (AI-2) signalling, upon host-cell interactions could hold key to further elucidate the mechanisms involved in increasing multispecies biofilm formation.)

The mechanisms that arise antimicrobial resistance in biofilm producers include i) altered growth kinetics of biofilm producers, ii) delayed uptake of antibiotics through matrix of biofilm, and iii) other morphological and physiological changes by biofilm production (Donlan and Costerton, 2002). Biofilms exhibit remarkably expanded resistance to antimicrobials and various other environmental stresses. Because of this, they are capable of surviving and evading the host immunity by providing physical protection with the help of extracellular matrixes (Van Gerven *et. al.*, 2018).

During stress, and nutrient deprived condition, biofilm producers have limited growth and antimicrobial uptake rate becomes slow. During this time, slow growth activated gene (rosS) is activated that also promotes more biofilm production (Adams *et.al.,* 2002). Similarly, the antibiotics diffusion pathway is altered and retards the diffusion rate of antibiotics, thus, antibiotics become less effective in the biofilm surface (Donlan and Costerton, 2002).

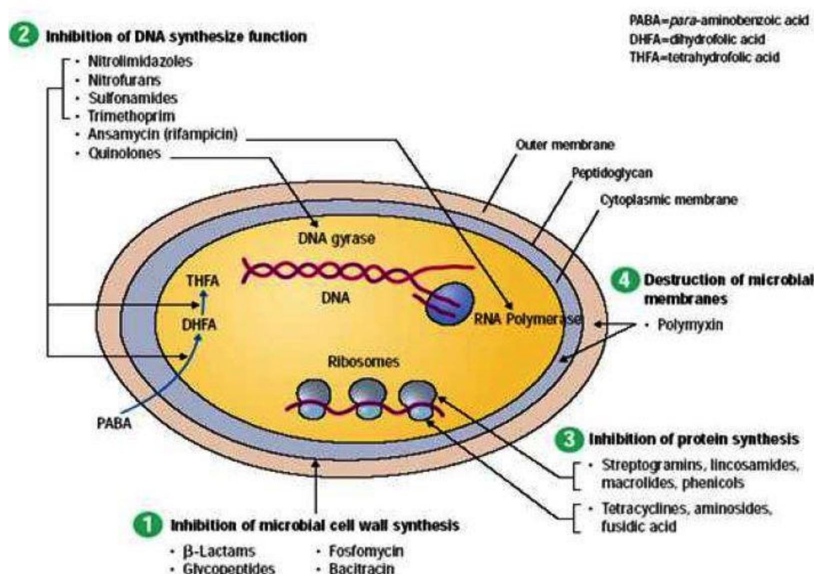
2.4 Antibiotics and mode of action of developed antibiotics

Antibiotics are the type of therapeutic agents synthesized by microorganisms or later modified chemically that are active against other microorganisms at low concentration. They act against both Gram's positive and Gram's negative bacteria according to their spectrum of actions. Hence, they can be categorized into broad and narrow spectrum.

Broad spectrum acts against both Gram's positive and Gram's negative bacteria where as narrow spectrum acts towards Gram's positive or Gram's negative bacteria only.

Similarly, on the basis of mechanism of action, antibiotics are further categorized into bacteriostatic and bactericidal. Bacteriostatic antibiotics inhibit the growth and replication of bacteria but not kill so they limit the population size which further is cleared by host immune system. They include tetracyclins, sulphonamides, macrolides. But, bactericidal antibiotics induce cell death and they include penicillins, carbapenems, vancomycin etc (Nemeth *et. al*., 2015).

In general an interlinkage between an antibiotic and its target site of the bacterial cells causes inhibition of cellular function(s) in leading to bacterial cell death. From the knowledge of drug target interaction (Cheng *et. al.,* 2012) and side chain alteration of the existing drugs, many effective antimicrobials have been developed by various pharmaceuticals with no side effects on host cells. The basic mechanisms of existing antibiotics to either cause cell death or to inhibit bacterial growth are as follows.



(Chakraborty, Pramanik, & Roy, 2012)

Figure 5: Mechanism of action of antibiotics

2.4.1 Inhibition of cell wall synthesis

The outer peptidoglycan layer (PG) of bacterial cells is composed of covalently cross linked different glycan chains attached with peptides by penicillin binding proteins. This stabilizes frangible protoplast membrane by providing mechanical strength and helps for maintaining osmotic pressure (Godlewska *et.al.*, 2009).

Moreover, binding of Glycopeptides to D-Ala-D-Ala dipeptide ends of peptidoglycan pentapeptide moiety blocks transglycosylation and transpeptidation steps of cell wall biosynthesis (Mc Dermott *et.al.,* 2003) leading to osmotic pressure. Similarly, β- lactams inhibit cell wall synthesis (Figure 6) by binding to anabolic enzymes causing alteration in cell shape, size and finally induces cellular stress with cell lysis (Cho *et.al.*, 2014).

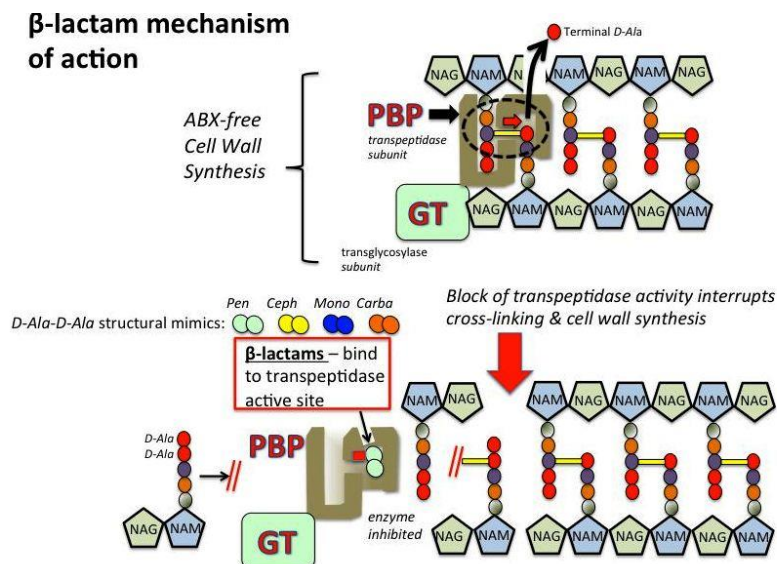


Figure 6: Mode of action of β- lactams antibiotics
(Source : http://tmedweb.tulane.edu/pharmwiki/doku.php/antibiotic_pharmacology)

2.4.2 Inhibition of nucleic acid metabolism

Antibiotics that inhibit DNA or RNA synthesis either interfere with nucleotides or nucleic acid biosynthetic pathway. Compounds that block enzyme function for nucleotide synthesis either act on DNA topoisomerases (*gyr*A, *gyr*B, *par*C, *par*E) or RNA polymerase (*rpo*B). An example includes rifampicin, antituberculosis drug, that interacts with bacterial RNA polymerase inhibiting the initiation process of only bacterial transcription (Piddock *et.al.,* 2000).

Sulfonamides bind with active site of dihydropteroate synthetase and synthesis of nucleotide precursor is blocked. Since, they are structural analogues of *p*-aminobenzoic acid (PABA), substrate of tetrahydrofolic acid synthesis, they compete for that enzyme (Mc Dermott *et. al*., 2003) to inhibit folic acid synthesis. Similarly, fluoroquinolones act by converting the target of two correlated enzymes, DNA gyrase and DNA topoisomerase IV into toxic enzymes. Hence, bacterial chromosomes are fragmented due to toxicity of enzymes (Aldred *et.al.,* 2014).

2.4.3 Inhibition of protein synthesis-translation

Protein synthesis is required for metabolic and physiological processes that ultimately results in multiplication and survival of bacterial cells. Microbial 70S ribosome consists of 30S and 50S ribosomal subunits. Each subunit is again divided into small ribosomal proteins and RNA units such as 5S and 23S rRNA in 50S subunits and 16S rRNA in 30S subunits (Schuwirth, 2005). On the basis of this division, protein synthesis inhibitors can be divided into 30S inhibitors and 50S inhibitors.

The 30S inhibitors like tetracyclins and aminoglycosides interact with 16S rRNA near the A-site that leads to misreading and premature termination of translocation of tRNA (Davies and Davis, 1968). Tetracyclines reversibly bind to 30S subunit of bacterial ribosome to block the attachment of acyl-tRNA to the ribosomal acceptor A–site

(Chopra and Roberts, 2001). In addition, a large pore is formed for penetration of antibiotics when aminoglycosides (positively charged) attach to the outer membrane (negatively charged) of the bacteria (Kapoor *et. al.*, 2017). The 50S inhibitors such as macrolides (erythromycin), oxazolidinones, streptograins hinder protein synthesis by preventing either protein translation initiation or peptidyl-tRNAs translocation (Cocito *et.al.*, 1997). Thus, resulting in a premature separation of incomplete polypeptide chains.

2.4.4 Alteration of bacterial cell membrane architect

The cell membrane forms an effective diffusion barrier for regulation of flow of molecules. But, the drugs like colistin and polymixin B have vital role for the membrane disruption such that other toxic substances enter the cell. This leads to rapid permeability changes, inhibition of specific metabolic pathways and ultimately selective cell death of bacteria (Poirel *et. al.*, 2017).

2.5 Virtual screening and molecular docking

Identification of drug target against bacterial pathogens is not an easy task. The computational methods provide a robust way to screen the molecules that can be used as scaffold of drugs. Computer Aided Drug Design (CADD) is cost effective and fast way of drug design process by which new drugs with unique target can be discovered (Kapetanovic, 2008). Computational methods are seen as most effective to observe the affinity, stability and selectivity of the protein based therapeutics.

The drug discovery cycle starts with target lead identification against which the drug is to be developed to the last stage of clinical trials Thus, is too long lengthy process and takes about ten to fifteen years (Kraljevic *et.al.,* 2004) and costs approximately about 2.6 billion US dollars. Since, not a single new antibiotics has been registered after 1984 ("A Scientific Roadmap for Antibiotic Discovery,"2016), the new age is searching the robust solution of antimicrobial resistance problem. Therefore, this demands the virtual screening method into play where there is limited time and resources.

Virtual screening (VS) is a new and robust computational technique applied for the *in silico* drug discovery process by filtering and assessing large number of chemical and/or compounds from combinatorial libraries. It reduces the number of real compounds to be selected as drug target and thus the time taken for evaluation by High Throughput Screening (HTS) in less cost. With the advent of new technologies, it provides and generates the more accurate data for selection of target compounds (Lionta *et.al.*, 2014). Therefore, it has become a cornerstone in modern rapid drug discovery and development process.

VS can be categorized into two methods that includes structure-based and ligand-based methods. The ligand–based method is employed when active ligand molecules are known but structural information is unavailable for the required targets (Meng *et.al.,* 2011). However, structure-based methods involve the close interaction between the

ligands and a target expecting the goal to sort out ligands with stronger binding affinity to the targets. Structure based drug design includes the molecular docking which has been regularly used since past three decades (Kuntz *et.al.,* 1982) to identify the best and novel drug targets.

2.5.1 Molecular Docking

Molecular docking allows to find the correct position and conformation of a ligand and its target receptor. During the interacting process, binding of a small lead molecule to its protein target is influenced by environment of protein that distributes charge over the ligands, presence of surrounding water molecules as well as mobility of both proteins and ligands. The various docking programs include DOCK, AutoDOCK, GOLD and others too (Alonso *et.al.,* 2006) according to the need of identification of targets.

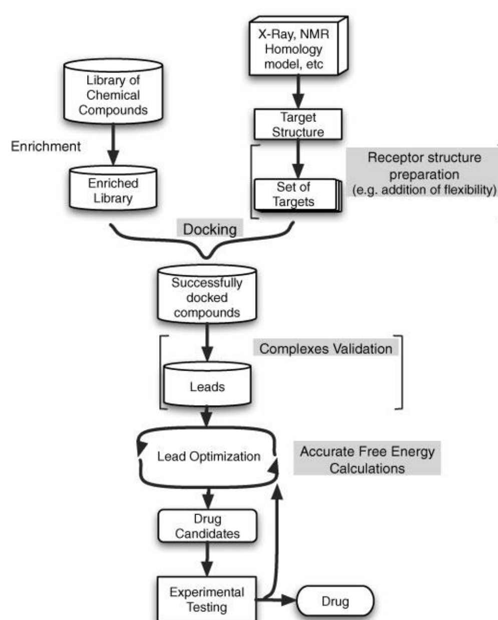


Figure 7: Schematic representation of the drug-design process (when the structure of the protein target is known or can be modeled) (Alonso *et.al.,* 2006)

2.5.2 Steps Involved in molecular Docking

i) Target Preparation

It is regarded as the important stage of the virtual screening. This acts as data bank of 3D structure of the target protein. If that structure is available then it is retrieved for those proteins that have the capacity to modify the disease and this phenomenon must have to be identified where target indentification becomes more important. If the 3D structure of the target protein is not available in the rcsb protein data bank (https://www.rcsb.org/), a reliable homology-modeling method is applied to construct it using PHYRE2 server (http://www.sbg.bio.ic.ac.uk/~phyre2) (Cerqueira *et. al.,* 2015b). The structure on the protein data bank is rich in cofactors, water molecules, activators, metal ions and ligands along with some protein subunit also. Thus, the binding site of the native ligand is crucial to develop any drug candidates.

ii) Binding Site Identification

Retrieval of 3D structure allows the evaluation of the druggability score that is the ability to understand the tendency of the receptor for efficient binding to the molecules having properties like drug. In this step, the molecule's capacity to cooperate with a specific binding pocket is determined that allows the easy positioning of the drug candidate in the binding sites after the ligands are co-crystallized clearly with the target protein (Cerqueira *et. al.,* 2015b). If the 3D structure with the ligand is not available then the binding site has to be determined.

iii) Compound Database Preparation

It is the database of the compounds that contains different drug-like small molecules with essential properties like stability and solubility, suitable functional group to react with biological targets. In addition, it is avoid of unwanted toxic moieties (Lionta *et.al.,* 2014). "Lipinski Rule of Five" (Lipinski *et.al.,* 2001a) is applied to confirm 'druglikeness'. It clearly states that,

- Molecular weight < 500
- Lipophilicity (logP) < 5
- Hydrogen bond donors < 5
- Hydrogen bond acceptors < 10

However, some drugs don't obey this rule and the extensions of this rule are produced.

iv) Filters

Filters are developed in order to enhance the quality of the database. The filters such as The Pan Assay Interference Compounds and the ALARMNMR have compound which are chemically reactive and assay-interfering and also not recognized by toxicophoric filters (Peach and Nicklaus, 2009). The filtering desired pharmacological and adsorption, distribution, metabolism, excretion and toxicological (ADMET) properties enhances the rapid drug design process. Chembioserver is used to filter a library of compound from the database (Baell and Nissink, 2018).

v) Library Design

After the compounds are screened by ADMET tests, a customized library is created but in some cases, several compound libraries are found. The libraries (T. Cheng *et.al.,* 2012) are divided into following categories:

- Generic virtual high throughput libraries, comprising large sets of compounds.
- Diversity-oriented libraries, comprising highly chemically diverse compounds.
- Target-oriented libraries, preparing by exact required target in mind.
- Molecular property diversity libraries are created by exact molecular property profiles.

vi) Docking and Scoring

It is the process of analyzing the binding affinity of one molecule to another to form a stable complex compound by predicting correct position and alignment by using

computational method (Hu *et.al.*, 2018). The main purpose of docking is to find out the conformation and the orientation to minimize the energy of the entire system. The energy minimization depends upon two programms listed as search algorithms (Dias and de Azevedo, 2008) and scoring function (Huang *et.al.*, 2010).
Search algorithm provides the efficient ways to position the ligand to the receptor that aids in minimization of the energy. In contrast, scoring function provides the rank of docking score according to the efficient binding affinity of the ligand and the receptor molecule and arranged in the order of the high scores after molecular docking (Robertson and Varani, 2006). It majorly acts for the comparision of the binding free energy of thousands of the protein-ligand complexes in the data base. The docking results should be differentiated to get true binding affinities and the interaction of the protein-ligand score signifies thermodynamics of bond formed after complex formation.

vii) Post Processing

After the docking results are obtained, post-processing is required to minimize the experimental errors and make the experiment more efficient and reliable. Sometimes, the docking results having high scored compound would have defective hydrogen-bonding networks, intra-ligand steric clashes, perverse and twisted amides and so on (Wichapong and Sippl, 2012).

2.6 Indole and its derivatives

Various organic compounds and their derivatives are used as scaffold for drug discovery because of their wide range of inhibitory action against bacteria, virus, fungi and other parasites. Indole is an aromatic, bicyclic organic compound which consists of benzene ring connected to a pyrrole ring with florid smell. It occurs naturally or can also be generated by bacteria from their key metabolic pathways. In addition, it also acts as signal molecule (Kim and Park, 2015) to spore formation and regulate the bacterial physiology.

It is biosynthesized via shikimate pathway in plants and bacteria. They produce indole as major product from tryptophan by the use of enzyme tryptophanase (TnaA) with pyruvate and ammonia as byproducts. The indole concentration might reach upto 5mM in the extracellular medium (Kim and Park, 2015) of the cell that is uptaken by bacterial membrane. Therefore, indoles act as toxic compounds when expressed in bacteria by using higher concentration than this because of their potent therapeutic capacity. Furthermore, one single compound of indole produces one indigo with one indirubin both having two moieties with same replacing group. Moreover, indigoids and isatin are produced when human cytochrome P4502A6 is present (Wu *et.al.,* 2005).

Figure 8 : Synthesis of indole through tryptophan (Wu *et.al.,* 2005)

Table 2 : Lists of the 1H-indole tested against the bacteria as possible drug compounds (Wu *et.al.,* 2005)

Substrates for indioid 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	5-Bromo-1H-Indole	1H-Indole
7- Methyl-1H- indole	4-chloro-1H-Indole	1H-Indolyl acetate
4- Fluoro-1H- indole	5-Iodo-1H-Indole	1H Methyl -1H-Indole
5- Fluoro-1H- indole	7- Bromo-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- Methoxyl-1H- indole		
6- Methoxy-1H- indole		
6- Methyl-1H- indole		
4-Nitrole-1H- indole		
Methyl-1H- indole-4-carboxylate		
5-Methoxy- 4-Methyl-1H-indole		
4- Methyl-1H- indole		
1-H- indole-7-carboxaldehyde		
7- Methoxy-1-H- indole		
1H- indole		

2.7 Natural Products

Natural products (NPs), also referred to as secondary metabolites, are the therapeutic agents that are derived from the medicinal plants (Newman and Cragg, 2012) and various soil microorganisms (Jakubiec-Krzesniak *et.al.,* 2018) for millennia. They are well thought of as an essential source of successful drug leads which originate from biodiverse flora and fauna. Out of 1,135 new drugs approved from 1981 to 2010 around 50 % were from NPs (Newman and Cragg, 2012). Therefore, many pharmaceutical companies now a days have reverted to NPs with, focus towards High Throughput Screening (HTS) and synthetic compound libraries (David *et.al.,* 2015) for identification of new drug leads.

In 2008, "Drugs from Nature Targeting Inflammation (DNTI)" consortium was formed (Fakhrudin *et. al.,* 2014) that united scientists to generate multiscope disciplines for drug discovery through NPs. That program aimed to identify and characterize natural products to seek anti-inflammatory activity by the combined use of computational tools, phytochemical analysis, biochemical pathway and different bioactivity models. Natural products exhibit antibacterial, antifungal, anti viral, anti inflammatory, anti cancer as well as anti diabetic properties because of its multidimensional therapeutic aspects. Natural products from the plants, as phytochemicals, have contributed a lot in drug discovery and development that still advance as sufficient source for identification of lead target structure (Newman and Cragg, 2012).

2.7.1 Phytochemicals

Phytochemicals are any non-nutritive biologically active compounds abundant in the plants, generally help them against their competitors, predators and other pathogens. They are produced either by primary or secondary metabolism (Molyneux *et.al.,* 2007) of the plants and also play a role as plant growth promoters. They have been used in medication and as poison since long time. The phytochemical, salicin as analgesic, also acts as anti- inflammatory agent. This was extracted and isolated from the bark portion of the white willow tree. Now a days, it is synthetically produced to become aspirin, over the- counter drug (Sneader, 2000).

There are three major groups of phytochemicals (http://blog.cancernet.co.uk/phytochemicals-types-food-sources/) that includes:-

2.7.1.1 Polyphenols

These are secondary metabolites found in edible as well as non edible plants parts. They are further divided into various classes (Manach *et.al.*, 2005).

Phenolic acids: These include hydroxycinnamic acids and hydroxybenzoic acids that act as antioxidant by retarding oxidative degradation of fatty lipids (El Gharras, 2009). Crude extracts of fruits, vegetables and plant materials are rich in phenolics. Hydroxybenzoic acids include ellagic acid, gallic acid and vanillic acid found in grape seed, blackberries, vanilla, pomegranate, raspberries, tea and others. Hydroxycinnamic acids include P-coumaric acid, caffeic acid, ferulic acid found in cinnamon, coffee, wheat bran, kiwi, blueberries, plums and others.

Flavonoids: These consist of flavones, flavonols, flavanones, flavanols, proanthocyanidins and isoflavones. These are the molecules having phenolic benzopyran structure and occur as glycosides in the plants only (El Gharras, 2009). They provide the colours in flowers, leaves and fruits and act as antioxidant molecules .

- Flavonols: It includes kaempferol and quercetin found in onions up to 1.2 g/ kg fresh weight (Manach e*t. al.*, 2005), leeks, buckwheat, broccoli, apples and others.

- Isoflavones: It includes daidzein, genistein and glycitein found in soyabeans within the range of 580-3800 mg/kg isoflavones fresh weight (Manach *et. al.*, 2005), beans, peas, alfalfa, chick, peanuts and others.
- Flavanones: It includes naringenin, hesperitin found in mint, tomatoes and many citrus fruit, a glass of orange juice might contain 40 -140 mg flavanone glycosides (Manach *et. al.*, 2005).
- Anthocyanidins: It is found in cherries, strawberries, red grapes, blueberries, blackberries, raspberries and others.
- Flavanolols: It includes the compounds silibinin, silymarin, aromadedrin found in milk, red onions, thistle.

Lignans: These are composed of two units of phenylpropane and found in linseed rich in secoisolariciresinol (up to 3.7 g/ kg dry weight) and low quantities of matairesinol (El Gharras, 2009). It includes sesamin, secoisolariciresinol, enterolactone and prominent in cereals, fruits and other fresh vegetables.

Stilbenes: It includes cinnamic acid and resveratrol found in wine (El Gharras, 2009), blueberries, peanuts and others.

2.7.1.2 Terpenoids

The terpenoids also known as isoprenoids are naturally occurring organic compounds derived from terpenes. Out of total natural products, 60% of them are of terpenoids. They serve as an important functions for photosynthetic pigments (carotenoids) in the plants. In addition, they have antimicrobial, antiviral, antiinflammatory, antifungal and immunomodulatory properties (Thoppil, 2011).

They are further categorized into two parts.

2.7.1.2.1 Caretonoides

- Alpha, beta and gamma carotene found in carrots, sweet potato, pumpkin.
- Lutein found in spinach, corn, eggs, oranges , pumpkin papaya, mango.
- Lycopene found in tomatoes, pink grapefruit, papaya.

2.7.1.2.2 Non-carotenoid terpenoids

- Saponins : chickpeas and soya beans.
- Limonene : citrus fruits.
- Perillyl Alcohol : cherries, caraway seeds and mint.
- Phytosterols: natural cholesterols : vegetable oils, grains, nuts, whole grains, legumes.

2.7.1.3 Thiols

These include allylic sulfides, glucosinolates and non-sulfur containing indoles. It contributes to the prevention of cardiovascular disease and cancer. Similarly, thiols activate the enzymes used in liver detoxification and help to block the enzymes that promote the growth of tumors. Additionally, in some case studies, allylic sulfides, have

been observed blocking the harmful activities of the toxins produced by bacterial and viral invaders (Jordan, 2005).

- Glucosinolates: Isothiocyanates (sulforaphane) and dithiolthiones (broccoli, asparagus, cauliflower, radish and mustard).
- Allylic sulfides: Allicin and S-allyl cysteine (leeks, garlic, onions).
- Indoles: Indole-3-carbinol (broccoli).

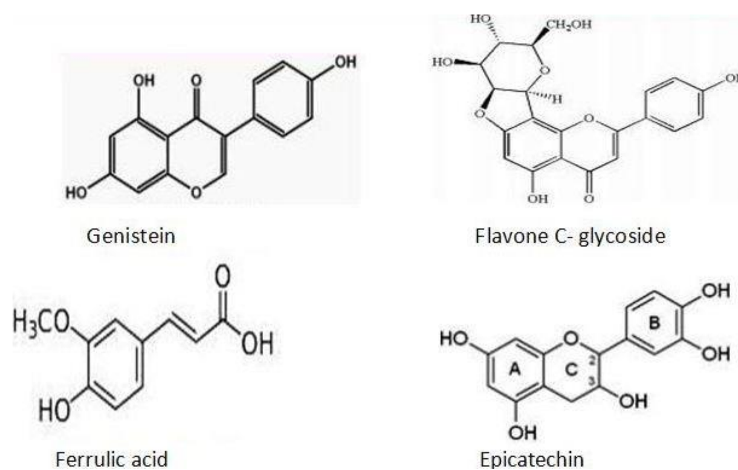


Figure 9: Different structures of phytochemicals (Dw, 2016)

2.8 Lignin

Besides with various phytochemicals from the plant origin can be used as antimicrobial agents, another approach could be the use of lignin and its degradation product to produce secondary metabolites. Lignin is one of the class of highly branched phenolic polymers of methoxylated phenylpropanoids connected via both ether and carbon-carbon bonds (Higuchi, 2003) and accounts for about 30% of total organic carbon. It has found to have noticeable diversity with complex irregular structure (Ralph *et. al.*, 2004). They either act as antimicrobial or act as inducer for more antibiotic production. Since lignin is the major constituents of plant cell wall, it provides structural support, transport nutrients and water inside cell and protect the cell from chemical and biological attacks (Lu *et. al.,* 2017). In addition, it can be viewed from phytochemicals aspect also.

Biosynthesis of lignin occurs via a complex biochemical pathway which is constructed from three phenylpropanoid monomers named as guaiacyl (G), *p*-hydroxyphenyl (H), and syringyl (S) units. These units are derived from coniferyl, *p*-coumaryl and sinapyl alcoholic precursors, respectively. Other units such as coumarates and ferulates are also identified along with these units (Molinari *et.al.*, 2013). Radical coupling reaction between the monomers form a cross-linked, branched phenolic polymer and due to which the lignin composition varies (Lu *et. al.,* 2017).

2.8.1 Lignin degradation mechanism and its products

Lignin degradation to small fragment units occurs via many chemical as well as biological methods such that these fragments can be alternatively used as chemical compounds

and biofuels. Oxidation, heat pyrolysis, hydrogenolysis and microbial degradation (Bugg *et.al.,* 2012) play an important role to give degradation products like coumarin, guaiacol, acetic acid, transferrulic acid, coumaric acid, vanillin, vannilic acid, furfuraldehyde and other monomers too. Biologically, slow lignin degradation is carried by various enzymes produced by fungi and microorganisms such as *Pseudomonas, Nocardia* and *Streptomyces* (Majumdar *et. al.*, 2014).

2.8.1.1 Role of enzymes in lignin breakdown

Lignin degradation and modification is the major mechanism to produce chemically active compounds as phytochemicals. *Phanerochaete chrysosporium* (white rot basidiomycetes) results in small white molecule rich in cellulose. Various extracellular oxidoreductases like, laccases, manganese peroxidase (MnP) and lignin peroxidase (LiP) produced by this fungi cause lignin biomass degradation in the cell wall of plant (Bugg *et. al.,* 2012). Manganese ion (Mn^{3+}) produced by MnP play a role as strong diffusible oxidizer on lignin components by lipid peroxidation reaction where as LiP degrades 90 % units of non phenolic lignin. It has been found that brown rot fungi oxidize lignin incompletely by the production of hydroxyl radicals (Kerem *et. al.*, 1999). Similarly, extracellular peroxidase secreted by *Streptomyces viridosporus* T7A cleaves the β- aryl ether lignin by oxidation to fragment the polymer of lignin (Bugg *et. al.*, 2012).

2.8.1.2 Catabolic pathways for breakdown of lignincomponents

The fate of oxidized lignin components by various enzymes are based upon the biochemical pathways that are essential for survival to the plants and bacteria as well. Knowledge and byproducts of such pathway might be crucial for application of such compounds for industrialization. The different types of catabolic pathways for lignin degradation can be listed as follows (Bugg *et. al.*, 2012).

- degradation of β- aryl ether
- degradation of biphenyl
- pathway for oxidative cleavage of protocatechuic acid
- phenylcoumarane and pinoresinol lignin components degradation pathway
- degradation of diarylpropane pathways
- ferulic acid degradation by bacterial strain

All of these pathways are important and regulated by their respective catabolic enzymes for regular growth and metabolism of the bacterial and plant cell wall. In diarylpropane degradation pathway, diarylpropane is taken as model compound where breakage of C_α -C_β linkage occurrs by oxidation in *P. chysosporium.* This results in phenolic aldehyde products where pathway is catalysed by LiP enzyme. The enzyme which eliminates formaldehyde and water from this compound has been extracted from the bacteria *Pseudomonas paucimobilis* TMY1009 (Kishi *et .al.,* 1991). The product formed from elimination of formaldehyde is lignistilbenewhich is then further oxidatively catalyzed by Lignostilbene dioxygenase (non-heme iron-dependent) to produce a pair of vanillin molecules and later converted into vanillic acid (Bugg *et. al.,* 2012).

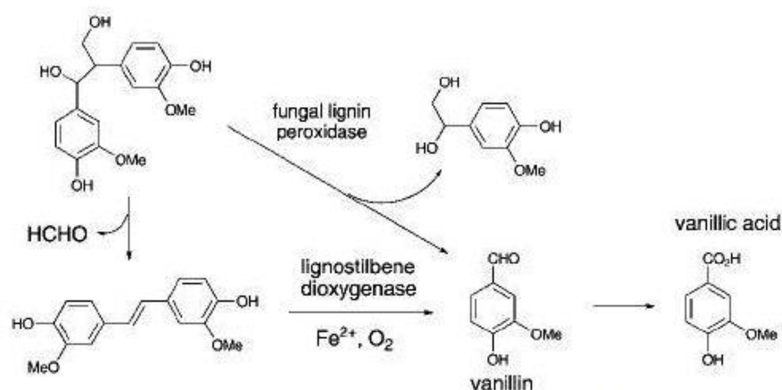


Figure 10: Fungal and bacterial pathways for the degradation of the diarylpropane degradation pathway in fungi and bacteria

2.9 Soil as inhabitant of saprophytic microbes

Soil is considered as most vast (Vos *et. al.*, 2013) and common inhabitant of almost all terrestrial plants and microorganisms along with various nematodes and insects. Various microbes that are dominant in soil include endophytic, symbiotic, parasitic, saprophytic and pathogenic bacteria as well as fungi, too. These microbes exhibit various physiological diversity to exist in own habitats with the involvement of various biogeochemical cycles by playing either as electron donors or as an electron acceptors. The growth and metabolism of the microbes depends upon the aboitic and biotic factors present in the soil.

Saprophytic microbes such as bacteria and fungi are also considered as prominent microbes of the soil. The absorptive mode of feeding on death and decayed organic composition (detritus) of plants present in soil is regarded as saprotrophic nutrition and organisms are known as saprotrophs. They excrete various biologically active enzymes for the conversion of detritus into more simple carbon sources that eases the cell for absorption. For example, they produce pectinases, cellulases and glucanases (Jun *et.al.*, 2011), that convert the cell wall materials, cellulose, hemicelluolse, lignin to the easily metabolizable compound by hydrolytic activity (Aranda *et.al.,* 2004). That can be used by other saprophytic bacteria as well as by soil *Streptomyces* species as a simple source of carbon by exhibiting carbon catabolite repression. However, the plant pathogen *Streptomyces scabies* (Chater and Chandra, 2006) is found to cause various plant diseases.

2.10 *Streptomyces*

Streptomycetes are gram-positive, aerobic, non-acid fast, saprophytic (Valli *et. al.,* 2012) mycelium forming soil bacteria belonging to the Actinobacteria and profuse producers of secondary metabolites. They are found to be with high GC content (70%) sporulating bacteria (Chater and Chandra, 2006) and possess earthy odor due to production of active volatile metabolite geosmin. Streptomycetes produce long-chain filaments that resemble the mycelia of fungi but are true bacteria. They are common inhabitants of marine and terrestrial soil but are infrequent pathogens with very few exception. They

possess more than 200 ABC transporters that permits these to rapid response towards environmental signals and utilize many complex carbohydrate sources including agar too (Bentley *et. al.,* 2002).

Streptomyces have been used for the production of secondary metabolites since two hundred years ago. Genetically, it has the ability to produce many bioactive secondary metabolites, such as antiviral alanosine (Murthy *et. al.*, 1966), antifungal amphotericin B (Caffrey *et. al.*, 2001), antitumoral mitomycin (Stulberg *et. al.*, 2016), immune suppressant rapamycin (Bolourian and Mojtahedi, 2018) and more specifically antibiotic streptomycin (Ohnishi *et. al.*, 2008) with addition of various pigments. Production of these bioactive compounds are initiated during idiotrophic growth, that coincides with the formation of aerial hyphae from the substrate mycelium (Stulberg *et. al.*, 2016).

2.11 Factors affecting Idiophytic growth of Streptomycetes for metabolites production

The transition period from vegetative to aerial mycelium formation and spore formation lead to the programmed cell death of *Streptomyces* (Daigle *et. al.*, 2015). In addition, during these interval, maximum cells undergo to stationary phase such that different genes are regulated allowing the production of different secondary metabolites. For the optimum production of secondary metabolites, various biotic as well as aboitic and environmental factors play a major role.

For regular growth and mass multiplication of *Streptomyces* in liquid media, first compromising factor is size and volume of inoculums. On the other hands, various composition of media including carbon, nitrogen, phosphorous sources, pH, temperature, incubation time and also agitation rate (oxygen intake) have crucial role for the bioactive metabolite production. Mostly, the cells that are in idiophytic phase are activated to generate many antibiotics.

Streptomyces thermoviolaceus has growth and metabolism temperature in the range of 30 to 55 °C but optimum temperature was found to at 45°C (James *et. al*., 1991). Similarly, Yegneswaran *et. al.,* (1991**)** had shown that there is increased ratio in both biomass yield and additional product by *Streptomyces clavuligerus* when oxygen saturation level is increased above the dissolved oxygen concentration. Several studies on *Streptomyces* species show that the optimal pH range is between 5.5 and 11.5 for maximum growth and sporulation (Kontro *et.al.*, 2005)**.**

2.12 Streptomycetes as Antibiotic producers

The novel era of antibiotics began after the introduction of first antibiotic, penicillin by Alexender Flemming in 1940s from fungi. Because of its active therapeutic potential, trend of developing more other antibiotics from fungi and other actinomycetes were developed to cure the infectious diseases. Among actinomycetes, about 7,600 bioactive compounds and secondary metabolites are produced by *Streptomyces* species till date. Streptomycetes are taken as potent organism for the production of different human and

animal health benefit antibiotics and they are still under exploration. The available data base about gene and genetic information of this organism act as complete source for rapid and target based production of new antibiotics.

The following figure depicts the name of the antibiotics developed by various fungi and actinomycetes strain from 1910s to till 2010 with discovery void from the time of 1987 (Silver, 2011).

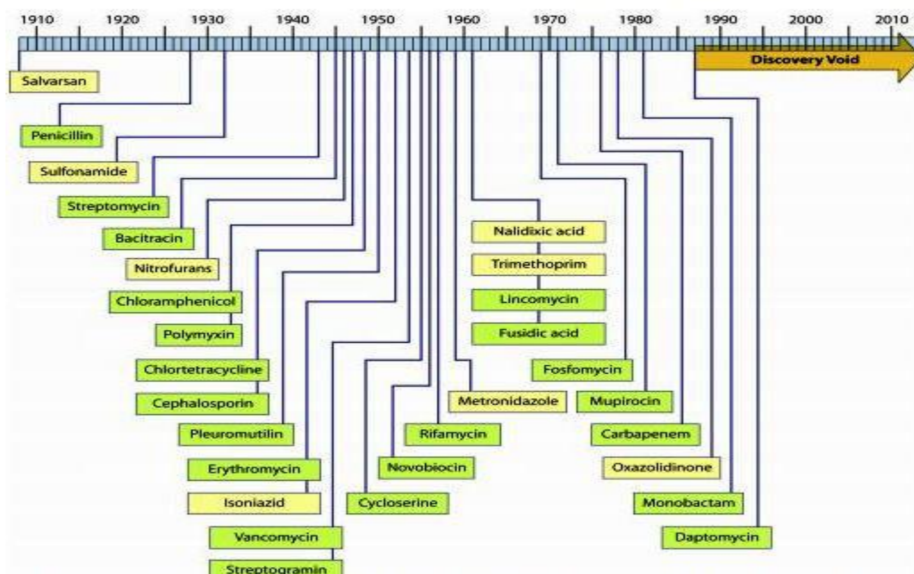


Figure 11: Discovery of antibacterial drugs from various *Streptomyces* species (Silver, 2011)

2.12.1.1 *Streptomyces coelicolor*

Streptomyces coelicolor is biotechnologically considered as the actinorhodin-producing representative organism (Omura *et. al.,* 2001) having a linear chromosome with a length of 8.7 Mb (Vockenhuber *et. al*., 2011). It contains 8,000 annotated open reading frames (ORF). The chromosome is mainly divided into three major parts. i) core, ii) left arm and iii) right arm.

The size of core is 4.9 Mb that encodes the essential constitutive genes (Bentley *et. al*., 2002). The regulatory function to control the metabolism, chemical differentiation and morphology in *S. coelicolor* is maintained by 10 % protein coding genes. The genome of this species encodes hundreds of transcription factors and about 70 sigma factors for the transcriptional regulation and control of the secondary metabolism (Reuther and Wohlleben, 2007). The left arm (1.5 Mb) and right arm (2.3 Mb) on either sides of the core region harbors the non essential along with species specific genes to code secondary metabolic pathways (Vockenhuber *et. al.,* 2011). The high GC content (74%) (Yakovchuk, 2006) and the lack of molecular RNA chaperone for covalent folding and assembly (Storz *et. al.,* 2005) controlling the post transcriptional level add to form highly structured and stable RNA species that would be critical in secondary metabolite synthesis.

2.13 Carbon Catabolite Repression (CCR)

In our surroundings, different forms of organic carbon sources are found in the soil. All the living organisms use reduced simple form of carbon sources for regular growth and metabolism. Furthermore, preferred carbon source utilization also aids in taxonomic identification of bacterial species.

Almost all of the bacteria and fungi use various organic substrates as source of carbon for their growth (Adnan *et. al.,* 2017). They can either co-metabolize or can preferentially utilize the carbon sources that are most easily accessible and allow the fastest growth. Carbon catabolite repression (CCR), is an unique conserved mechanism that uses the preferred reduced carbon source, in most cases as glucose (Görke and Stülke, 2008) for growth by restraining the expression and activities of other enzymes for the use of secondary carbon sources. About 5-10 percentage of all the yeast and bacterial genes are involved in CCR (Liu *et. al*., 2005). Jacques Monod in 1942 first observed the selective carbon-source usage was the glucose–lactose utilization in diauxie growth in *Escherichia coli* to understand the mechanism of carbon source utilization.

As the preferred available carbon source determines the growth rate of bacteria, it is important for competition with other species in the same environment. Similarly, it enables both pathogenic and free-living microorganisms to access the new source of nutrients for expression of virulence genes (Mendez *et.al.,* 2008). But, CCR is not regulated in some pathogenic bacteria such as *Chlamydia trachomatis* and *Mycoplasma pneumonia* (Görke and Stülke, 2008) since they have small genome size and highly adapted to nutrient–rich host environments (Nicholson *et.al.*, 2004; Halbedel *et. al.,* 2007). However, reverse CCR is observed in *Bifidobacterium longum* (Parche *et.al.,*2006), *Pseudomonas aeruginosa* and *Streptococcus thermophilus* (Görke and Stülke, 2008). In these bacteria, glucose utilization genes are repressed until the other preferred carbon sources are excess.

2.14 Carbon Catabolite Repression (CCR) in *Streptomyces*

As with the different species of bacteria, CCR is also exerted by various *Streptomyces* species. In *Streptomyces clavuligerus,* maltose and glycerol restrain the cephalosporin production where as succinate and alpha ketoglutarate activate the production of such antibiotics in high amount (Aharonowitz and Demain, 1978). In *S. coelicolor*, CCR of genes of glucose kinase (glk) enzyme acts as major player in the use of secondary carbon sources that is not involved in the metabolism for repressing other sugars. The mutants lacking this enzyme, cannot regulate CCR because of incapacity of utilizing glucose or other readily usable carbon sources. Besides this, complex protein operon DasABC encodes an ABC transporter (Seo *et.al.,* 2002) that is involved in sugar import. But the mutant *bld*B is unable to develop aerial mycelium and hence, insensitive to glucose repression. This suggests Glk A acts as the limited source for morphological differentiation through the primary metabolism (Pope *et.al.,* 1998). In addition, the

shifting and phosphorylation of glucose occurs via simultaneous reaction of GlkAand GlcP (Romero-Rodríguez *et. al.*, 2017) along with various transcription factors and regulatory genes.

In *S. coelicolor*, unlike Enterobacteria, cyclic AMP receptor protein (CRP) doesnot bind with cAMP. The inducer, glycerol 3-phosphate of glycerol operon (*gylCABX*) decides the repression degree that might be able to apply for the repressor GylR on promoter region of that *gylCABX* operon. After addition of glucose, it decreases the expression of glycerol operon, due to modulation of intracellular glycerol 3-phosphate levels by GlkA. In *Streptomyces*, the phosphotransferase system (PTS) helps in phosphorylation, transportation of carbon sources and CCR of *N*-acetylglucosamine (GlcNAc)and D-fructose. In *S. coelicolor*, this mechanism is found to be inducible. The mutants Null *pts*H is found to have repression in fructose by glycerol kinase enzyme (Romero-Rodríguez *et.al.,*2017).

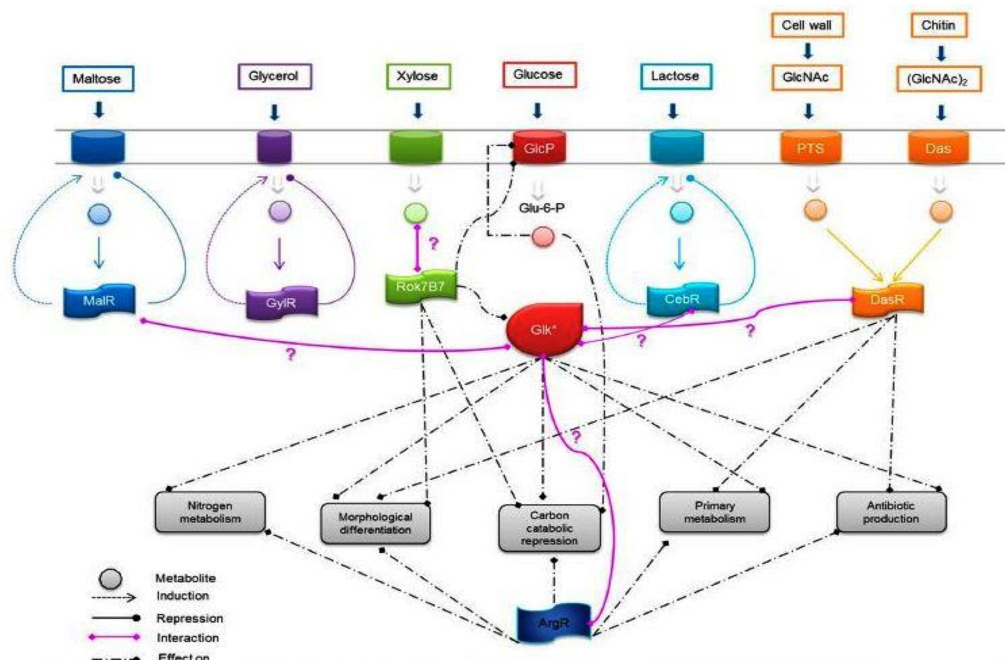


Figure 12**:** Regulators for CCR mechanism in *Streptomyces*. (MalR induces and represses the maltose utilization by interacting with Glk (*pink line*). Likewise, GylR induces and represses glycerol transport. CebR acts as induction and repression for β-galactosidase. Xylose and its metabolic products activate the Rok7b7, where as the absence of Rok7B7 increases expression of Glk and GlcP showing its implication in CCR. Glucose and its metabolic products exert an effect on GlcP and CCR that is independent to Glk. Das Rresponds to various signals of the cell wall or the presence of chitin. It is found that DasR, Glk and ArgR have pleiotropic effects on different cellular pathways and regulated network occurs.)

2.15 Antibiotics produced by *S. coelicolor*

Actinorhodin (ACT)((3’- carboxymethyl-5,5’,10,10’-tetrahydroxy-1,1’-dimethyl-6,6’,9,9’-tetraoxo-3,3’,4,4’6,6’9,9’-octahydro-1H,1’H-8,8’-bi(benzo(g)isochromen)-3-yl)acetic acid) (Bystrykh *et.al.,* 1996), the red/blue or acid base indicating pigment, originally isolated in Germany (Brimble *et.al.,* 1997) is a benzoisochromanequinone polyketide antibiotics

produced by *S. coelicolor* which is also known as pH indicator. It turns into red when pH lowers from 8.5 and above that pH (Brockmann & Hieronymus, 1955) that gives blue colonies on the agar media. A blue zone would be recognized if a converter strain could use precursor (Cole *et. al.,* 1987) that was secreted by a donar strain to produce actinorhodin. Polyketide antibiotics include daunurubicin (anticancer agents) and tetracyclin (Zhan, 2009). This antibiotics was alsoproduced by *S. coelicolor* A3(2)

(Gorst-Allman *et.al.,* 1981). But, according to Mc Nicolas (McNicolas, 1991), blue colonies of the *S. coelicolor* A3(2) derivative M145 was found with no actinorhodin production.

Secondly, it involves in the redox–cycling reactions. Because of it's redox potential (Mak and Nodwell, 2017), electrons are generated by the reduction of the quinone groups that reacts with molecular oxygen to yield superoxide radical anions (O_2^-). The dismutation (Lee *et.al.,* 2015) of these radicals produces reactive oxygen species (ROS) such as hydrogen peroxide(H_2O_2). Hence, this redox activity can be described for actinorhodin pigment for the change in colony morphology on the agar media and it can be sensed by the SoxR regulon (Lee *et. al*., 2017) that is involved in the responses to peroxides and reactive electrophile species. Similarly, it catalyzes the conversion of L-cysteine and L-ascorbic acid with the simultaneous production and accumulation of toxic hydrogen peroxide (Nishiyama *et. al.,* 2014) that acts as bactericidal agent.

2.16 Biosynthesis and Regulation of the Transcriptional Activator of Actinorhodin

Actinorhodin (ACT) is synthesized during stationary phase of the growth (Gramajo *et.al.,* 1993) that acts as one of the model system for the exploration of secondary metabolism by *S. coelicolor.* ACT biosynthesis is determined by five transcriptional units in the *act* gene cluster.

During biosynthesis of ACT, a 16- carbon linear polyketide is generated by type II polyketide synthase (PKS) (Act I ORF 1-3) to synthesize the final product. After carbon backbone is produced from acetyl CoA and malonyl CoA (Craney *et.al.,* 2013) in primary metabolism, ACT biosynthetic cluster encodes an activator (*act* II –ORF4) that activates a pathway specific biosynthetic genes. The *act* II –ORF4 binds two of the three intergenic regions (*act* VI- ORFA/ *act*VI-ORF1 and *act*III/ACTI- ORF1) that includes the proteins involved in antibiotics production. This binding overlaps the -35 regions of the promoter, such that recognition by RNA polymerase is easier (Craney *et.al.,* 2013). Thus, activator gene act as global regulators for either activation or repression of expression.

As a global regulator for signal transduction pathway, serine/ threonine kinase (AfsK) phosphorylates the DNA binding protein AfsR that is attuned by binding of the protein KbpA (Umeyama and Horinouchi, 2001). Therefore, it promotes the interaction with the promoter of the afsS gene by activating the expression of the ACT biosynthetic genes.

The sensor kinase PhoR senses the afsS expression hence leads to phosphorylate the response regulator PhoP.

This enhances expression of AfsS leading to increased production of ACT. Hence, this provides a way to assimilate metabolic and environmental signals. Similarly, *S*-adenosyl-L-methionine (SAM) also activates signal for the AfsK pathway (Jin *et.al.,* 2011).

2.17 Cytochromes P450 and its role

Cytochromes P450 (CYPs) are also known as hemeproteins because of presence of heme as a cofactor. They are attached to the membranes of cell and absorb the light at 450 nm when complexed with carbon monoxide in reduced condition (Lynch and Price, 2007). They are regarded as terminal oxidase enzymes of electron transfer system and have an active role in synthesis of adenosine triphosphate molecules. Among the various CYPs system, bacterial system uses ferrodoxin reductase and ferrodoxin to shift electrons to P450 where as electron for CYPs in human is provided through NADPH-P450 reductase and mostly act as the drug and various steroid metabolizing enzymes.

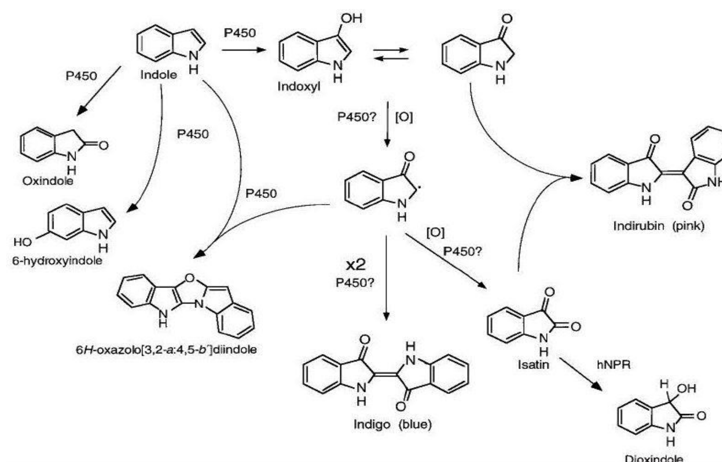


Figure 13 : Pathway of cytochrome P450 2A6 mediated metabolism of indole (Gillam *et.al.,* 2000).

In the presence of cytochrome P450 2A6 (in liver) and CYP 2A13 in lungs, indole is converted into indirubin that detoxifies the toxins and nicotins too. Similarly, CYP P4502A6 metabolizes the xenobiotic in body but its catalytic activity is inhibited by flavones and flavanone. The cytochrome P450 2A6 converts indole into indigo (blue) and indurubin (pink). The nascent oxygen acts as catalyst for this irreversible reaction. Isatin acts as intermediate compound for the formation of pink colored indirubin (Gillam *et.al.,* 2000).

In *Streptomyces* species, P450 genes are involved in biosynthesis of most important secondary metabolites, pigments and antibiotics, too. It has been found that *S. coelicolor* A3(2) contains 18 P450 genes. Its genome possesses two members of the CYP158A enzymes: CYP158A1, 2, and 3. Along this, *S. coelicolor* CYP 154C1 was found to interact with macrolide antibiotics indicating that the heme protein permits the substrate to bind with active site. This suggests the strong basis for the CYP catalytic mode toward that

antibiotic ring system. Inverted linear position of heme of CYP154A1 helps for proper orientation of propionic acid side chains (of pyrrole ring) to the heme (Lamb *et. al.,* 2006). Similarly, albaflavenone is also biosynthesized by CYP170A1 that helps in catalyzing the last stairs of its synthesis. It also acts to determine other catalytic activity (M.-A. Cho *et. al.,* 2018) as well. The P450 enzyme also helps for sporulation in Streptomycetes. Again, in *S. coelicolor*, new *bld* gene named as *sco3099* encodes CYP107U1. Deletion of this gene generates the mutant which is defective to form spore through aerial hyphae. This suggests that P450 enzyme has major role in developmental biology of *S. coelicolor* (M.-A. Cho *et.al.,* 2018).

2.18 The Resazurin Assay

The low toxic blue dye, Resazurin or Alamar blue,7-Hydroxy-3*H*-phenoxazin-3-one 10-oxide, is known as the colorimetric cell permeable redox indicator (De Fries and Mitsuhashi, 1995) for both aerobic and anaerobic respiration (Chen *et. al.,* 2015). It was first used to quantify bacterial content in milk by Pesch and Simmert in 1929 (Pesch and Simmert, 1929).

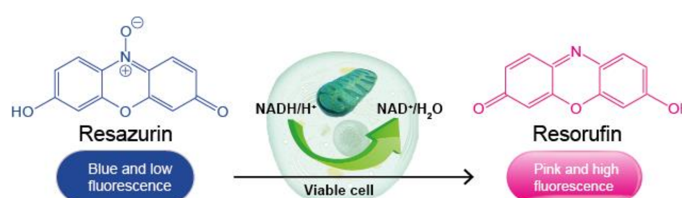


Figure 14: Conversion of resazurin to resorufin

It can be dissolved in physiological buffers and double distilled water and added directly to cells in culture in a homogeneous format with desired final concentration. The viable cells or bacterial cells of culture with active metabolic state can reduce irreversibly resazurin into the highly red fluorescent product resorufin (Bueno *et. al.,* 2002) and finally to a colorless and non-fluorescent molecule, hydroresorufin by oxidoreductase (Karuppusamy and Rajasekaran, 2009). The large change in perceived color of resorufin is measured by the quantification of Kreft's dichromaticity index which is the highest value of resazurin solution (Kreft and Kreft, 2009). This index measures the resorufin when the thickness or concentration of observed sample increases or decreases. Resorufin fluorescence signals can be easily detected using a fluorescence spectrometer, and they provide quantitative measurements of cell proliferation and cell inhibition.

Hence, this assay has been applied for cell culture, cell survival and cell proliferation ranging from eukaryotes to prokaryotes (Kurin *et. al.*, 2012). Moreover, It has huge application in the determination of antibiograms, contamination and detection of bacterial biofilm formation (Franzblau *et. al.,* 1998; Bucaloiu *et. al.*, 2012)

The conversion of resazurin to resorufin is enzymatically activated by NADPH dehydrogenase or NADH dehydrogenase. Since, NADPH or NADH is the reductant for this bio chemical conversion, resazurin/diaphorase/NADPH system can be used to

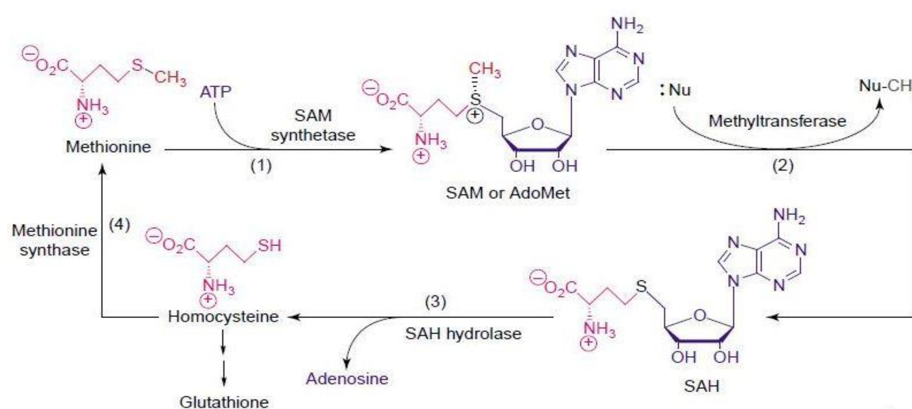
detect for NADH, NADPH or diaphorase level generation (Andrade *et. al*., 2010).

2.19 *S*-adenosylmethionine (SAM) and riboswitch

One of the bacterial *de novo* thiol metabolites, *S*-adenosylmethionine or SAM, which is also known as AdoMet, is considered as an important biological methyl donor reagent (Yan *et. al*., 2010). It is key substrate in various biochemical pathways. It acts as co substrate and plays role in methylation, aminopropylation as well as in trans sulfuration (Beri *et. al*., 2017). It is produced by anabolism and later consumed in the liver of animals.

DNA methylation is critical for gene expression regulation where as phospholipids methylation retains the membrane receptors and fluids. SAM falls in the second position that acts as enzyme substrate after ATP. Ludwig and Matthews in 1997 found that cobalamin cofactor methylation of methionine synthase afford a complete repair mechanism pathway for this enzyme.

The complete cycle of SAM contains various intermediate products with the involvement of different catalytic enzymes. SAM is converted to the *S*-adenosyl homocysteine (SAH) by the enzyme methyl transferase. SAH is known as a strong negative regulator of SAM-dependent methylases (methyl transferase), which is hydrolyzed to homocysteine and adenosine by S-adenosy lhomocysteine hydrolase (SAH hydrolase) (EC 3.3.1.1) and the homocysteine again is converted back to methionine through transfer of a methyl group from 5-methyltetrahydrofolate, by one of the two classes of methionine synthases. In another way, homocysteine might be converted to glutathione. Thus generated methionine can then be transformed back to SAM-e or AdoMet, completing the cycle. In the rate-limiting step of the SAM cycle from conversion of homocysteine to methionine, MTHFR (methylenetetrahydrofolate reductase) irreversibly reduces 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (Fontecave *et.al.,* 2004).



(Fontecave *et. al*., 2004).

Figure 15: The S-adenosylmethionine (SAM) cycle. Nu acts as the nucleophile substrate for the enzyme methyltransferases. Reaction of methionine and ATP forms the SAM by SAM synthetase (1); Methyl group of SAM is transferred to the nucleophiles by methyltransferases (2); Homocysteine is produced from S-adenosyl homocysteine (SAH) (3); Methionine is regenerated by methylation of homocysteine activated by methionine synthase (4).

2.20 Riboswitch

For the regulation of Central Dogma of every cell, DNA, RNA and proteins are required in a proper functioning states. Riboswitches are the 3D structures of short regions of 5'-UTR of mRNAs containing specific conserved domains for ligand binding along with a different variable sequences. They act as common ways of genetic regulation at RNA level in prokaryotes (Barrick and Breaker, 2007) since they allow the non coding RNAs regions to become functional for genetic switches (Serganov and Nudler, 2013).

In general, the riboswitch contains two functional domains named as aptamer domain and expression platform. Aptamers are single stranded oligonucleotide that recognize the ligands (effector molecules) functioning as ligand binding pocket. Ligand binding occurs through very high affinity and specificity. The second domain, expression platform changes its structure in the response of changes in aptamer and regulating gene expression of downstream sequences. After overlapping of these two domains, folding of RNA occurs in the expression platform to represent either switch on or off of the mRNA (Garst *et. al.,* 2011). Hence, riboswitch mechanism can be highly implemented to develop antibiotics and design unique molecular biosensors too (Serganov and Nudler, 2013).

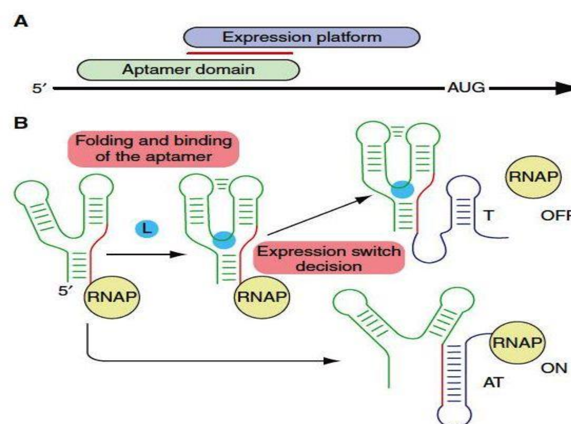


Figure 16: A schematic representation for organization and mechanism of the typical riboswitch (Garst *et. al*., 2011)

Various riboswitches have been validated till date according to their function on mRNA level. Some of them include cobalamin riboswitch, SAH riboswitch, SAM riboswitch, tetrahydrofolate riboswitch, glutamine riboswitch and others. Cobalamin riboswitch binds with adenosyl cobalamin or aquocobalamin that regulates the biosynthesis of cobalamin and further transportation of it (Polaski *et.al*., 2017).

2.21 SAM Riboswitch

The SAM riboswitch or the S-box leader sequence is found upstream of genes coding for biologically active proteins that are involved in either methionine or cysteine biosynthesis for metabolic regulation. They actively participate in the various biological mechanism, such as attenuation of transcription process, initiation of translation as well as antisense production, also. There are different SAM

riboswitches (Garst *et. al.*, 2011).

SAM I riboswitch allows the formation of an intrinsic terminator stem loop upon SAM binding. That ultimately results in the termination of transcription (Mandal *et. al.,* 2003). However, in the absence of this riboswitch, an antiterminator loop structure is formed that activates transcription by producing complete functional transcript. The derivative SAM IV differs in the scaffolding beneath the binding nucleotides (Sudarsan *et. al.*, 2008).

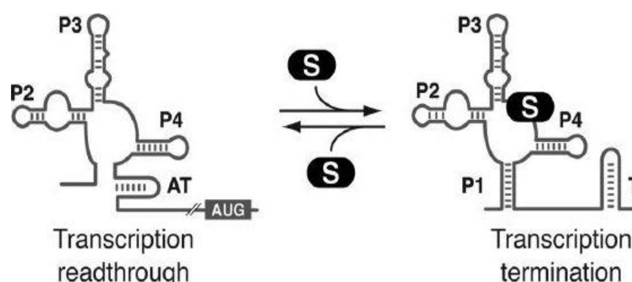


Figure 17: Transcriptional control of the SAM-I riboswitch (Heppell *et. al*., 2011)

SAM-II riboswitches are typically short sequences of oligonucleotides. These sequences form an H-type pseudoknot after binding with SAM which is entirely distinct sequence and structural features from those of SAM-I and SAM-IV. Though, the pseudoknot ends 2 nt upstream of the Shine-Dalgarno (SD) sequence, it is sufficient to occlude the ribosome binding in "off " nonfunctional state (Gilbert *et. al.,* 2009). These type of riboswitches are mainly found in α-proteobacteria (Corbino *et. al.*, 2005).

SAM-III is also a translational riboswitch that regulates gene expression by sequestering ribosome binding site (RBS) or SD sequence. When SAM binds with SD sequence, aptamer is formed by base pairing of SD base sequence with an anti-SD sequence. As a result, this prevents the binding of ribosome in the ribosome binding site. The tertiary structure and binding pocket of SAM-III aptamers are distinct as compared to other SAM riboswitches (Lu *et. al.*, 2008). But, it is narrowly distributed mainly in the order Lactobacillales (Fuchs *et. al.*, 2006).

2.22 Reporter Gene Assay

There are various methods of assaying drug targets by high-throughput screening techniques. The reporter gene assay is considered as sensitive and versatile method that allows to choose the cellular biosystem employed (An and Tolliday, 2009). The combined unit of reporter gene consists of a promoter and the required reporter gene. The choice and number of copies of promoter as well as nature of the reporter gene determines the activities of reporter gene (Ponglikitmongkol *et.al.*, 1990).

A reporter gene codes for a valuable product that is easily measureable. These genes are used when the product of gene of interest is difficult to assay quantitatively. The reporter gene generates a signal that can be easily identified. Hence, the products of reporter gene might be either extracellular or intracellular. Intracellular reporter genes include β-galactosidase, chloramphenicol acetyltransferase (CAT), luciferase and green

fluorescent protein (GFP). These products are retained inside the cell. But extracellular products are released into the extracellular matrices without destroying the cells and includes secreted placental alkaline phosphatase (SPAP) (New *et.al.*, 2003).

Reporter genes are linked to other sequences to make the reporter protein or the reporter protein itself is fused to another protein known as fusion protein. Most of the reporter genes are accommodated downstream of the promoter region but that is closed towards the gene which is under study for simultaneous expression of these genes (Debnath *et .al.,* 2010). However, there are some reports that focus on the chromosome independent accommodation of reporter genes in a vector. In that case, other highthrough put techniques can be used to identify the gene of study (Pardy, 1994).

2.22.1.1 *Luc* Reporter Gene System

In nature, the luciferase reaction occurs in the peroxisomes of fireflies (*Photinus pyralis*). The reaction fluorishes a yellow-green light by using luciferin as substrate and requires ATP, Mg^{2+}, and O_2 (Pardy, 1994). The emitted glow is taken as an important assay for luciferase activity that helps to monitor expression of regulatory elements. *Luc* can be introduced into living cells and its expression has no adverse effect on the metabolic activity of transgenic cells. According to Nordeen (Nordeen, 1988), *luc* reporter gene is considered as an ideal method for detection of low-level gene expression in both prokaryotes and eukaryotes. After the addition of ATP and luciferin to the cell lysates, expression of luciferase is measured by analyzing bioluminescence with the help of spectroscopy.

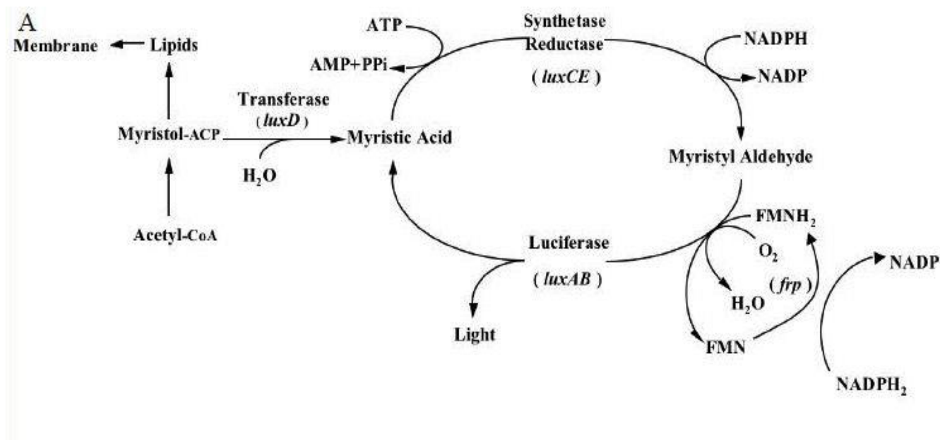


Figure 18: Reaction of the bacterial luciferase gene cassette

2.22.1.2 β- galactosidase and *Lac Z* Reporter Gene System

The *lac*Z gene of *Escherichia coli* is used to quantify transcriptional and translational activities that are associated with gene of interest in many different organisms. The enzyme β galactosidase expressed from *lac* Z gene hydrolyzes the β galactosides, including lactose. The colonies that express *lac* Z appear blue color on the culture media incorporated with X-gal (5-bromo-4-chloro-3-indoxyl-beta-D-galactopyranoside).

Enzymatic activity is measured by photometric analysis by measuring the hydrolysis of substrate *O*-Nitrophenyl-β-D-galactopyranoside (ONPG) by the enzyme β galactosidase in cell free extracts (Schaefer *et. al.*, 2016). Some of the plasmid carries only the alpha region of Lac Z protein because of large size of complete *Lac Z*. When alpha region of *lac Z* expresses an inactive protein, it can combine with beta – gal after complementing with lac Z protein that is devoid of alpha region (Juers *et. al.*, 2012). This is possible when plasmid with alpha region of *lacZ* is transformed into bacteria with mutated alpha region. These bacterial cells will convert to β galactosidase positive in the presence of plasmid.

After the cloning of insert into alpha region of *lac Z* vector, alpha region become dysfunctional. In this case, intragenic complementation does not occur and negative β galactosidase is shown by the bacterial cells that carries plasmids having foreign DNA. The β galactosidase negative cells can be distinguished from positive ones after incubating bacteria on media supplemented with X- gal and *lac* promoter inducer IPTG (isopropyl-beta-D-thiogalactopyranoside) that is followed by the selection of white colorless colonies (Debnath *et. al.*, 2010).

2.22.1.3 Promoter reporter assay

Various reporter genes can be applied to assay the function or nonfunctional activity of a specific promoter in a cell. The reporter gene is arranged in such a way that the target promoter controls the reporter gene and its products activity is quantitatively measured by enzymatic assay. The results are analyzed relative to the activity of promoter either they are of constitutive, inducive, strong or weak promoter. This assay provides a rapid method to analyze transgene and protein expression (Debnath *et. al.*, 2010).

2.23 Vitamin B12 biosynthesis pathway as a probable drug target

Vitamin B12 (cobalamin) is a cobalt-containing tetrapyrrole, which is produced mainly by bacteria (Martens *et. al.*, 2002) and some plants, and an essential cofactor for biochemical reactions. It catalyzes the various transmethylation and rearrangement reactions. It is the most complex vitamin of all the other vitamins, it is very expensive in terms of energy to synthesize since there are more than 30 biochemical steps required (Scott and Roessner, 2002). Thus, this vitamin can be considered highly valuable for survival of bacteria and as a probable therapeutic target, that might become a potential solution to the increasing antimicrobial resistance problems.

A total of eight SAM mediated methylation has been reported to transfer the methyl groups to the uroporphyrinogen framework, although only seven are observed in the final product (Warren *et. al.*, 2002).

The transformation of uroporphyrinogen III into tetrapyrrole containing vitamin B12 requires peripheral methylations of carbon, contraction of ring with the loss of the C-20 *meso*-position, cobalt chelation, carboxylic acid side chains amidation, decarboxylation of the acetic acid side chain on ring C, attachment of aminopropanol,

adenosylation and attachment of the lower base in the form of α-ribazol with the central corrin ring (Warren *et. al.*, 2002).

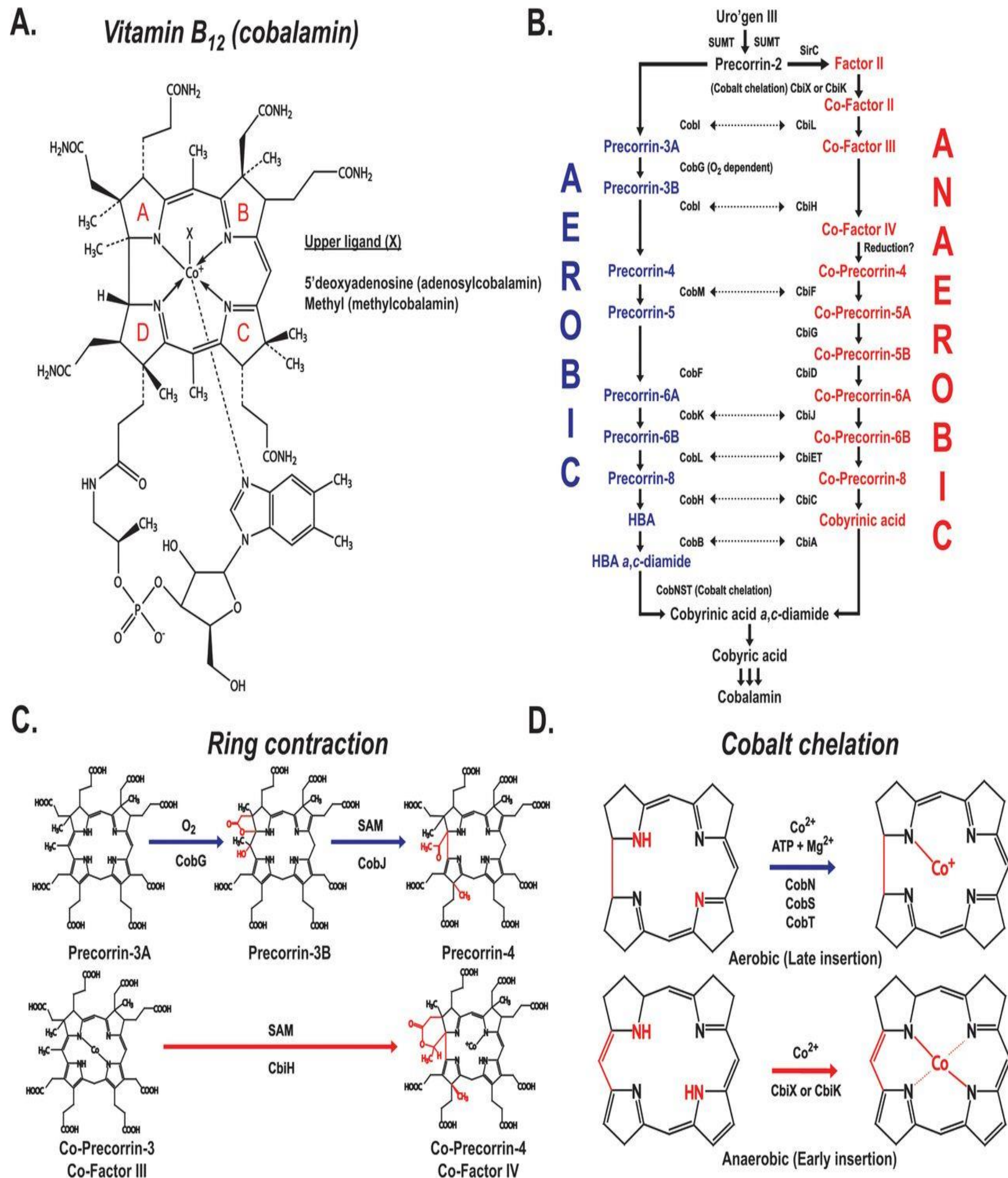


Figure19: Summary of the aerobic and anaerobic corrin ring contraction and cobalt chelation inVitamin B12 biosynthesis pathways (Moore and Warren, 2012).

(A) Structure of vitamin B12 (B) Aerobic and anaerobic pathways. Similarities shared between Cob and Cbi enzymes are indicated by broken arrows. (C) Ring contraction. (D) Simplified diagram of *coba*lt chelation at tetrapyrrole ring.

3 MATERIALS AND METHODOLOGY

3.1 Materials Required for Confirmation and Antimicrobial Sensitivity Test (AST) of test organisms

Test organisms, Nutrient Agar plates, *Salmonella – Shigella* agar plates, Mannitol Salt Agar plates, Mac-Conkey agar plates, Luria Bertani (LB) broth, Gram staining reagents, different biochemical media, Mueller Hinton Agar (MHA) plates, antibiotic discs

3.1.1 Collection and selection of the test organisms

Different bacteria were obtained from Central Department of Biotechnology (CDBT) laboratory and Animal Health Research Division (AHRD). They were different strains of *Pseudomonas aeruginosa, Klebsiella pneumoniae, Acenetobacter baumannii, Escherichia coli, Staphylococcus aureus* and *Salmonella* Typhi.

Similarly, different American Type Culture Collection (ATCC) bacteria named as *Pseudomonas aeruginosa* 27853, *Klebsiella pneumoniae* 700603, *Escherichia coli* 25923, *Staphylococcus aureus* 29213 *were obtained from* National Public Health Laboratory, Teku, Kathmandu.

3.1.2 Identification and reconfirmation of the test organisms

Among the collected test organisms, *Acenetobacter baumannii* (*Aceneto9*) and *Klebsiella pneumoniae* (*Kleb*52) were provided as already sequenced strain. Hence, further identification and confirmation was carried out for those two strains too.

For the identification of the test organisms, the cryopreserved strains were inoculated on LB broth and then subcultured by streaking on the Nutrient Agar (NA) plates. Then, single colony was transferred on respective selective agar plates. *S*. Typhi strain was streaked on *Salmonella – Shigella* (SS) agar plates, *S. aureus* on Mannitol Salt Agar (MSA) plate, where as other Gram negative bacteria were streaked on Mac-Conkey (MA) agar plates for further confirmation.

3.1.2.1 Microscopic Observation of the test organisms

Microscopic observation of test organisms was done through Gram staining. A thin and uniform smear of sample was prepared on the microscopic slide by mixing the sample with distilled water and allowed for air dried. Heat fixation was done to fix the smear to the slide. Then, three drops of crystal violet was flooded over the heat fixed smear and allowed to stand for one minute. It was then rinsed with tap water and three drops of Gram's Iodine was flooded and allowed to stand for one minute. Then, rinsing was done by tap water and decolourizing agent (acid alcohol) was flooded and washed by tap water immediately. After that, counter stain was flooded and allowed to stand for fourty five seconds. It was rinsed with tap water again and allowed for air dry. The prepared slide was observed under microscope at 40X and 100X (oil immersion) to observe colour, shape and arrangement of the colonies.

3.1.2.2 Biochemical Tests of the test organisms

After Gram staining, different biochemical tests were performed to identify the test organisms.

Indole Test: A sterilized culture tubes containing 5 ml of tryptophan broth were taken. The tubes were aseptically sub-cultured by taking the growth from 18 to 24 hrs tests culture from Luria Bertani broth. The tubes were then incubated at 37°C for 24 hours. Three drops of Kovac's reagent was added to observe the presence or absence of ring to compare with negative control.

Methyl Red –Voges- Proskauer (MR-VP) test: Using test organisms taken from an 18-24 hour pure culture, the MR broth was inoculated. They were then incubated aerobically at 37 °C for 24 hours. Then, 1 ml of the broth was aliquoted to a sterilized culture tube. The remaining broth were incubated for an additional 24 hours. Then 3 drops of methyl red indicator was added to aliquot and the red color was observed immediately.

Using test organisms taken from an 18-24 hour pure culture, the VP broth was inoculated. They were then incubated aerobically at 37 °C for 24 hours. Then, 1 ml of the broth was aliquoted to a sterilized culture tube. The remaining broth were incubated for an additional 24 hours. 5% alpha-naphthol and 40% potassium hydroxide were added in the ratio of 3:1 and mixed well to aerate. A pink-red color at the surface within 30 min was observed. The tube was shaken vigorously during the 30-minutes period.

Citrate utilization Test: The Simmon's citrate agar slant was prepared and cooled in slanted position (long slant, shallow butt). The uninoculated medium would be a deep forest green due to the pH of the bromothymol blue. A light inoculum picked from the center of a well-isolated colony was stabbed in butt and streaked the slant back and forth. It was then incubated aerobically at 37°C for up to 24 hours. A color change from green to blue along the slant was observed in case of positive result.

Urease Test: The urea broth was autoclaved at 121°C under 15 psi pressure for 15 minutes and distributed 5 ml per sterile culture tube. Then 40 percentage urea crystals was aseptically added to each tubes and inoculation of fresh test organisms was done. The cap was left on loosely and incubated the tube at 37°C in ambient air for 48 hours to 7 days. In case of positive, the development of a pink color was examined for as long as 7 days.

Oxidase Test: The strip of Whatman's No. 1 filter paper that was soaked in a freshly prepared 1% solution of tertramethyl-p-phenylene-diamine dihydrochloride were freeze dried and stored in a dark bottle tightly sealed with a screw cap. For use, a strip was removed, laid in a petridish and moistened with distilled water. The colony to be tested was picked up with a wooden stick and smeared over the moist area. A positive reaction was indicated by an intense deep-purple hue, appearing within 10 seconds, a "delayed positive" reaction by colouration in 10-60 seconds, and a negative reaction by absence of colouration or by colouration later than 60 seconds.

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% hydrogen peroxide (H_2O_2) was poured in the glass slide. The evolution of oxygen bubbles was then observed.

Triple Sugar Iron Agar (TSIA) Test: The TSI 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.

3.1.3 Antibiotic Sensitivity Test of the test organisms

Preparation of inoculums

After the preliminary confirmation of the test organisms, all bacteria were tested in vitro to determine susceptibility towards different antibacterial drugs by agar disc diffusion method as described by Kirby-Bauer and WHO (Biemer, 1973). The 18 hours overnight cultures of test organisms were inoculated in the LB broth and turbidity was observed. Then, required turbidity of the bacterial suspension were adjusted by comparing with 0.5 McFarland standard.

Antibiotic sensitivity testing (AST)

The susceptibility test of abovestrains of bacteria was carried out using MHA plates and was tested *in vitro* for susceptibility to different antibacterial drugs produced commercially by HiMedia company; Colistin (10 mcg and 25 mcg), Ertapenem (10 mcg), Imipenem (10 mcg), Meropenem (10 mcg), Oxacillin (1 mcg), Ofloxacin (5 mcg), Gentamicin (10 mcg), Methicillin (5 mcg), Vancomycin (30 mcg) and others.

For antibiotic sensitivity test, agar disc-diffusion testing method was followed as published by the Clinical and Laboratory Standards Institute (CLSI) for bacteria with slight modification. First, test organism suspension equivalent to that of a 0.5 McFarland standard was evenly spread onto dried MHA plates by dipping a sterile cotton swab twice into the suspension and the agar rim was made finally by the sweep of the swab. MHA plate was incubated at 37°C for half an hour to activate the test organism. Then, antibiotics discs (about 6 mm in diameter) (as mentioned above), were aseptically placed on the agar surface. The plates were incubated at 37°C for eighteen hours. Diameters of inhibition of growth zones were measured and compared with CLSI chart as either sensitive or intermediate or resistant. Methicillin, carbapenem and colistin resistant bacteria were carried out for further analysis.

3.1.4 Biofilm formation by test organisms

Brain Heart infusion agar with sucrose was autoclaved. Then, autoclaved congo red indicator solution (8 gm/ml) was mixed at 55°C and poured to the plates. The test organism was inoculated by streaking and incubated at 37°C for 24 hours aerobically. After incubation, black colony morphology was observed as biofilm producers.

3.1.5 Quality control

The quality of test culture and biochemical media was checked by using above obtained ATCC strains. For every new batch of experiment, they were used to ensure correct staining reaction, biochemical tests and AST.

3.1.6 Glycerol stock preparation

The ATCC and test organisms were preserved by glycerol stock preparation in Luria Bertani broth (LB) containing 25% glycerol. For that, organisms were inoculated in 1 ml sterile LB and incubated overnight followed by aseptic addition of equal volume of 50% sterile glycerol. The resulting broth was mixed properly by shaking well and then stored at -20°C.

3.1.7 Molecular Identification of Test Bacteria

3.1.7.1 Genomic DNA extraction of test Bacteria

Bacterial DNA was extracted from the TIANGEN DNA secure kit protocol (http://www.tiangen.com/asset/imsupload/up0688479001433140730.pdf) but slight modification was done. Three ml of bacterial culture suspension was pipetted in a centrifuge tube and centrifuged for one minute at 10,000 rotation per minute (rpm). To the pellet, 200 microlitres(µl) of Buffer GA (for Gram negative bacteria) was added and mixed thoroughly by vortexing. For Gram positive bacteria, that step was replaced by lysozyme treatment. For that, 180 µl of enzymatic lysis buffer [(20 m M Tris-Cl, p^H 8.0; 2 mM sodium EDTA; Triton X-100; lysozyme (final concentration of 20 mg/ml)] was added. Then, it was incubated for 30 minutes at 37°C. Lysozyme was prepared with buffer, otherwise the lysozyme wouldnot active. 4 µl of RNase A (100 mg/ml) was mixed by vortexing for 15 seconds and incubated for 15 minutes at 37° C. After incubation, 2 µl of proteinase K was added by vortexing and incubated for 25 minutes at 37°C. 220 µl of Buffer GB was added to the sample, vortexed for 15 seconds and incubated at 70° C for 10 minutes to yield a homogenous solution. It was briefly centrifuged to remove the drops from the inside of lid. 200 µl of absolute ethanol was added to the sample and again mixed thoroughly by vortexing for 15 seconds. It was then, briefly centrifuged to remove the drops from the inside of lid. The mixture was pipetted into the spin column CB_3 (in a 2 ml collection tube) and centrifuged at 12,000 rpm for 30 seconds.

Flow through was discarded and spin column was placed into the collection tube. After that, 500 µl of Buffer GD (absolute ethanol had been added) was added to spin column CB_3 and centrifuged at 12,000 rpm for 30 seconds. After flow through was discarded and spin column was replaced into the collection tube, 600 µl of buffer PW (absolute ethanol had been added) was added to spin column CB3 and centrifuged at 12,000 rpm for 30 seconds. Flow through was discarded and spin column was placed into the collection tube and step was repeated. After that, centrifugation was done at 12,000 rpm for 2 minutes to dry the membrane completely. It was air dried for 20 minutes. The spin column CB3 was placed in a new centrifuge tube and 30 µl of Buffer TE was pipetted

directly to the centre of the membrane. It was incubated at room temperature (RT) for ten minutes and centrifuged for two minutes at 12,000 rpm. The final eluted supernatant was taken as extracted genomic DNA. One µl of the extracted genomic DNA was loaded in the nano drop and concentration of DNA was noted.

3.1.8 Polymerase Chain Reaction of the Test Bacteria

The genomic DNA extracted from the different multidrug resistant test organisms, (specially methicillin, carbapenem and colistin resistance) were used as template for the following PCR programmes. PCR products were then visualized on Gel Doc system using 1% agarose gel.

a. 16S rRNA PCR : Universal 16S rRNA bacterial gene primers were used to amplify the gene from genomic DNA using the following PCR program (Table 3) and reaction mixture (Appendix:8.1).

Table 3: PCR condition for 16S rRNA gene primer

Steps	Temperature(°C)	Time	Cycle
Initial Denaturation	95	2 mins	1
Denaturation	95	30 sec	
Annealing	52	35 sec	35
Extension	72	60 sec	
Final Extension	72	10 min	1
Hold	4	∞	

b. *Salmonella* specific 16S rRNA PCR: The genomic DNA of *Salmonella* Typhi was used as template for *Salmonella* specific 16S rRNA PCR. Following PCR program was carried out (Table 4) using reaction mixture (Appendix: 8.1) to obtain desired amplicon size.

Table 4: PCR condition for *Salmonella* specific 16S rRNA PCR

Steps	Temperature(°C)	Time	Cycle
Initial Denaturation	95	2 mins	1
Denaturation	95	30 Sec	
Annealing	52	35 Sec	30
Extension	72	45 Sec	
Final Extension	72	10 min	1
Hold	4	∞	

***c. bla* NDM and *mcr*-1 PCR for carbapenem and colistin resistance genes**

The genomic DNA extracted from all Gram negative test organisms were used as template for *bla*NDM and mcr-1 PCR. Following PCR program was carried out (Table 5 and 6) using reaction mixture (Appendix: 8.1) respectively to obtain desired amplicon size.

Table 5 : PCR condition for *bla* NDM PCR for carbapenem resistance genes

Steps	Temperature (°C)	Time		Cycle
Initial Denaturation	95	2 mins		1
Denaturation	95	30 sec		
Annealing	56	30 sec		14
Extension	72	1 min 30 sec	+ 0.5 (°C) per cycle	
Denaturation	95	30 sec		
Annealing	49.7	30 sec		19
Extension	72	1 min 30 sec		
Final Extension	72	5 mins		
Hold	4	∞		

Table 6 : PCR condition for *mcr*-1 gene

Steps	Temperature(°C)	Time	Cycle
Initial Denaturation	95	5 mins	1
Denaturation	95	1 min	
Annealing	52	30 Sec	35
Extension	72	45 Sec	
Final Extension	72	10 mins	1
Hold	4	∞	

3.1.9 Sanger Sequencing of 16S rRNA genomic DNA PCR amplicon

The isolated strains *Sal4, Pseudo34 and Pseudo39* were sent for Sanger Sequencing in Xcelris Laboratory Limited, India and BLAST was performed using the NCBI tool to find the 16S rRNA gene similarity with respective organisms.

3.2 Computational part

3.2.1 Homology Modeling of target protein

Comparative modeling allows the sequence alignment between the template sequence and target sequence to construct the 3D structure. Since, the 3D crystallography structure of target protein of uroporphyrinogen III methyltransferase of *Salmonella enterica* subsp. enterica serovar Typhimurium, also known as CobA, was not available in rcsb database, the structure of the target protein using its template was generated by using Phyre2 server (http://www.sbg.bio.ic.ac.uk/~phyre2).

3.2.2 Protein and Inhouse Ligand Database Preparation

Prior to molecular docking, it is necessary to prepare the proteins and ligands for the efficient and accurate docking results. Protein (Uroporphyrinogen III methyltransferase) can be prepared by adding hydrogen atoms, merging non-polar bonds, adding Kolman's charges in mgltools. Similarly, ligand (Kinase Inhibitors) was prepared in Openbabel GUI

(O'Boyle *et. al.*, 2011a) available in PyRx interface by energy minimization and converted to pdbqt file format, a useable file format for docking afterwards.

3.2.3 Insilico ADME/Tox Screening

ADME/Tox screening was performed to cut off the ligands which do not pass the ADME/Tox test before doing the virtual screening. The toxic profile and drug likeness based on the binding energies were calculated using OSIRIS program (Sander *et. al.,* 2015). OSIRIS calculated various principal descriptors such as molecular weight, cLogP, cLogS, druglikeness and toxicities like mutagenicity, tumorigenicity, reproductive and irritant effects in the lead molecules on the basis of functional groups present in their structures.

3.2.4 Binding Site Analysis and Prediction

Usually, binding site of protein is highly conserved among the closely related proteins. A highly conserved group of amino acid residues within the binding site determines the functional activity of a protein. Active site identification includes the superimposition of the model with template that provided integrity of the homology model and assisted in positioning conserved active site residues. The binding site residues of the lead proteins were predicted by 3DLigandSite (Wass *et. al.,* 2010a) server (http://www.sbg.bio.ic.ac.uk/3dligandsite/). It is based on the meta-server approach to investigating the ligand binding site in target protein structure using comparison with complementary binding-specific substructure and sequence profile alignment.

3.2.5 Structure Based Virtual Screening

Virtual screening was performed using Autodock Vina in a virtual screening software, PyRx (Dallakyan and Olson, 2015a) against the lead target proteins with the prepared ligands. The highest binding energy among the ligand database molecules were taken as lead compounds for further analysis and processing.

3.3 Isolation and screening of putative *Streptomyces* from different areas of Nepal

3.3.1 Materials required for isolation and screening of *Streptomyces*

Soil samples, International *Streptomyces* Project 2 (ISP2) and International *Streptomyces* Project 4 (ISP4) agar plates and broth, autoclaved distilled water, test tubes, Cycloheximide 50 µg/ ml and Nalidixic acid(NA) 30 µg/ ml.

3.3.1.1 Study Area

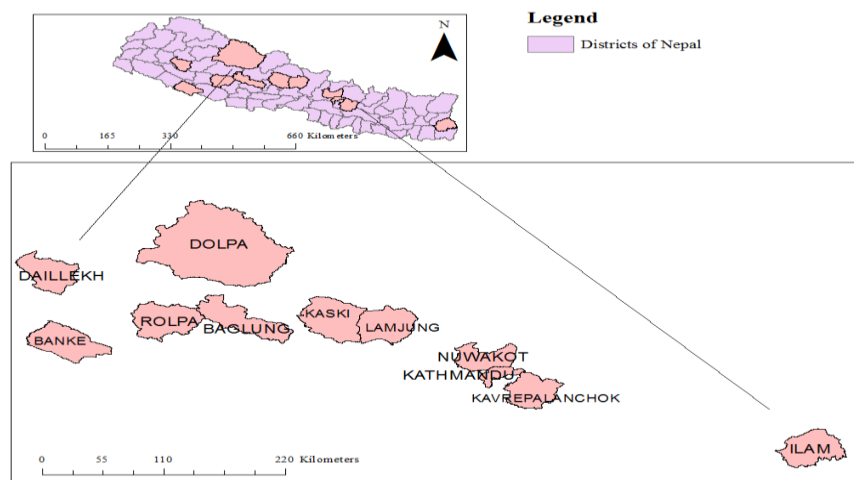


Figure 20 : Major Soil Sample collection sites of Nepal

The main focused study area of my thesis work was various geographical locations of Nepal because of its well known natural habitat of variety of microorganisms such as *Streptomyces* with novel secondary metabolites for the production of antibiotics.

3.3.1.2 Soil Sample collection

Soils were collected as sample for the isolation of putative *Streptomyces* from various geographical location of Nepal from 2016 to 2018. For that, selected sites were agricultural and forest soils, soils from preserved areas, rhizospheric soils of the plants, dry soils and muddy soils too. The soil samples were taken from the depth of about 15 cm after removal of upper surface soil. The samples were closely packed in polyethylene bags with proper labeling and stored in the refrigerator at 4°C at CDBT.

3.3.2 Preliminary isolation and screening of *Streptomyces*

The primary isolation and enumeration of putative *Streptomyces* were performed by serial dilution technique. ISP2 and ISP4 agar plates were mainly used among all the ISP medias (Shirling and Gottleib, 1996) supplemented with cycloheximide 50 µg/ml and Nalidixic acid (NA) 30 µg/ml as an antifungal and inhibitor of Gram negative bacteria respectively.

For preliminary screening of putative *Streptomyces*, soil samples were dried on hot air oven at 65 °C for about one hour to kill vegetative cells of bacteria. One gram of soil sample was suspended in 9 mL of sterile autoclaved distilled water in test tube and slightly vortexed for mixing. 1 ml was then transferred to 9 ml sterile autoclaved distilled water of second test tube to make 10^{-2} dilution. The dilution was carried out upto 10^{-4} dilutions in the similar way. Aliquots (100 µl) of 10^{-4} were spread on the ISP agar plates under aseptic conditions and dried. The plates were then incubated at 30^{0} C for 7 to 15 days with regular monitoring. Based on the colony morphology, the *Streptomyces* cultures were subcultured on ISP2 medium by streaking and incubated at 30^{0}C for five to seven days until the pure culture was obtained. Colony morphology and different cultural characteristics with pigmentation were recorded.

Criteria for isolate selection

Inclusion Criteria: Only the auxenic isolates obtained as chalky appearance with different colours on substrate and aerial mycelium and earthy odour were included for further analysis in this study.

Exclusion Criteria: Mucoid, contaminated with bacteria and fungi and the samples' code lacking with proper labeling on the agar plates were excluded.

3.3.3 Microscopic Observation of the putative *Streptomyces*

Microscopic observation of putative *Streptomyces*was done through Gram staining. For gram staining, same protocol was followed as that of the test organisms.

3.3.4 Biochemical Tests of the putative *Streptomyces*

Different biochemical tests of the putative *Streptomyces* were performed as that of the test organisms. But, incubation was done at 30^0C. Besides that, other biochemical tests performed were as follows:

Nitrate Reduction Test: Nitrate Broth was autoclaved in sterile culture tubes and pure colony of the putative *Streptomyces* was inoculated aseptically. The tubes were incubated at 30^0C for 48 hours. 5 drops of nitrate solution A and B were added and red colouration was observed. If necessary, a pinch of zinc powder was added and looked for the development of red colour.

Oxidative/ Fermentative test: The culture tubes with Oxidative/ Fermentative medium was autoclaved and the putative *Streptomyces* was stabbed aseptically with a sterile inoculating wire. One of the medium was sealed with 1 ml of sterile paraffin oil to create anaerobic condition. Both the tubes were incubated at 30^0C for 48 hours and colour development was observed.

Starch Hydrolysis Test: The starch agar medium was autoclaved and pure colony of the putative *Streptomyces* was inoculated by making a line in the centre of the plate by using a sterile inoculating loop. The plate was incubated at 30^0C for 48 hours in an inverted position. After the pure colony was observed on the inoculated line, Gram's Iodine solution was flooded around the line and clear zone was observed.

Gelatin Hydrolysis test: The nutrient gelatin (nutrient agar containing 1% gelatin) was prepared in the culture tubes and the putative *Streptomyces* was inoculated with the inoculating needle by stabbing the inoculum to 1/3 part of the tube. The tubes were incubated at 30^0C for 48 hours and liquefaction was observed.

3.3.5 Molecular Identification and Confirmation of isolated putative *Streptomyces*

3.3.5.1 Genomic DNA Extraction

Genomic DNA was extracted from the putative *Streptomyces* strains from Tiangen Kit as mentioned above.

3.3.5.2 Polymerase Chain Reaction (PCR) of putative *Streptomyces*

The genomic DNA extracted from putative *Streptomyces* were used as template for the following PCR programmes. PCR products were then visualized on Gel Doc system using 1% agarose gel.

a. 16S rRNA PCR : Universal 16S rRNA bacterial gene primers were used to amplify the gene from genomic DNA of putative *Streptomyces* (PCR program as in Table 3 and reaction mixture in appendix 8.1).

b. *Streptomyces* specific PCR

After putative *Streptomyces* were amplified by the Universal 16S rRNA bacterial primers, it was further confirmed by *Streptomyces* specific PCR using two sets of primers. Following PCR program was carried out (Table 7) respectively using reaction mixture (Appendix: 8.1) to obtain desired amplicon size.

Table 7: PCR condition for *Streptomyces* specific gene PCR (Set I and Set II)

Steps	Temperature(°C)	Time	Cycle
Initial Denaturation	98	5 mins	1
Denaturation	95	1 min	
Annealing	54 (Set I)	40 secs (Set I)	30
	58 (Set II)	40 secs (Set II)	
Extension	72	2 mins	
Final Extension	72	10 mins	1
Hold	4	∞	

3.3.6 Secondary metabolite production from preliminary confirmed *Streptomyces*

For the secondary metabolite production, 50 ml ISP2 and ISP4 broth (incorporating with glass beads) were inoculated with putatitive *Streptomyces* and incubated at 30°C for 7-10 days in a shaker (150 rpm). During this period, the cultures were regularly checked to prevent it from contamination. After 10 days, the crude extracts were stored at 4°C until further use.

3.3.7 Antimicrobial susceptibilty testing of the crude extract

Test Organisms: The strains which were confirmed as methicillin, carbapenem and colistin resistant from antibiotic sensitivity testing of my study were used as test organisms.

Agar Well Diffusion Method: This method was used to evaluate the antimicrobial activity of crude extract. In this method, the test organism suspension (0.5 McFarland standard) was evenly spread onto dried MHA plates by dipping a sterile cotton swab twice into the suspension and the agar rim was made finally by the sweep of the swab. Then MHA plate was incubated at 37°C for half an hour to activate the test organism. A

hole of diameter of 6 mm was punched with a cork borer or a tip and 100 µl of the crude extracts of *Streptomyces* were introduced into the well. Then, plate was incubated at 37°C for 24 hours. The antimicrobial extracts diffused in the agar medium and zone of inhibition of growth of the test bacteria was measured by comparing with zone of inhibiton given by the substrates only.

Since, insignificant or very small zone of inhibition was observed, original ISP media was modified by altering the constituents of the media. The compounds that resemble with phenolic and lignin degradation putative drug molecules obtained from virtual screening with target identification with antimicrobial property were added to modified ISP media. The indole and phenolic molecules obtained from lignin degradation pathway such as furfural, transferulic acid, vanillic acid, coumarin, vanillin, furfuraldehyde, acetic acid, guaiacol and tannic acid were used as supplemented sources as source of carbon and nitrogen in the modified media.

Table 8: Modified media composition for *Streptomyces*

S.N.	Macronutrients	Gram/ Litre
1	Di- potassium phosphate	0.5
2	Magnesium sulphate	0.2
3	Sodium chloride	0.1
4	Calcium chloride	0.02
5	Ferrous sulphate	0.1
6	Ammonium sulphate	2.0
7	Potassium chloride	0.1
8	Indole / lignin degradation product	5 miliMolar

S.N.	Micronutrients	Gram/ Litre
1	copper sulphate penta hydrate	0.4
2	Zinc sulphate	0.12
3	Phosphorous acid	1.4
4	sodium molybdate	1.0
5	Manganese II sulphate mono hydtrate	1.5

After modification of the media, putative *Streptomyces* were again cultured on 50 ml modified media with indole only, lignin degradation product only and indole plus lignin degradation product. The cultures were incubated at 30°C for 7-10 days in a shaker (150 rpm) for secondary metabolite production and kept at 4 °C until use.

In my study, the concentration (5mM) of lignin degradation product was calculated after performing toxicity test of that compound. For that, autoclaved five culture tubes with modified media were taken. The first culture tube contained 20 ml of media and rest four contained 10 ml of modified media. 10 mM furfural was added to the first culture tube and serial dilution was performed upto last tube by transferring 10 ml media such that concentration of furfural became half in each dilution. 1 ml media was taken from each diluted tube for blank measurement in spectrophotometer. Then 100 µl of

overnight grown *Streptomyces* suspension (0.5 McFarland standard) strain was inoculated in each tube and incubated at 30°C for 2 days in a shaker (150 rpm). Then optical density was measured at 600 nm by using blank for each concentration.

Agar well diffusion method was again performed for antimicrobial test. All total 45 strains of Streptomyces were inoculated in modified media supplemented with indole only, lignin degradation product only and indole plus lignin degradation product. Among these, the crude non concentrated extracts of six strains containing indole plus tannic acid showed the zone of inhibition against carbapenem and colistin resistance *S.* Typhi strain. Out of six strains, the strains PA2, PA3, MA1 and KA10 showed the highest zone of inhibition.

3.3.8 Molecular analysis and phylogenetic tree construction

The isolated putative strains (PA2, PA3, MA1 and KA10) that showed the zone of inhibition was sent for Sanger Sequencing in Xcelris Laboratory Limited, India. After the chromatogram of sequenced *Streptomyces* were obtained, they were further analyzed by BLAST program. On the basis of similarity shown to other strain in BLAST program, phylogenetic tree was constructed by the use of Clustal W for further analysis.

3.3.9 Salinity test of the sequenced *Streptomyces* strain PA3

Among four strains of *Streptomyces* were identified as positive strains for antimicrobial property, salinity test was performed for only one strain. At first, culture tubes with 5 ml ISP2 broth were taken and sodium chloride from 1% to 14% were added in each tube respectively. Then single pure colony of the sequenced *Streptomyces* strain PA3 was inoculated aseptically and incubated at 30°C for 2 days in a shaker (150 rpm) and absorbance was measured at 600 nm.

3.4 Revival and subculture of *Streptomyces coelicolor* strain M145 (SCP1-/SCP2-)

S. *coelicolor* strain M145 (SCP^{1-}/SCP^{2-}) was revived from cryopreserved stock in ISP2 broth and then pure single colony was obtained by streaking on ISP2 agar and starch casein agar (SCA) plates.

3.4.1 Secondary metabolite production from *S. coelicolor* strain M145 (SCP1-/SCP2-) and isolated *Streptomyces* strain PA3 for resazurin assay

Pure colonies of S. *coelicolor* strain M145 (SCP^{1-}/SCP^{2-}) and isolated strain PA3 were inoculated on first set of autoclaved 50 ml modified media with 5 mM lignin degradation product (compounds mentioned as above) only and second set of modified media with 5 mM lignin degradation product plus 5 mM two amino acids valine and tryptophan respectively.

Similarly, pure colonies of these two strains were also inoculated on first set of autoclaved 50 ml modified media with different 5 mM carbon sources such as glucose,

sucrose, starch, sorbitol, maltose, mannitol, lactose, pectin and cellulose. Second set of modified media contained 5 mM two amino acids valine and tryptophan respectively along these carbon sources.

The media were then incubated at 30°C for 7-14 days in a shaker (150 rpm) for secondary metabolite production and kept at 4 °C until use.

During that period, after eight days, growth of those two strains were measured in the interval of two days upto twelve days at 600 nm.

3.4.1.1 Resazurin Antimicrobial Assay

The test organism suspension equivalent to that of a 0.5 McFarland standard was prepared by dilution with LB broth. For resazurin assay, a 96 well microtiter plate was taken (Appendix 8.2 and 8.3). Then, 48 µl of bacterial culture along with 50 µl extract and 2 µl 1% resazurin solution was added in each well to maintain the final volume as 100 µl in each well and final resazurin concentration as 0.02%. The wells having only LB broth and resazurin solution without test organism were taken as positive control and the wells containing only resazurin and test organism without antimicrobial extracts were taken as negative control. 50 mg/ml ampicillin and kanamycin solution were used as positive control of antibiotics. It was then incubated at 37˚C until the color change in negative control was observed as pinkish color. Finally, absorbance reading was taken at 551 nm. The percentage inhibition of the test organisms was calculated by evaluating the strength of the extracts as follows:

% inhibition = O.D reading with test extract/ (Average of positive controls − Average of negative controls) X 100 %

3.5 Cloning of the essential CobA gene of the Multidrug Resistance Pathogens

Materials, reagents and chemicals used for gene cloning

TECHNE 5Prime-G/02 PCR machine was used for amplification of DNA. Restriction enzymes used were *EcoR*I HF (Thermo-scientific), *Bam H*I (TakaRa CloneTech), *Spe*I (TakaRa CloneTech) and PCR Master Mix (2X) of Zymogen company. T4- DNA ligase and its buffer were also obtained from TakaRa CloneTech, China. The primers amplifying the DNA were purchased from Macrogen, Korea. The Gel Doc was used to visualize Ethidium Bromide (EtBr) stained DNA. All the chemicals and reagents were obtained from Hi-media and Thermo-fischer company.

Cloning vector

pUC19 vector (from TakaRa CloneTech) with size of 2686 bp was used as cloning vector that possesses an ampicillin resistance gene as a selection marker for transformed bacteria.

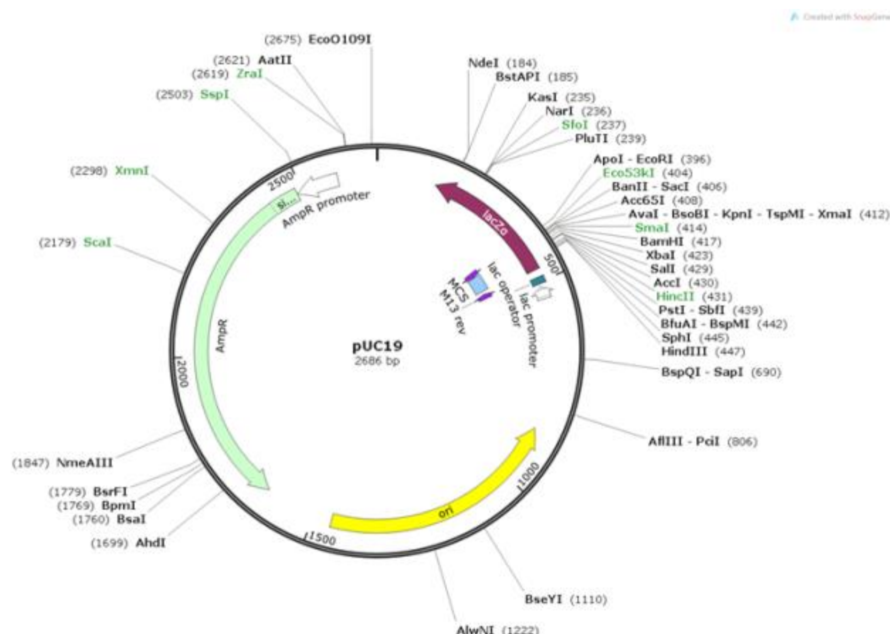


Figure 21: Vector map of pUC19 cloning vector with promoter and multiple cloning sites (MCS).

3.5.1 Primer designing and primer sequence

The cobA gene specific set of promoter primer {(CobA F100), (CobA F200), (CobA F600) and CobA R} was designed against *P. aeruginosa* (PA 96 genome) manually using different online tools named as OligoCalc (The oligonucleotide properties calculator), M-fold (the m-fold web server) and IDT (The integrated DNA technology). The restriction site of *EcoR*I (***G AAT TC***) was created in forward primers where as sites of *BamH*I (***GG ATC C***) and *Spe*I (***AC TAG T)*** were designed in reverse primer respectively.

Similarly, the *Lac Z* gene specific set of ORF primer (Lac Z F and Lac Z R) was designed manually by using *E coli* CFT073 with restriction sites *Spe*I and *Bam H*I respectively.

A set of cobA and *Lac Z* gene specific primer sequences for full length amplification is as follows.

Table 9 : Primers and their sequences of CobA and *Lac Z* gene

Primer	Sequence	Mer	Tm	Amplicon (bp)
CobA F100	5'- GAA CT***G AAT TC***C GCC TCG GCA AGC AAC T -3'	29	73.3	2522
CobA F2O0	5'-CAA TA***G AAT TC***G GCG AAC GCA TCC TCG GC -3'	29	73.3	2645
CobA F600	5'-GCC GAG ***GAATTC*** GAT GAA ATC AAC CCG CTG -3'	30	73.5	3058
Cob A R	5'-CAT T***GG ATC C***GT AAC CAT T***ACTAGT***TC TCC TCA GGC ATT CG -3'	41	77.9	-
Lac Z F	5'- CGG C ***AC TAG T***AT GAC TAT GAT TAC GGA TTC -3'	30	69.4	3075
Lac Z R	5'-ATA C***GG ATC C***TT ATT TTT GAC ACC AGA CCA AC -3'	32	70	-

Bacteria used in the study

Escherichia coli: *E. coli* DH5α was used as host bacteria for cloning of plasmid DNA and construct was prepared. The genotype of the DH5α is F – Φ80 lacZ ΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK–, mK+) phoA supE44 λ– thi-1 gyrA96 relA1.

Similarly, ATCC strain of *E. coli* 25923 and *P. aeruginosa* 27853 were used for *Lac Z* gene amplification and CobA gene amplification.

Culture media: *E. coli* DH5α was cultured in LB medium (broth and agar) with or without antibiotics as per requirements. Similarly, *P. aeruginosa* 27853 was cultured in LB broth for genomic DNA isolation.

3.5.2 Transformation of pUC19 in *E. coli* DH5α

Preparation of competent cells (Calcium chloride method)

A loopful of *E. coli* DH5α from glycerol stock was inoculated in 5ml LB broth. After overnight incubation at 37°C (200 rpm), 1 ml of this culture was inoculated in 50ml of LB broth in 250 ml conical flask and incubated at 37°C (200 rpm) till the $O.D._{600}$ reached 0.4. Culture was then aliquoted in two chilled falcon tubes and chilled in ice for 10 minutes. Then centrifugation was done at 4000 rpm for 10 minutes to harvest the cells and supernatant was discarded. Pellet was washed with autoclaved chilled distilled water (pellet was suspended in autoclaved ice-cold distilled water and centrifuged at 4000 rpm for 10 minute and supernatant was discarded). Pellet was re-suspended in 30 ml solution of mixture of chilled 80mM Magnesium chloride and 20 mM Calcium chloride. Suspension was again centrifuged at 4000 rpm for 10 minutes and pellet was re-suspended in 1 ml 100 mM Calcium chloride. Suspension was aliquoted 200 µl each in fresh ice chilled eppendorf tube and directly used for transformation by heat shock.

Heat shock

200 µl of competent cell was taken in a fresh ice chilled eppendorf tube and 1 µl (100 ng) plasmid (pUC19) was mixed with it. The mixture was chilled in ice for 30 minutes. The tube was then placed in pre-heated water bath for heat shock at 42°C for 90 seconds. Immediately after heat shock, 1 ml of LB media was added in tube and incubated at 37°C and 200 rpm agitation for 1 hour. After incubation, tube was centrifuged at 8000 rpm for 1 minute and supernatant was discarded. Pellet was suspended in 100 µl of fresh LB medium and plated on LB Agar supplemented with 100 µg/ml ampicillin. The plates were then incubated at 37°C overnight.

3.5.3 Selection of Transformants

Plasmid was provided with ampicillin resistance gene as selection marker, indicating that only those colony harboring pUC19 plasmid will grow on LBA ampicillin plate. The colony growing on LB-ampicillin agar media were sub-cultured in LB-ampicillin agar plate and in LB ampicillin (50 µg/ml) broth.

Confirmation of Transformants

Transformants were selected on the basis of ampicillin resistance on LBA plate. Isolated colonies were randomly selected among transformants and sub-cultured of LB broth supplemented with 50µg/ml ampicillin for plasmid extraction by alkaline lysis method.

Plasmid Extraction by Alkaline lysis method

Cell culture and cell harvesting

Isolated colony from LB-ampicillin plate were sub-cultured in 50 ml LB ampicillin (100µg/ml) broth overnight at 37°C and 200 rpm. The culture was aliquoted in 1.5 ml eppendorf tube and centrifuged at 13000 rpm for 1 minute. Supernatant was discarded completely followed by draining with micropipette. 500 µl STE buffer was added and mixed gently by vortexing. Then tube was centrifuged at 13000 rpm for 1 minute and supernatant was discarded.

Cell lysis and plasmid recovery

Cell pellet was suspended in 200 µl of Alkaline lysis solution-I with pipette and vortexing. Then 200 µl of freshly prepared alkline lysis solution-II was added and inverted gently for five times (at this stage denaturation of cell components occurs indicated by viscous nature developed in suspension). Immediately after this, 200 µl renaturation solution i.e., alkaline lysis solution-III was added and inverted few times and incubated in ice for 5 minutes (at this point renaturation of nucleic acid occurs).

Plasmid precipitation

After 5 minutes incubation in ice, tubes were centrifuged at 13000 rpm for 8 minutes. The supernatant was then transferred in fresh tube with the help of micropipette. 1 µl of RNase solution was added and incubated at 37°C for 30 minutes. Double volume of supernatant i.e., 1 ml of ice cold isopropanol was added to the solution and left in ice for 30 minutes allowing precipitation of nucleic acids. Then mixture was centrifuged at 13000 rpm for 10 minutes and supernatant was discarded. Pellet was washed with 1 ml of 70% ethanol and centrifuged again for 5 minutes at 13000 rpm. Supernatant was discarded and completely drained with help of micropipette and allowed to air dry for about 15 minutes. Then pellet was dissolved in 20µl of nuclease free water (NFW) and used for visualization by 0.8% agarose gel-electrophoresis at 60 volt for 60 minutes. Concentration of isolated plasmid was quantified by nano drop reading.

3.5.4 CobA promoter and Lac Z gene PCR amplification

The genomic DNA samples isolated from ATCC strain of *E. coli* 25923 and *P. aeruginosa* 27853 were quantified spectrophotometrically and used as template DNA for PCR amplification of cobA promoter and *Lac Z* gene respectively. PCR amplification was performed by preparing the reaction mixtures as follows (Table 10) and PCR condition was optimized. Then 3 µl of PCR product was subjected to 1% agarose gel

electrophoresis along with 1kb DNA (Thermoscientific) in a separate well as described previously.

Table 10: Reaction mixtures for CobA and *Lac Z* gene amplification

Components (stock concentration)	Volume (working concentration)
Template(100ng/µl)	2 µl (174 ng)
Forward primer (10pM/µl or 10µM)	2 µl (1 µM)
Reverse primer (10pM/µl or 10µM)	2 µl (1 µM)
DMSO	0.2 µl
Master Mix (2X)	10 µl (1X)
NFW	3.8 µl (To adjust final volume)
Total	20 µl

PCR Product Purification

After confirmation of correct amplicon size from gel electrophoresis, PCR product was purified by using Foregene Kit with slight modification. First, four times the buffer BD (corresponding volume of isopropanol was added) of the PCR product volume and one volume of buffer BD-S was added. The solution was then transferred to the spin column, held for 1 minute and centrifuged at 12,000 rpm for 1 minute.

After flow through was discarded from the collection tube, 700 µl of buffer WB1 was added to the spin column, held for 1 minute and centrifuged at 12,000 rpm for 1 minute. Flow through was discarded and step was repeated. Spin column was placed back into the collection tube. It was centrifuged at 12,000 rpm for 2 minutes and residue was discarded from collection tube. Spin column was moved to a new 1.5 ml centrifuge tube and 30 µl of NFW was added to the middle of the silica gel membrane. It was incubated at room temperature (RT) for 5 minutes and centrifuged for 1 minute at 12,000 rpm. The final eluted supernatant was taken as purified PCR product and used for restriction digestion.

3.5.5 Restriction digestion of CobA promoter and pUC19 vector

Purified PCR product of CobA F100 promoter (first Insert) and pUC19 plasmid (vector) was restriction digested with enzymes *Eco R*I and *Bam H*I as given below.

Restriction digestion of insert (Optimized) Restriction digestion of vector (Optimized)

Insert (Cob A)	20 µl(130 ng)
*Eco*R I (2U/µl)	0.3 µl
*Bam*HI (3U/µl)	0.3 µl
Buffer K(10X)	2 µl
NFW	7.4 µl
Total	30 µl

Vector (pUC19 plasmid)	30 µl(110 ng)
*Eco*R I (2U/µl)	0.5 µl
*Bam*HI (3U/µl)	0.5 µl
Buffer K(10X)	5 µl
NFW	14 µl
Total	50 µl

Reaction mixtures were incubated at 37 ˚C for 3 hours for complete digestion. After incubation, tubes were kept at 65ºC for 30 minutes to stop the digestion reaction and

directly used for 0.8 % low melting agarose gel electrophoresis along 1 kb DNA ladder from thermoscientific.

0.8 % Low melting Agarose Gel electrophoresis.

0.8% low melting agarose was prepared by melting 0.4 gm of agarose in 50ml 1X TAE. Digestion product of insert and puc19 vector were subjected to gel electrophoresis using 1kb DNA ladder from Thermoscientific. The gel run was performed for 80 minutes at 60 V and visualized in Gel Doc (low intensiy at 254 nm). The bands of insert and vector of desired size were cut out and stored in fresh centrifuge tube for further experiment.

Gel Elution for Insert and vector purification from low melting agarose gel

Bands of insert and vector seen on required size were cut out with scalpel and placed in fresh centrifuge tube. Weight of the gel piece were taken and it was used for elution of insert and vector using the Kit.

The weight of gel piece was calculated as-

Weight of gel piece = final weight (tube + gel) – initial weight (empty tube)

Restriction digested products purification from QIA quick® Gel Extraction Kit

Restriction Digested Insert and vector DNA were extracted from QIA quick® Extraction Kit, (Cat No. 28704) with slight modification. After weight of gel piece was calculated, 3 volume of buffer QG was added to 1 volume of gel (100 mg nearly equals to 100 µl). The mixture was heated at 50^0 C for 10 minutes. During that time, tube was vortexed every 2-3 minutes to help dissolve gel. Then, 1 gel volume of isopropanol was mixed and sample was applied to spin column having collection tube. After centrifugation at 13,000 rpm for 1 minute, flow through was discarded and column was placed to the same tube. It was left at RT for 5 minutes after 750 µl buffer PE was added. Then it was centrifuged at 13,000 rpm for 1 minute and column was placed to the same tube after discarding the flow through. It was centrifuged at 13,000 rpm for 1 minute and residue was discarded from collection tube. Spin column was moved to a new 1.5 ml centrifuge tube and 30 µl of NFW was added to the middle of QIA quick membrane. It was incubated at RT for 5 minutes and centrifuged for 1 minute at 13,000 rpm. The final eluted supernatant was taken as purified digested product and used for ligation reaction.

Gel Electrophoresis for Quantification of insert and vector

Eluted gene insert and vector was then visualized in 0.8% agarose gel as mentioned above along with 1kb DNA ladder for calibration and quantification. Quantification is based on the intensity of the band with respect to the ladder bands. The concentration of the digested vector and insert was estimated based on the intensity of band observed.

3.5.6 Ligation of insert and vector

DNA insert and plasmid vector i.e., CobA F100 gene and pUC19 vector prepared from double digestion were subjected to the ligation reaction activated by T4 DNA Ligase enzyme. The enzyme forms a covalent linkage between insert and vector similar to that of the phosphate backbone of DNA. Ligation mixture was prepared as follows.

Ligation Reaction Composition

Components (stock concentration)	Volume (working concentration)
T4 ligation buffer (10X)	2 µl (1X)
Insert (CobA F100 gene)	9 µl (105 ng)
Vector (pUC19)	3 µl (250 ng)
T4 DNA Ligase	0.5 µl
NFW	5.5 µl (To adjust final volume)
Total	20 µl

3.5.7 Transformation after ligation

The ligation mixture was incubated overnight at 4^0 C. After overnight incubation, whole of the ligation mixture was used for transformation into competent *E. coli* DH5α host bacteria by heat shock method. Briefly, 10 µl of ligated product added to 150 µl of *E. coli* DH5α competent cells. The transformation was performed in duplicate sets.

Screening of Transformants

Plasmid Extraction

Ampicillin resistant transformed colonies grown on plate were selected and inoculated to 10 ml of LB/ampicillin (100 µg/ml) broth and incubated at 37^0 C with shaking at 200 rpm. The plasmid was isolated as described previously by alkaline lysis method.

3.5.7.1 Restriction digestion

Isolated possible ampicillin resistance plasmid DNA was digested with restriction enzyme *Spe* I to confirm cloning of CobA promoter to pUC19. The restriction digestion mixture was prepared as follows and incubated at 37^0 C for three hours to allow digestion. Reaction mixture was subjected for 1% agarose gel electrophoresis and required size (total size of insert plus vector) was compared with 1 kb ladder run on the adjacent well.

Restriction digestion mixture (Single Digestion)

Transformed plasmid (pUC19)	4 µl(130 ng/ µl)
Spe I (10U/µl)	0.2 µl
Buffer M (10X)	1 µl
NFW	4.8 µl
Total	10 µl

Transformed plasmid (pUC19)	4 µl(130 ng/ µl)
Bam H I (3U/µl)	0.2 µl
Buffer K (10X)	1 µl
NFW	4.8 µl
Total	10 µl

3.5.7.2 Confirmation of CobA gene ligation into pUC19 vector post transformation in colonies by PCR

Transformation of vector plasmid with target gene was confirmed by full length PCR using the primer set CobA F100 and CobA R using plasmid extracted from transformant colony as template. After the required amplicon size was obtained from PCR, the prepared ligated plasmid (pUC19+CobA F100) construct was named as **"pAG101"** and used as vector plasmid for the *Lac Z* gene cloning.

3.5.8 Restriction digestion of *Lac Z* gene (second insert) and "pAG101"

After *Lac Z* gene PCR product was purified and transformant **"pAG101"** was obtained, they were proceeded for double restriction digestion reaction with *Spe*I and *Bam* HI. The reaction mixtures were as follows.

Restriction digestion of insert (Optimized)

Insert (LacZ)	25 µl (142 ng)
Spe I (10 U/µl)	0.5 µl
*Bam*HI (3U/µl)	0.5 µl
Buffer K(10X)	5 µl
NFW	9 µl
Total	50 µl

Restriction digestion of vector (Optimized)

Vector ("pAG101")	24 µl(179 ng)
Spe I (10 U/µl)	0.5 µl
*Bam*HI (3U/µl)	0.5 µl
Buffer K(10X)	5 µl
NFW	10 µl
Total	40 µl

Reaction mixtures were incubated at 37 ˚C for 3 hours for complete digestion. Then, tubes were kept at 65°C for 30 minutes to stop the digestion reaction. Whole mixtures were directly used for 0.8 % low melting agarose gel electrophoresis along with 1 kb DNA ladder from thermoscientific.

Digested products were visualized through gel electrophoresis on 0.8% low melting agarose, required size of bands were eluted from gel extraction kit and DNA was quantified as mentioned above.

3.5.9 Ligation Reaction

Lac Z DNA insert and **"pAG101"** vector prepared from double digestion were subjected to the ligation reaction. Ligation mixture was prepared as follows.

Components (stock concentration)	Volume (working concentration)
T4 ligation buffer (10X)	2 µl (1X)
Insert (Lac Z gene)	10 µl (285 ng)
Vector ("pAG101")	4 µl (192 ng)
T4 DNA Ligase	0.5 µl
NFW	5.5 µl (To adjust final volume)
Total	20 µl

3.5.10 Transformation after ligation

The ligation mixture was incubated overnight at 16^0 C. After overnight incubation, whole of the ligation mixture was used for transformation into competent *E. coli* DH5α cell by heat shock method. Briefly, 10 µl of ligated product added to 150 µl of *E. coli* DH5α competent cells. The transformation was performed in duplicate sets.

3.5.10.1 Confirmation of Transformants

Plasmid Extraction

The transformed colonies grown on ampicillin plate were selected and inoculated to 10 ml of LB/ampicillin (100 µg/ml) broth and incubated at 37^0 C with shaking at 200 rpm. The plasmid was isolated as described previously by alkaline lysis method.

Restriction digestion

Isolated ampicillin resistance plasmid DNA was digested with restriction enzyme *Spe*I to confirm cloning of *Lac Z* to "pAG101". The restriction digestion mixture was prepared as follows and incubated at 37^0 C for three hours to allow digestion. Reaction mixture was subjected for 1% agarose gel electrophoresis and required size (total size of *Lac Z* insert plus "pAG101") was compared with 1 kb ladder run on the adjacent well.

Restriction digestion mixture (Single Digestion)

Transformed plasmid "pAG101"	8 µl (130 ng/ µl)
Bam H I (3U/µl)	0.2 µl
Buffer K (10X)	1 µl
NFW	1.8 µl
Total	10 µl

Confirmation of *Lac Z* gene ligation into "pAG101" vector post transformation in colonies by PCR

Transformation of vector **"pAG101"** plasmid with target gene was confirmed by full length PCR using the primer set *Lac Z* F and *Lac Z* R using plasmid extracted from transformant colony as template.

4 RESULTS AND DISCUSSION

Streptomycetes as the producers of novel antibiotics (Lo Grasso *et.al*., 2016) could be considered as the life saving bacteria against the infectious diseases. World is currently facing the various health issues including rapid emergence of multidrug resistant bacteria (Nikaido, 2009). Hence, addressing such issue is becoming a tremendous task for all researchers and strakeholders. Foremost, it is prudent to identify the resistant organisms and their resistance patterns before venturing in tosolving this global threat. Moreover, XDR tuberculosis (Klopper *et. al.,* 2013) and other enriched MDR bacteria *Haemophilus influenza* (Nag *et. al.,* 2001), *Streptococcus pneumonia* (Whitney *et.al.*, 2000) are also getting more resistance towards existing antimicrobial drugs. In the present study, WHO prioritized pathogens (WHO (February 27, 2017)) that have been collected from different facilities and are available at CDBT were of focus.

4.1 Characterization and revival of bacteria from cryostock

The characterization of the pathogenic bacteria was done collectively with Safalta Mallick to make sure that the designated pathogens are the same organism. It has been suggested that the robust surveillance mechanisms followed by rapid but accurate diagnostic tools are of the need to tame the resistance pattern (Bhattacharya, 2013). There is high probability that in the hospital settings or diagnostic laboratories where skilled human resources could be lagging could mistake with the organism if they have some proximity. Thus, pathogen identification was done. This was because while looking for probability of developing rapid PCR based molecular diagnostic tool the presence of some other organism than that designated propelled us to look in parallel so that it could be verified. This suggests that the laboratory conditions of some of the diagnostic laboratories around Kathmandu Valley have to be updated. Though in the present works identification of the laboratory from where the particular strain was obtained is not known.

The bacterial strains (ATCC and pathogens) that have been stored as cryostocks were retrieved in LB broth, NA plates and respective selective agar plates for respective organisms. After streaking of a loop full of slightly thawed cryopreserved stock of the respective organisms in respective media containing plates, the single colony isolated (Figure 22) was taken as the starting material for further works. The respective media in some cases act as selective media, thus giving purity of the strain. Once the single colonies of respective bacteria were isolated, the subsequent works if otherwise not mentioned specific media, the works were carried out in LB or LA media. The ATCC bacterial strains were also proceeded as quality control strain for each experiment to verify the result obtained for the tests bacteria.

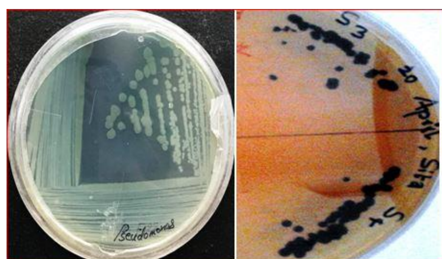


Figure 22: Isolation and revival of *Pseudomonas* on NA and *Salmonella* on SS agar

4.1.1 Gram stain and biochemical tests of isolated bacteria

Gram staining and biochemical tests are considered to be preliminary tests for the identification and confirmation of bacteria. This helps to distinguish bacterial families with their respective genus on the basis of Bergey's Manual (https://www.cabdirect.org/cabdirect/abstract/19872040344). The results of these tests (Table 11) demonstrate Gram stain and various biochemical tests for the respective pathogens and in concordance to be reported forthe respective pathogens.

Table 11: Gram stain and biochemical tests for identification of bacterial strain

S. N.	Test	Organism					
		Salmonella	*Klebsiella*	*Pseudomonas*	*Acenetobacter*	*Staphylococcus*	*Escherichia*
1	Gram stain	N	N	N	N	P	N
2	Indole	N	N	N	N	N	P
3	MR	P	N	N	N	P	P
4	VP	N	P	N	N	P	N
5	Citrate	P	P	P	P	P	N
6	Oxidase	N	N	P	N	N	N
7	Catalase	P	P	P	P	P	P
8	TSIA	Alk/acid	Acid/acid	Alk/Alk	Alk/alk	-	Acid/Acid

N: Negative, P: Positive, Alk: Alkaline

4.2 Antibiotic Susceptibility Test (AST) of the test pathogens

The preliminarily confirmed bacterial strains were tested for their antibiotics sensitivity against different classes of antibiotics. Out of total 32 bacterial isolates, total 16 isolates (4 strains of *Salmonella* (*Sal1, Sal3, Sal4, Sal6*), 1 strain of *Escherichia* (*Esch1*), 2 strains of *Pseudomonas* (*Pseudo34*, *Pseudo39*), 1 strain of *Staphylococcus* (*Staph1*), 1 strain of *Acenetobacter baumanii* (*Aceneto9*) and 7 strains of *Klebsiella* (*Kleb2, Kleb3, Kleb40, Kleb41, Kleb52, Kleb57, Kleb66*)) were found to be resistance to at least one of the antibiotics used in the discs (Figure 23).

Figure 23: Antibiotic susceptibility tests for *Sal4* and *Staph1* where, 1.MRP(10), 2.ETP(10), 3.G(30), 4.VA(30), 5.CL(25), 6.OX(1), 7.OF(5), 8.P(10), 9.PI(100), 10.IPM(10), 11.MET(5), 12.PIT(100/10)

Moreover, the focus was to study multi-drug resistant bacteria that are resistant to three or more antibiotics. MDR characteristics which were also carbapenem (either of meropenem, ertapenem, imipenem) and colistin resistance for Gram negative or methicillin resistance for Gram positive bacteria were selected for the present study. Among these 16 isolates, 8 isolates, 4 strains of *Salmonella*, 2 strains of *Pseudomonas, Aceneto9* and *Kleb66* were found to be both carbapenem and colistin resistance where as *Esch1* and other 6 strains of *Klebsiella* were found to be carbapenem resistance only. Similarly, the strain *Staph1* was found to be methicillin resistance (Figure 23).

Hence, seven selected strains showing resistant to the colistin, carbapenem and methicillin mainly *Sal4, Esch1, Pseudo34, Pseudo39, Kleb52, Aceneto9* and *Staph1* respectively were taken for further investigation. All of these seven strains were found to be biofilm producers indicating more harder to treat as biofilms prevent penetration of antibiotics to the cell (Donlan and Costerton, 2002). Among these *Klebsiella* strains, *Kleb52* and *Acenetobacter* (*Aceneto9)*, 16S rRNA genomic sequence were already sequenced and were determined to be *Klebsiella pneumoniae* and *Acenetobacter baummannii* respectively, by other group members working in bacteriophage mediated killing of these pathogens.

4.3 Molecular Identification of Test Bacteria

a. Sanger Sequencing of 16SrRNA genomic DNA

The biochemical tests that suggested the test pathogens were further confirmed through molecular characterization. In order to identify other isolates, 16S rRNA genomic DNA PCR amplification was performed using universal 16S rRNA PCR primer for amplification and the amplicon size of about 1500 bp upon comparsion with 100 bp ladder (Figure 24 A) confirmed the isolates were bacteria and this amplicon could be sequenced for sequence comparision.

Furthermore, the genomic DNA of *Salmonella* Typhi were additionally amplified by using *Salmonella* specific 16S rRNA genomic DNA amplifying primers that had been designed in earlier works at NARC, Animal Health Research Division, Khumaltar (generous gift

from Aarogya Gyawali). The amplicon size of 406 bp observed (Figure 24B) further confirmed that the isolated strains were *Salmonella* sps.

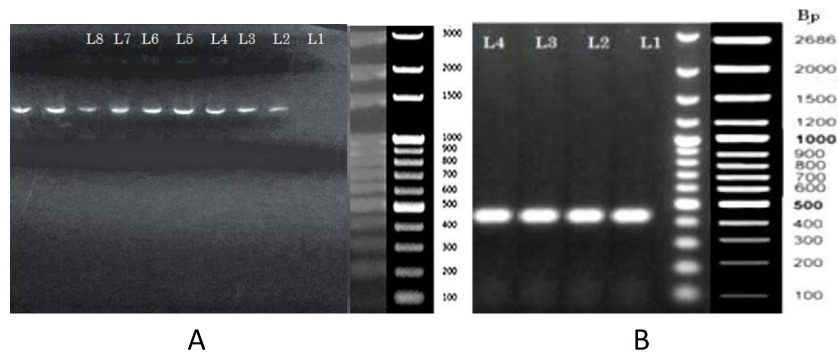


Figure 24 :A) Bands of PCR product of MDR (1500 bp) on 1% agarose (From Right Side: L2: *Sal4*, L3: *Esch*1, L4: *Pseudo34*, L5: *Pseudo39*, L6: *Kleb52*, L7 : *Aceneto*9 and L8 :*Staph1);* B): L1: Product of *Sal1*, L2: PCR Product of *Sal3*,L3 : PCR Product of *Sal4*,L4 : PCR Product of *Sal*6)

For the further molecular confirmation of the MDR bacteria, *Sal4, Pseudo34* and *Pseudo39* were taken for 16S rRNA genomic DNA sequencing. Upon comparing the chromatogram (Figure 25) sequences with other sequences of different *Salmonella* species, using BLAST tool of NCBI, it revealed high similarity with *Salmonella enterica* Typhi LT2 strain.

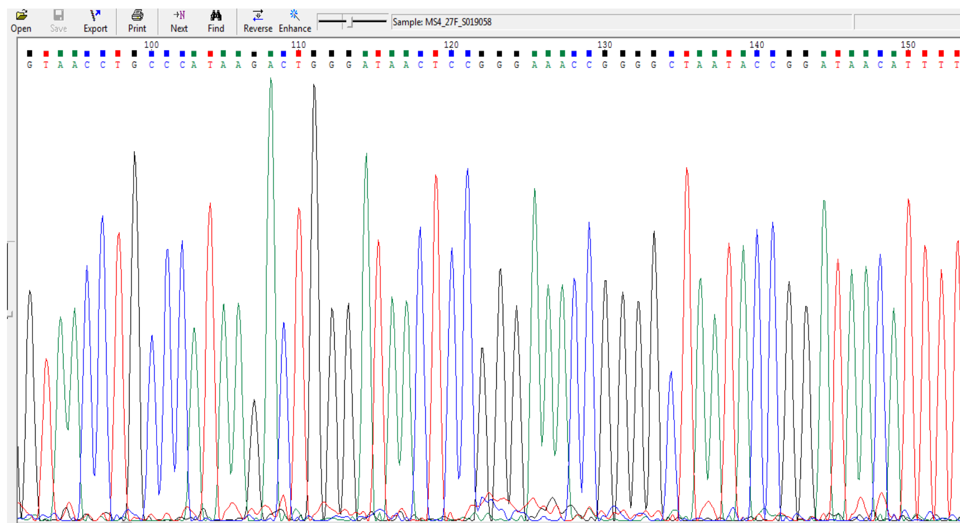


Figure 25: Chromatogram of isolated *Salmonella sps.* that revealed to be *Salmonella enterica* Typhi LT2 strain.

Similarly, upon BLAST of other *Pseudo34* and *Pseudo39* sequence, they were found to have high similarity with *Pseudomonas aeruginosa*.

b. bla NDM and *mcr*-1 PCR for carbapenem and colistin resistance genes

The organisms when found to be the respective organism from biochemical and ribosomal 16S rRNA genomic sequence analysis and were found to be MDR isolates with carbapenem and colistin resistant were further subjected to PCR for investigation for elucidating their mechanism through possession of genetic marker in these resistance organisms by amplifying *bla* NDM and *mcr*-1 genes, respectively for carbapenem and colistin resistance, using primers that have been designed for amplification of these

genes. Carbapenem resistance have been reported to have developed by harboring *bla* NDM gene. All six Gram negative isolates' plasmid DNA when PCR amplified using *bla* NDM primers gave amplicon size of about 860 bp (Figure 26) indicating that they harbored cabapenem resistance marker in the plasmid. This is of additional concern as the plasmid is easy genetic material transferable trait and if these organisms can be in heteroresistance (Nicoloff *et. al.,* 2019) community they could potentially transfer the trait to other sensitive members.

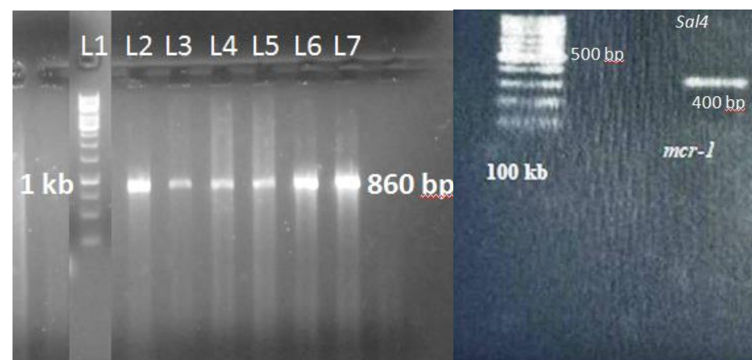


Figure 26: *bla* NDM and *mcr*-1 PCR for carbapenem and colistin resistance genes of Gram's negative bacteria

However, upon amplification of plasmid based *mcr*-1 gene, the PCR product with amplicon size of about 400 bp was obtained only from *Salmonella* strain by using *mcr*-1 gene specific PCR primers (Figure 26). One of the mechanisms of colistin resistance is due to breakdown of the molecule by plasmid, *mcr*-1 gene product, phosphoethanolamine (pEtN) transferase protein. Thus, those strains that showed colistin resistance in AST but did not have *mcr*-1 gene in their plasmid can be presumed to have gained resistance to colistin by other mechanisms. It could be by harboring other resistance marker such as mic-1 gene as colisitin resistance is also known to be by phosphoethanolamine (pEtN) transferase (Hu *et. al.,* 2016) that are commonly found in chromosome or any genetic materials obtained from horizontal gene transfer from other *Enterobacterieceae* strains but present study couldnot reveal these. Moreover, the plasmid based *mcr*-1 gene also pose higher threat of passing the trait to other organisms as mentioned above for plasmid. Since the primary focus of the study is to look for antimicrobial products that could potentially be inhibitory to carbapenem and colistin resistance MDR pathogens, *Salmonella* strain labelled as *Sal4* was taken to investigate further.

Though WHO is focusing only the carbapenem resistance Gram negative bacteria, cases of colistin resistant pathogens must also be addressed to solve the antimicrobial resistance health issues. Both carbapenem and colistin are used as last line of defense agents to cure the infections mainly caused by Gram negative bacteria. Out of many classes of carbapenems, meropenem, ertapenem and imipenem are used to treat both Gram positive as well as Gram negative bacteria because they are categorized as broad spectrum antibiotics (Nicolau, 2008). Finding of both carbapenem and colistin resistance

in Nepalese clinical samples and poultry samples is of grave health concern. Thus, the project was mainly focused in the carbapenem and colistin resistance Gram negative and methicillin resistance *Staphylococcus aureus* to find putative molecule or some products that could be further developed as new line of antibiotics.

4.4 Computational Screening for Antibacterial potential

4.4.1 Drug Target Protein Identification

Several literatures suggest that some essential proteins already have been discovered as an important antibiotic targets by already available antibiotics. However, the resistance developed against those drugs by pathogens suggest that drugs against the same targets could be resisted by similar mechanism or resistance could develop easily. Thus, there is urgent need of new drugs with novel new targets preferably working in multiple essential enzymes or something that is specific to bacteria, only. Compared to hit and trial method fast and robust computational method for computer aided drug discovery (CADD) could potentially narrow down putative drug like molecule that could be further explored to develop antibiotics. This is now appearing to be common practice to search for ligands that could potentially act as drug through molecular docking (Das, 2017).

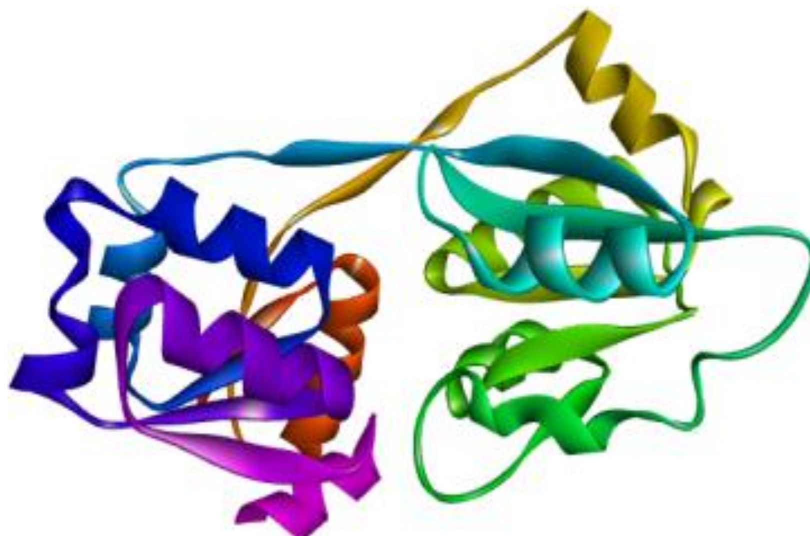


Figure 27: Structure of uroporphyrinogen III methyltransferase (*Salmonella enterica* subsp. enterica serovar Typhimurium)

Uroporphyrinogen III methyltransferase (CobA) of various human pathogens has been validated as an essential protein from gene essentiality check (Suprim Tha, 2018). Hence, CobA (Figure 27) was used as potential drug target in this study for *in silico* screening. For *in silico* molecular docking the three dimensional (3D) structure of the protein mainly these known structures are found in RCBS site (https://www.rcsb.org/) in pdbqt format, is critical. If the structure is not available, then the 3D structure has to be made using different available computer tools such as MODELLER, IntFOLD, RaptorX, Phyre2(https://ipfs.io/ipfs/QmXoypizjW3WknFiJnKLwHCnL72vedxjQkDDP1mXWo6uco/wiki/List_of_protein_structure_prediction_software.html). Since *Salmonella* sps. CobA

protein structure is not available in the RCBS site it had to be drawn and among these tools, Phyre 2 software was used to create present structure (Figure 27).

As we know that this essential gene utilizes *S*-adenosylmethionine (SAM) as the substrate for catalyzing methylation of corrin ring for corrin ring contraction during biosynthesis of vitamin B12 and SAM is also found in every living cells. In every methylation reaction, SAM acts as methyl group donor (Fontecave, 2011). This enzyme is mainly involved in conversion of uroporphyrinogen III into precorrin-1 and then precorrin-1 into precorrin-2. During this biochemical reaction *S*-adenosyl-L-homocysteine (SAH) is released (Roth *et. al.,* 1996). Vitamin B12 is critical during lipid biosynthesis in prokaryotes (Romine *et. al.,* 2017). Hence, if this gene is blocked or its expression can be attenuated, this could consequently be lethal to bacterial survival. Thus, screening of ligands that can compete with SAM at SAM binding pocket was envisaged and relevant chemical ligand library had to be prepared.

4.4.2 Ligand database and protein target preparation

Ligand library preparation is critical in reducing time and increasing number of hits. Though with the advancement in computational power with super computers vast array of chemicals could be analyzed. Moreover, having rationale of probable chemical interaction from particular group of ligands to that of the native ligand to the protein could be one of the ways for making ligand library. Since SAM has adenosine moiety, indole derivatives were thought as potential ligand since indole has been reported to be drug scaffold which shows antimicrobial properties (Sravanthi and Manju, 2016). In addition, kinase requires ATP for its phosphorylation activity (Deutscher and Saier, 1983) and due to adenosine moiety of SAM it was presumed that kinase inhibitors that have been developed as drug candidates in different human diseases (Arslan *et. al.,* 2006) could be potential drug candidate molecule.

This also supports the notion of repurposing the drugs that have been developed for one ailment treatment for possible use in other therapeutic interest (Xue *et.al.,* 2018). Thus, the online database that has molecule in pdbqt file format was taken for ligand library preparation. The inhouse ligand library created consisted of 6,555 compounds (data not shown). Among them, 59 compounds were screened as Indole derivatives and remaining 6,496 were kinase inhibitors. Indole derivatives were searched and filtered from Zinc databases (https://zinc15.docking.org/) and Kinase inhibitors were downloaded from UORSY (https://uorsy.com/downloads/) database.

These ligands were selected because indole mimics with different structures of proteins and binds to enzymes in a reversible manner (Thandavamurthy *et.al.,* 2014). It acts as an excellent scaffold in drug discovery with different noble mechanism of action (Sravanthi and Manju, 2016). Therefore, these candidates were assumed to be the best ligand sources for virtual screening in our study. In addition, kinase inhibitors can act as potential inhibitors because of the ATP binding pocket in the cleft between the C- and N-lobes of kinases. These mimic the hydrogen bond interactions normally formed by the

adenosine ring of ATP (J. Zhang *et.al.*, 2009). Due to this reason, protein kinase inhibitors have been used as an important drug targets in cancerous cells (Arslan *et.al.*, 2006) along with wide range of potential therapeutic targets. Similarly binding of SAM in CobA shows strong binding of adenosyl moiety in the protein (Suprim Tha, 2018). Thus, this ligand library was primary focus for molecular docking in the target protein to screen those ligands which have higher binding energy than native ligand SAM.

4.4.3 *In silico* ADME/Tox Analysis

After in house ligand database preparation, the compounds were proceeded for molecular docking but before that the drugability of the compound has to be screened to prevent future failure during the drug development process. With available different online tools to screen drugability of the compounds, mainly Lipinski rule of five (Lipinski *et.al.,* 2001a) and online tools for absorption, distribution, metabolism and excretion (ADME)/Toxicity test to rule out those molecules that don't pass these tests. Since all the compounds might not pass the ADME and toxicity test, so, ADME/T analysis would screen those compounds that might not pass in later stage of drug development and is done prior to docking. The main reason for that is their inability to reach the target and also due to the toxicity issues. Hence, ADME/T test was done before virtual screening. The ligands in the library developed (6,555 ligands) were subjected to *insilico* ADME/Tox analysis to generate toxicity profile of the ligands and druglikeness were predicted using the software OSIRIS Data Warrior.

ADME/T includes the criteria as follows:

Molecular weight – 200 to 500 Daltons
cLogP - -3 to 6
cLogS - greater than -4 i.e -4 to -2
Hydrogen bond donors – 0 to 5
Hydrogen bond acceptors – 0 to 10
Topological Polar Surface Area (tPSA) – 0 to 140
Rotatable bonds – 10 or less
Druglikeness – Positive value

The cLogP similarity of a compound is the logarithm of its partial coefficient between n-octanol and water $\log(c_{octanol}/c_{water})$ which is a measure of the compound's hydrophilicity. Those compounds with high logP value means drug cannot be absorbed properly. From 6,555 ligands in the primary library 1,788 ligands were filtered through ADME/T analysis that were predicted to have druglikeliness. Once the putative druglike ligands were screened, the CobA enzyme was prepared for docking. For docking the active binding site of the enzyme has to be determined as this is the site where native ligand SAM would bind.

4.4.4 Binding Site Analysis and Generation of Grid Box

Since the 3D structure of CobA protein of *Salmonella* sps. is not known finding the binding site had to be done computationally as there is no experimental data. The

development of certain computational tools has now assisted insuccessfully exploring the cavities for prediction of binding affinity and scoring with ligand(s) through molecular docking (Wei *et.al.*, 2002). For the protein-ligand binding site prediction, 3DLigandSite (http://www.sbg.bio.ic.ac.uk/3dligandsite/) was used that aids in identifying the consensus binding site areas (Wass *et.al.,* 2010b). The binding site included amino acids (Table12), once the binding site location is identified then the site has to be within the gird box in PyRx software that is used for molecular docking. To minimize the error on the grid box size, constant grid size as 25, 25 and 25 for X, Yand Z axis was fixed which is the default setting of PyRx. This grid box size was used to perform interaction studies with the target ligands through virtual screening.

Table 12: Predicted binding sites as predicted by 3DLigandSite in uroporphyrinogen III methyltransferase (*Salmonella enterica* subsp. enterica serovar Typhimurium) predicted via Phyre2 webserver

Residue	Amino acid	Residue	Amino acid
7	ARG	91	THR
8	PRO	110	GLU
32	ILE	111	ILE
33	GLU	134	GLY
34	PHE	135	ASN
35	VAL	158	TYR
59	LEU	159	GLN
60	SER	160	ARG
61	GLN	185	THR
62	HIS	186	SER
63	ALA	189	MET
87	ILE	236	ALA

4.4.5 Virtual Screening and Analysis of Protein-Ligand Complex

Virtual screening advances in the searching of new ligands on the basis of biological structures and their binding site residues (Shoichet, 2004). A ligand dataset of Indole derivatives and Kinase Inhibitors consisting of 6,555 compounds derived from respective database when subjected to ADME/T analysis narrowed these compounds to 1,788 ligands that were then virtually screened for binding capacity with CobA protein using AutoDock Vina and PyRx. However, since there is no experimental 3D structure with native ligand bound to the protein first of all the binding energy of native ligand SAM had to be calculated and whose binding energy was found to be -8.7 KJ/mol. Thus the ligand molecules that had binding energy higher than SAM were of interest and the top ten highest binding energy demonstrating ligands (Table 13) were taken for further analysis. The top hit compounds chosen were from both categories, indole derivatives and kinase inhibitors and it was presumed that these molecules could be taken as the scaffold for drug development.

Table13: Summary of results of docking analysis of top hits (Binding energy as KJ/mol)

S.N.	Zinc ID	Binding energy(KJ/mol) for *Salmonella* CobA	Chemical name
1	ZINC000040086879	-11.2	N-[3-[4-[4-(trifluoromethyl)benzoyl]piperazine-1-carbonyl]phenyl]pyridine-3-carboxamide
2	ZINC000026610689	-10.9	1-(4-phenylpiperazin-1-yl)-2-[4-[2-(trifluoromethyl)benzoyl]piperazin-1-yl]ethanone
3	ZINC000013003651	-10.4	N-(3-acetamidophenyl)-3-(4-benzylpiperazin-1-yl)sulfonyl-benzamide
4	ZINC15219763_uff_E=554.59	-10.4	(1R,4R)-4-(1H-indol-3-ylmethyl)-1-methyl-2,4-dihydro-1H-pyrazino[5,4-b]quinazoline-3,6-dione
5	ZINC000040467636	-10.3	N-(3-oxo-4H-1,4-benzoxazin-6-yl)-5-phenyl-1H-pyrazole-3-carboxamide
6	ZINC000058421664	-10.3	N-[2-(4-methylpiperazin-1-yl)ethyl]-1-phenyl-3-(p-tolyl)pyrazole-4-carboxamide
7	ZINC000023788096	-10.2	[4-(1H-indole-2-carbonyl)piperazin-1-yl]-phenyl-methanone
8	ZINC000024983707	-10.2	(4-methoxyphenyl)-[4-[3-(pyrimidin-2-ylamino)benzoyl]piperazin-1-yl]methanone
9	ZINC000010922671	-10.1	N-[1-(carbamoylmethyl)-4-piperidyl]-1-phenyl-3-(p-tolyl)pyrazole-4-carboxamide
10	ZINC000030256368	-10.1	4-(3-allyl-4,5-dimethoxy-benzoyl)-N-(4-fluorophenyl)piperazine-1-carboxamide

Since human require vitamin B12 from their dietary sources (https://www.sciencedirect.com/topics/food-science/vitamin-b12), it can be presumed that there is no CobA analogue in human or animal. Furthermore, the uroporphyrinogen III differs from heme biosynthesis from delta-amino levulenic acid (D-Ala or ALA), (Layer *et.al.,* 2010) the common precursor for heme and corrin ring (Layer *et.al.,* 2010), thus, would not affect the heme biosynthesis in human and animal. Hence, it can be presumed that the compounds acting against CobA of bacteria would presumably won't be toxic to animals since these compounds were virtually screened from ADME/T analysis. Among the ten compounds with the highest binding energy the highest binding top three compounds were selected (Table 14) and their respective names were derived from respective databases.

Table14: Summary of top three ligands with their chemical names

ZINC ID	Compounds' chemical name
ZINC000040086879	N-[3-[4-[4-(trifluoromethyl)benzoyl]piperazine-1-carbonyl]phenyl]pyridine-3-carboxamide
ZINC000026610689	1-(4-phenylpiperazin-1-yl)-2-[4-[2-(trifluoromethyl)benzoyl]piperazin-1-yl]ethanone
ZINC15219763_uff_E=554.59	(1R,4R)-4-(1H-indol-3-ylmethyl)-1-methyl-2,4-dihydro-1H-pyrazino[5,4-b]quinazoline-3,6-dione

Among the top scoring three compounds two compounds, N-[3-[4-[4-(trifluoromethyl) benzoyl] piperazine-1-carbonyl] phenyl]pyridine-3-carboxamide (ZINC000040086879) and 1-(4-phenylpiperazin-1-yl)-2-[4-[2-(trifluoromethyl) benzoyl] piperazin-1-yl] ethanone (ZINC000026610689) were kinase inhibitors and one compound, (1R,4R)-4-(1H-indol-3-ylmethyl)-1-methyl-2,4-dihydro-1H-pyrazino [5,4-b]quinazoline-3,6-dione) *(*ZINC15219763_uff_E=554.59) was indole derivative and their respective molecular structures are shown (Figure 28).

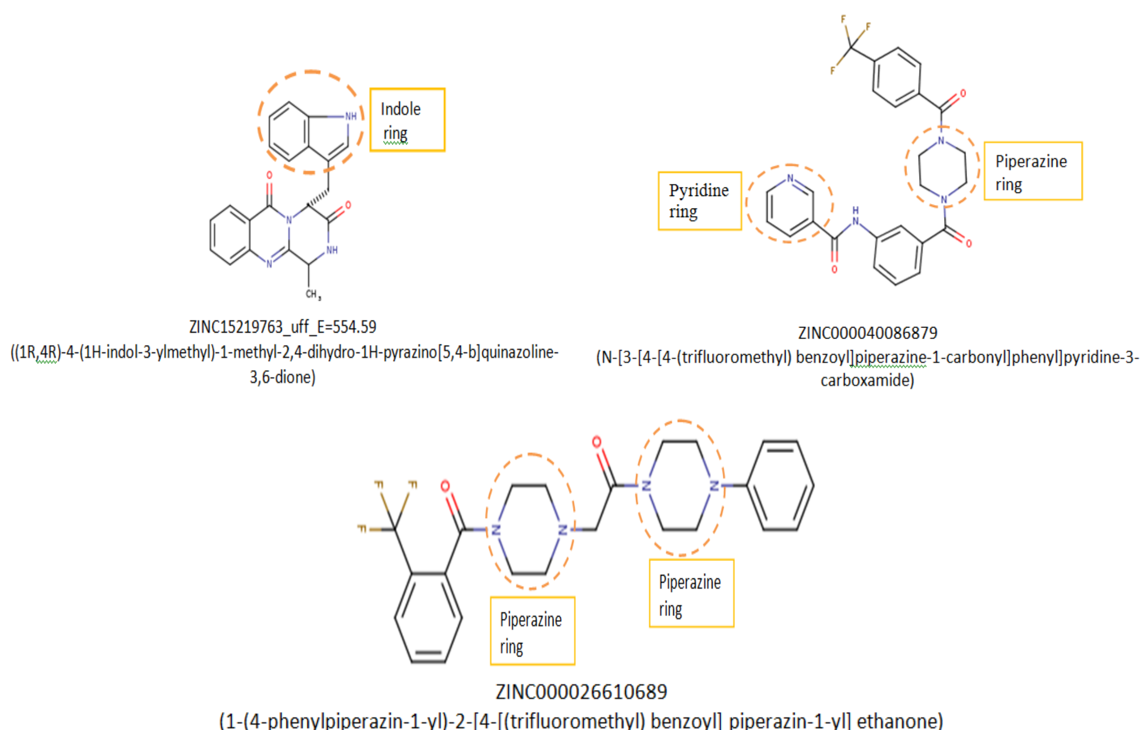


Figure 28: Molecular structure of lead compounds that could be developed as putative drugs

As it has been reported that the virtual screening could advance the searching of new ligands on the basis of biological structures and their binding site residues that have been extrapolated in computational system (Shoichet, 2004). Going through the molecular structures of the respective molecules it was found that the kinase inhibitors had piperazine as interlinking molecule of the scaffold (Figure 28). As piperazine is an organic compound with six-membered ring containing two opposing nitrogen atoms in opposite sides. It has been sought as an important heterocyclic compound for the development of new potential drug candidates and its derivatives have been reported

to act as antidepressant, antihelmentic, anticancer, antibacterial (Meher *et. al.,* 2013) and antimycobacterial, too, the compounds screened sounds to be promising.

Shaquiquzzaman *et. al.* (2015) have reported antibacterial activity by various piperazine derivatives against MRSA, *K. pneumonia*e, erythromycin and ampicillin resistant *Streptococcus pneumoniae* and vancomycin resistant *Enterococcus faecalis* further supporting that these molecules could be explored as antibiotics against the MDR superbugs. In addition, some derivatives were also reported to have antibacterial activity against *E. coli., P. aeruginosa* and *S. aureus* with zone of inhibition of 15, 16 and 18 mm, respectively (Shaquiquzzaman *et.al.,* 2015). Thus, although they were not able to explain the mechanism of inhibition, it can be presumed that the probable mechanism of killing of these pathogens could be inhibiting methyl transfer reaction mediated by methyl transferases that use SAM as methyl donor.

As reviewed by Hamada the pyridine which has benzene ring as skeleton, can be used as potential inhibitor for many targets because of its water solubility, basicity, hydrogen bond forming ability, stability as well as their small molecular size. In addition, they act as ring component of amides, amines and heterocyclic rings containing nitrogen atoms. It has a conjugated system of six π-electrons which are delocalized over the heterocyclic ring. Hence, this pyridine derivative can show an excellent activities against number of biological targets with changing substitutions on the pyridine nucleus (Hamada, 2018). So, based on the principle of Structure Activity Relationship (SAR), piperazine and pyridine derivatives could be suggested as a potent drug's candidates (Akhtar *et.al.,* 2017).

Since, indole is the parent substance of huge number of important organic compounds that occur originally in nature, Sravanthi and Manju reported that indole derivatives have been used as drug scaffolds as bacterial antibiotics. Similarly, they have mentioned that these molecules also have potential towards anti-oxidants, anticancer, anti-inflammatory, anti-diabetic, anti-tuberculosis and antiviral (Sravanthi and Manju, 2016). Thus, one of the compounds screened being indole derivative could also be taken as potential drug scaffold for drug development as an antibiotics.

Hence, this could provide sufficient clues that the ligands narrowed down, both indole and kinase inhibitors, could be the putative drug targets against pathogenic MDR *Salmonella enterica*. However, from this result, we cannot determine that whether these top drug candidate ligands are of broad or narrow spectrum. This demands the virtual screening of these ligands compounds against Uroporphyrinogen III methyl transferase gene of other WHO prioritized pathogens.

During drug discovery period, the issue of Drug Metabolism is an essential parameter. These putative drug compounds must be eliminated from our body easily without any or very little toxic effects after the biotransformation inside the body. It is hypothesized that our putative drug compounds are metabolized by Cytochrome P450 enzymes which oxidizes benzene into soluble and simple covalently bound metabolites in human liver

(Gillam *et.al.,* 2000) since they have passed the toxicity test prior to docking. In addition, indole has been reported to be metabolized by human cytochrome P450 2A6 (CYP2A6) and CYP2E1, but mostly by CYP2A6 (Gillam *et. al.,* 2000). In addition, one of the metabolite of CYP2A6 is indirubin and some of the indirubin derivatives have been found to be kinase inhibitors (Wu *et.al.,* 2005). Thus, this also clearly indicates that the putative compounds could be taken as lead molecule to explore for drug development as antimicrobial agent.

Hence, the result of virtual screening suggests that the organic benzene ring compounds consisting purine moiety and indole ring can be used as potential antimicrobials or can also be used as positive enhancer for antibiotics production. Therefore, these top hit putative drug compounds can be forwarded for further drug development stages. To support these results of *in silico* approach, different *Streptomyces* strains were subjected for production of antibiotics by incorporating benzene ring compounds on the basis of carbon catabolite repression mechanism and different antimicrobial tests were performed.

4.5 Soil Sampling and Isolation of *Streptomyces* from Nepalese soil

It has been suggested that about 60-70% of the etiological agents of infectious diseases are becoming resistant to the existing drugs. Many patients and tertiary care hospitals' staffs are affected directly (Khuntayaporn *et. al.*, 2012). Hence, from various soil samples, isolation of the novel potent isolates that can produce compound(s) that could be effective against multidrug resistant bacteria is thought due to the diverse ecological niches of Nepal and could potentially reduce mortality, morbidity, antibiotic use and health treatment care costs (Duin and Paterson, 2016).

To meet the aim of the antimicrobial production, soils from the different geographical locations of Nepal that were collected and and available in CDBT laboratory was taken as starting material. As it has been reported that one gram of soil contains 10^3 to 10^6 bacteria (Fierer *et.al.,* 2007) among the soil microorganism, *Streptomyces* are the second most abundant soil bacteria because they are considered as copiotrophic bacteria (Hashimoto and Hattori, 1989) that needs more carbon sources in their habitat for their growth and metabolism and they are known saprophytes (Valli *et. al.,* 2012) that can feed in decaying plant materials acquiring nitrogen, carbon and other nutrients. Though the preference habitat is marine sources (Dharmaraj, 2010), *Streptomyces* are also found in terrestrial (Elleuch *et. al*., 2010) and as Himalaya was made after lifting of Seabed (https://pubs.usgs.gov/gip/dynamic/himalaya.html) it can be presumed that the Nepalese soil could have both marine and terrestrial *Streptomyces* sps. In addition, the copiotrophic nature of these organisms suggests that they require high amount of reduced carbon source and the richness of organic carbon is found to be in rhizospheric areas as the root exudates consists of different forms of reduced carbon sources (Dennis *et. al.,* 2010). Moreover, *Streptomyces* sps. are capable to keep the mutualistic

relationship with the plants by the production of natural antimicrobials by suppressing the pathogenicity of the plant pathogens (Jauri *et.al.,* 2018). Hence, assuming the abundance of *Streptomyces* on the vicinity of the rhizospheric area of the plants it was thought that for searching of novel isolates could be possible from various soils of Nepal.

As recommended by International *Streptomyces* Project (ISP) (Shirling and Gottlieb, 1966), specially ISP2 and ISP4 growth agar media were used for isolation of putative *Streptomyces.* The ISP2 contains glucose as carbon source that facilitates the growth of almost all organisms since it is easily utilized for energy generation. Thus, the use of nalidixic acid and cycloheximide reduced the other bacterial and fungal contamination, respectively. In contrast, ISP4 media is rich in soluble starch which is polysaccharide.

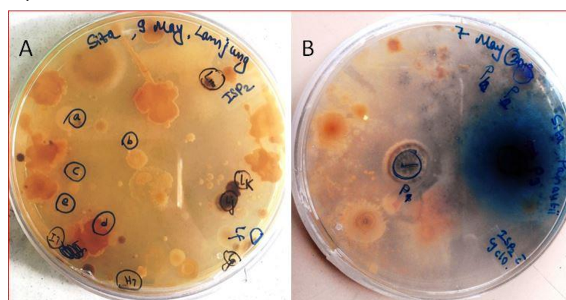


Figure 29: Primary cultures of soil sample in ISP2 media (A: soil sample from Lamjung, B: soil sample from Panauti)

As *Streptomyces* sps. is spore forming, soil sample was subjected to pasteurization for ten minutes to kill the vegetative cells present in the soil. After this the medium was serially diluted and from the serial diluted soil samples, 63 putative strains of *Streptomyces* were isolated from 19 different soil samples. Single and pure colony were selected based on the slow growing nature of the colonies that had chalky, heaped, cottony, non mucoid colony, earthy smell producing, showing different colors on aerial and substrate mycelia and in some case, pigmentation around the colonies on the agar media too (Figure 29).

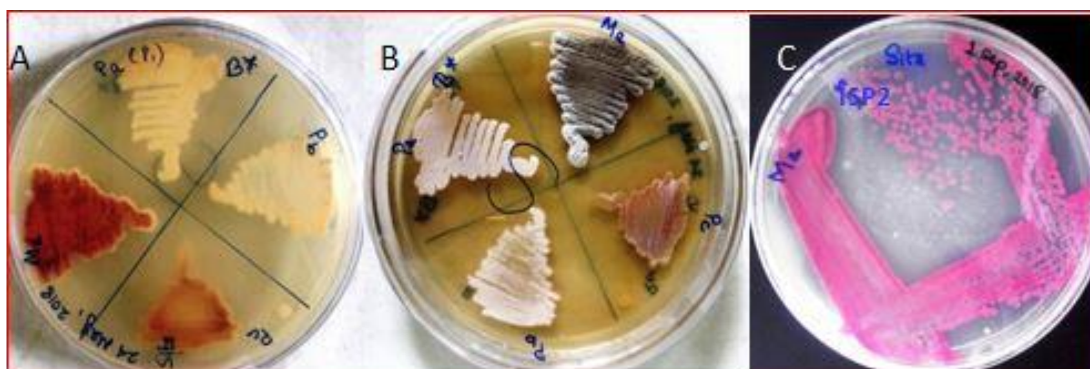


Figure 30: Pure culture isolates of 4 putative *Streptomyces* isolates (A: Substrate mycelial growth, B: Aerial mycelial growth, C: Single pure colony of putative *Streptomyces* strain MA1)

Pure auxenic colony was obtained after sub-culturing in both ISP2 and ISP4 agar media (Figure 30). As ISP4 media contains starch as reduced carbon sources and can be screening media because this reduced carbon source allows the growth of starch

utilizing organisms, mostly *Actinomycetes* (Zainal *et. al.,* 2016) and eliminates other organisms that depend on simple reduced sugars for energy generation. From this view, ISP4 medium can be regarded as more selective to isolate putative *Streptomyces* because of complex composition of carbon, nitrogen and other nutrient sources in this medium because failure of revival of some isolated putative strains (from ISP2) in this medium supports this hypothesis. From 63 isolates from ISP2 medium 10 isolates were screened as putative *Streptomyces* sps. in ISP4 medium. The interest was to have *Streptomyces* sps. that can thrive in complex reduced carbon sources with the hypothesis that they could be able to give antibiotics if grown under some stress conditions. The following table (Table 15) presents some characteristics of the some putative isolated strains.

Table 15: Morphological characteristics of some putative isolates on ISP2 agar medium

S.N.	Soil sample	Isolate code	Substrate mycelium colour	Aerial mycelium colour	Morphology
1	Kolputaar 1	K13	Orange	White	Powdery, chalky, smooth
2	Kolputaar 2	K21	Dark green	Grey	Smooth, chalky
3	Panauti	PA1	Yellow	Light pink	Chalky, smooth
4	Panauti	PA2	Yellow	Light pink	Chalky, smooth
5	Panauti	PA3	Dark pink	Grey	Heaped, powdery, smooth, highly pigmented
6	Baglung	BA1	Black	White	Flat, powdery, chalky
7	Baglung	BA2	Grey	Creamy white	Smooth, powdery, disc shaped
8	Mahendrapul	MA1	Pink	White	Powdery, chalky, smooth
9	Kanchanpur	KA10	Yellowish green	Grey	Heaped, Glabrous, chalky
10	Lamjung	LA3	Orange	Light pink	Powdery, chalky, concave

4.5.1 Morphological and biochemical characterization

Furthermore, further confirmations of putative *Streptomyces* were done by Gram staining and various biochemical tests. Morphological test allows to differentiate the bacteria for their cell wall characteristics and shape while biochemical tests allow to differentiate bacteria for their biochemical characteristics based on metabolism of different compounds.

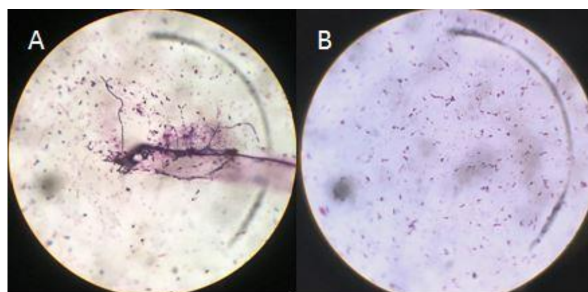


Figure 31 : Gram staining of two putative *Streptomyces* (A: BA1 and B: BA2)showing Gram positive cells

Morphological characterization of isolated putative *Streptomyces* sps. were done by Gram's staining. Almost all of the isolated putative strains were found to be Gram positive (Figure 31) and the the isolates which were seen as Gram negative were ruled out for further experiments. The Gram positive isolates were rod shaped (Figure 31). This is in concordance to be reported for *Streptomyces* sps (Valli *et. al.,* 2012). The Gram positive isolates were then subjected to different biochemical test. They were found to exhibit different characteristics (Table 16).

Table 16: Different biochemical tests of some putative *Streptomyces*

S.N	Test	Putative Isolate code					
		PA1	PA2	PA3	MA1	KA10	91
1	Starch hydrolysis	-	-	+	+	+	-
2	Urease	+++	+	-	-	+++	-
3	Indole	-	-	-	-	-	-
4	Catalase	+	+	+	+	+	+
5	Oxidase	-	-	-	-	-	-
6	MR	-	-	-	-	-	+
7	VP	-	-	-	+	+	-
8	Citrate	+	+	-	+	+	+
9	TSI	R/R	Y/Y	R/R	R/R	R/R	R/R
10	Gelatin	-	-	++	+	++	++
11	Nitrate Reduction	++	+	++	++	+++	-
12	Oxidation/Fermentation(O/F)	F	Use of peptone	F	F	F	Saccharolytic

4.5.2 Molecular Characterization of Putative *Streptomyces*

The putative *Streptomyces* which were preliminary confirmed were characterized by the amplification of 16S rRNA genomic region through PCR using universal 16S rRNA PCR primers to confirm that the isolates were bacteria but not fungi. The amplicon size of about 1500 bp of gene was obtained after visualization on gel electrophoresis (Figure 32 A) and confirmed the isolates were of bacterial prokaryotic family.

After confirmation as bacteria, the putative isolates were further confirmed by *Streptomyces* specific PCR by using two sets of primers. The first set contains the forward and reverse primers as StrepB(F) and Strep E (R) with product size 520 bp where as second set contains StrepB(F) and Strep F(R) as forward and reverse primers with product size of 1070 bp (Rintala *et.al*., 2001). As confirmed from UV visualization of

ethidium bromide reacted PCR amplified DNA at respective sizes of both primer sets (Figure 32 B), the isolates that were confirmed as bacteria through 16S rRNA genomic DNA amplification were in fact *Streptomyces* sps. suggesting that the isolation and screening protocol used were effective in screening *Streptomyces* sps. and further robust screen protocol could be devised to minimize selection time.

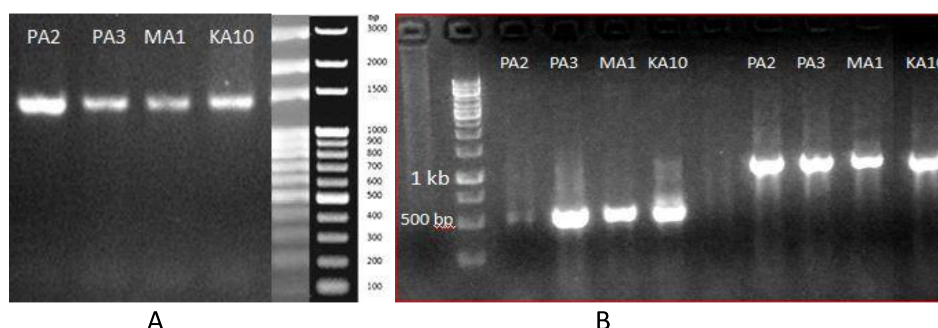


Fig 32: A) Bands of PCR of 16S rRNA of putative *Streptomyces* with product size 1500 bp on 1% agarose by using universal primer ; B) Bands of PCR of putative *Streptomyces* with product size 520 bp and 1070 bp on 1% agarose by using *Streptomyces* specific primer

4.5.3 Secondary metabolite production from preliminary confirmed *Streptomyces*

The different 45 isolated strains with colored colonies in one of the two agar media were cultured in ISP2 and ISP4 broth medium (pH 7) for screening of antimicrobial potential activity. The selection of colored colonies included the criteria on the basis of report that isatin (Rehman *et. al.,* 1997) and indirubin (Al-Dhabi *et. al.,* 2012) (both coloured compounds) act as antimicrobials. After incubation for secondary metabolite production, none of the non concentrated crude extracts (supernatants only) of isolates showed the significant positive results against tested sensitive as well as MDR tests bacteria in plate AST.

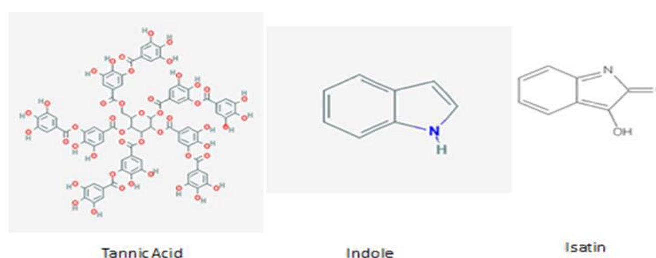


Figure 33 : Structure of tannic acid, indole and isatin

Gillam *et.al.* (2000) reported that human cytochrome P450 2A6 gives indigo and indirubin upon metabolism of indole and different indirubin have been found to be kinase inhibitors (Wu *et. al.,* 2005) and indirubin of plant and microbial extracts have potential antimicrobial capacity (Al-Dhabi *et. al.,* 2012). It was presumed that indole could be used as scaffold to induce antimicrobial production in the isolates as *Streptomyces* sps. posses several P450s (M.-A. Cho *et.al.,* 2018). Since, almost all *Streptomyces* are naturally antibiotic producers, it was hypothesized that the constituents of the antibiotic production media might not be suitable for that. With this

hypothesis, antibiotic production medium was modified by altering the carbon sources as well as ratio of various micro and macronutrients. These altered carbon sources and macronutrients provide carbon source and growth factors to *Streptomyces* along with to create a stressed condition to produce secondary metabolites in high amount.

Since, *Streptomyces* are slow growing saprophytes (Lerat *et.al.,* 2009) in nature, they should compete for the available carbon sources for nutrients. In competitive niche environment, they cannot get access to utilize the easily metabolizable carbon sources that are preferred reduced carbon sources for fast growing organisms (Kim *et. al.*, 2010). In addition, they cannot also utilize pentose sugars since these are also regulated by CCR simultaneously with hexose sugars. The fast growers exhibit diauxic growth to utilize simple carbon sources (Chu and Barnes, 2016) making pentose too unavailable. This suggests that *Streptomyces* in the soil must use plant degradation products such as lignin, cellulose, hemi cellulose and pectin (Barder and Crawford, 1981) to compete with the fast growers within the same niche and most probably have to produce antibiotic to prevent competition from plant and soil pathogens, too.

Some of the *Streptomyces* are indole negative. Such saprophytes cannot convert the amino acid tryptophan into the indole for metabolism but presence of indole in the niche environment by indole positive organisms reflects the need for metabolizing indole or tryptophan to antimicrobial agents to compete with these indole producing organisms. Thus, the presence of indole could help to produce more antimicrobials and potentially the indole could be utilized as carbon sources, too. Moreover, some *Streptomyces* also produce indole and its derivatives as antimicrobial agents (Igarashi Y. *et. al.,* 2004) suggesting potential use of indole or tryptophan in developing antibiotics. Furthermore, the CADD results also revealed presence of indole or similar structure in compounds having higher binding energy to CobA protein that utilizes SAM for methylation reaction.

Taking these into account, media modification was thought the way to create stress to *Streptomyces* for more antimicrobial production. Thus, medium was modified by incorporating certain concentration of various lignin degradation products in the media along with indole. The carbon sources of ISP2 and ISP4 media were replaced with these various complex carbon sources such as furfural, transferulic acid, vanillic acid, coumarin, vanillin, furfuraldehyde, acetic acid, guaiacol and tannic acid that might act as phytochemicals for *Streptomyces* sps. to utilize as reduced carbon sources for their survival. Before incorporating these compounds to the modified media, toxicity test of some of these compounds were performed for the isolates. It was found that about 5 mM concentration of these compounds was toxic to the cells. This provides the clue of using those lignin degradation compounds at that threshold concentration to create stress condition so that the isolates might produce antibiotics as it has been reported that *Streptomyces* produce antibiotics upon stress (van der Meij *et.al.*, 2017). After modification of the media, color pigment producing *Streptomyces* isolates were again cultured on 50 ml modified media supplemented with indole only, lignin degradation

product only and indole plus lignin degradation product. Due to the CCR (Görke and Stülke, 2008) presence of other reduced carbon source could limit the active transport of indole but indole is stress modulating molecule (Gaimster *et.al.*, 2014) that can easily disturb membrane stability by increasing permeability because of its binding to phospholipids of the membrane (Norman and Nymeyer, 2006) and diffuse easily to the cytosol. The isolates have to metabolize indole to overcome the stress. In addition, presence of indole could mimic the presence of indole positive pathogens such as *E. coli* indicating that the isolates have to compete for survival because of only availability of rarely utilized lignin degradation product. Hence, it was presumed that the isolates have to produce a molecule by utilizing indole that is toxic to indole positive fast growing organisms.

After secondary metabolite production, the plate AST of extracts (supernatants only) showed no zone of inhibition against *Esch1, Pseudo34, Pseudo39, Kleb52, Aceneto9* and *Staph1*. Among these, the non concentrated crude extracts (supernatants only) of six isolates upon co-culturing with indole plus tannic acid showed the measurable zone of inhibition against carbapenem and colistin resistance biofilm producer *Salmonella* Typhi LT2 strain (Figure 34). Out of six *Streptomyces* isolates, the four isolates PA2, PA3, MA1 and KA10 showed the highest zone of inhibition. The extracts (indole plus tannic acid) of these four isolates were tested against other carbapenem and colistin resistance *Salmonella* Typhi strains, too and showed the zone of inhibition by killing bacteria completely. This suggests that these isolates produce the antimicrobial substance that are highly specific to *Salmonella.*

Figure 34: Antimicrobial extracts (indole plus tannic acid) test of Streptomyces against Salmonella Typhi LT2 strain (1: PA2, 2: PA3, 3: MA1, 4: KA10, 5: Negative control without extracts)

Moreover, the cytochrome P450 or CYPome of *Streptomyces* (Chun Y *et. al.,* 2007) can be explored for drug discovery (Lamb *et.al.,* 2006). They can utilize indole and its derivative (Gillam *et.al.,* 2000) from external sources, too, to produce antimicrobial compounds because of active participation of CYPome. The human cytochrome P450 2A6 metabolizes indole into colored compound indigo (blue), indirubin (pink) and other products and source of indole could be from metabolism of tryptophan through gut microflora. This suggests that indole could be easily transported in human through gut providing a sufficient clue that indoles are metabolized in humans with no toxicity. Thus,

screening of antimicrobials from colorful isolates is more preferred and as advantageous way in pharmaceuticals for the production of novel antimicrobials against fungal, parasitic and other pathogens. The appearance of pink color around the well after diffusion of extracts in the plate with test bacterial lawn also supports our hypothesis that indole is converted to indirubin types for having antimicrobial potentials. Hence, it can be presumed that the produced antibiotic compounds could be of similar nature with attachment of extra benzene ring to indole moiety.

Among the various lignin degradation products that act as phytochemicals were used in modified media, other compounds did not show antimicrobial property that might be due to lateral diffusion of extracts in the agar media as well as low concentration of crude extracts loaded into the well. Furthermore, primary metabolism also plays a major role in production of secondary bioactive metabolites. The media composition and its constituents concentrations are closely related to the metabolic activities of the metabolite producing organism that influence the biosynthesis of secondary metabolite. Moreover, the effect of various factors such as medium volume, agitation rate, pH of the medium, temperature and required incubation time on secondary metabolites production have also been frequently reported (Harir *et.al.,* 2018). Thus, the lack of antimicrobial activity seen in these compounds could be because of different factors that could not be explained here.

Measuring the zone of inhibition diameter and plotted for bar graph of these extracts killing the *Salmonella* strain were way above the zone of inhibition observed for resistant marker. Out of them the crude extracts of PA2 (with indole and tannic acid) had the highest zone of inhibition against *Sal3* strain.

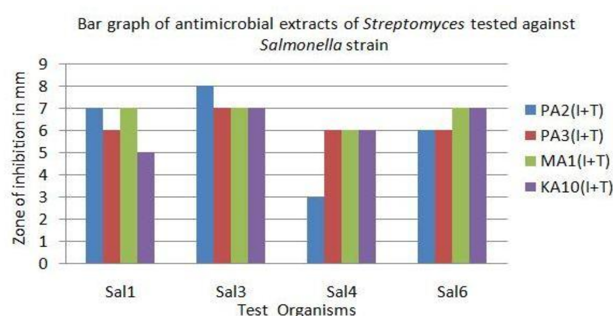


Figure 35: Bar graph of zone of inhibition of antimicrobial extracts of Streptomyces tested against*Salmonella* strain after subtracting from that of negative control test (I: Indole and T: Tannic acid)

4.5.4 Molecular Analysis

The four *Streptomyces* strains (PA2, PA3, MA1 and KA10) that showed the zone of inhibition against carbapenem and colistin resistance biofilm producer *Salmonella* Typhi LT2 strain were proceeded for 16S rRNA genomic DNA sequencing (Figure 36). Upon comparing the chromatogram sequences (Appendix Section) with other sequences of different *Streptomyces* species, using BLAST tool of NCBI, they revealed high similarity

with the *Streptomyces* strains. The isolated strain *Streptomyces* PA2 was found to be 82.18% similar with *Streptomyces cameroonensis* strain JJY4, PA3 was found to be 98.77% similar with *Streptomyces violaceolatus* strain NBRC 13101, MA1 was found to be 94.54% similar with *Streptomyces coelicoflavus* strain NBRC 15399 and KA10 was found to be 80.79% similar with *Streptomyces malaysiensis* strain NBRC 16446. In addition, phylogenetic tree (Figure37) was constructed with the help of various tools such as Sequencher, Bioedit and Mega 7 on the basis of similarity between different *Streptomyces.*

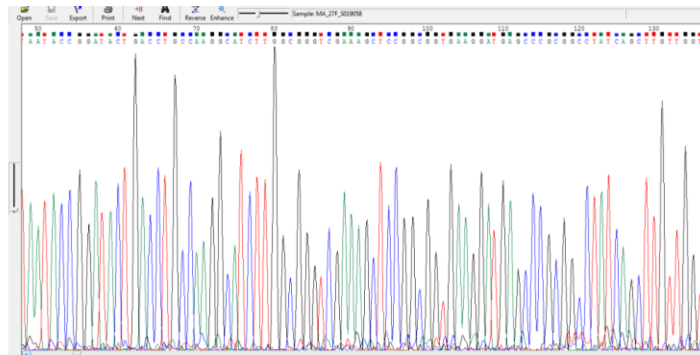
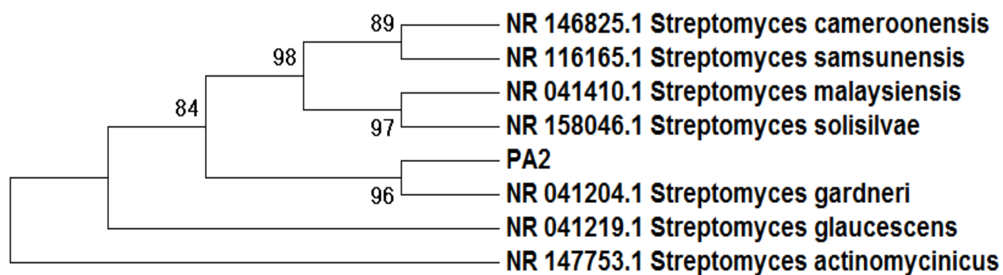
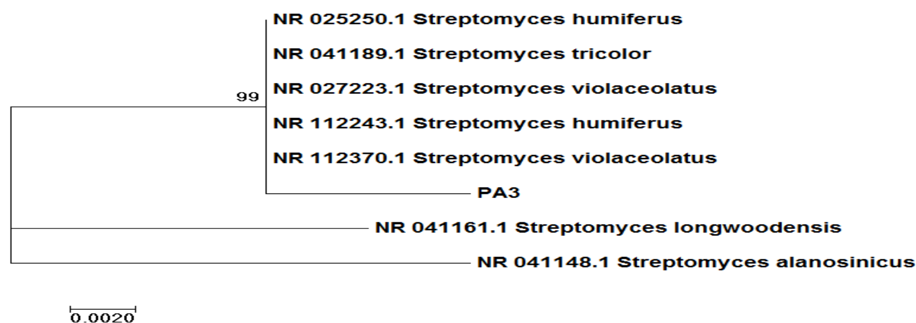


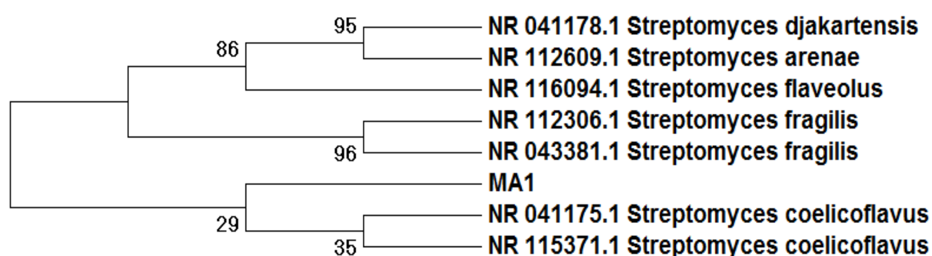
Figure 36: Chromatogram of isolated *Streptomyces* strain MA1 that revealed to be *Streptomyces* sps.



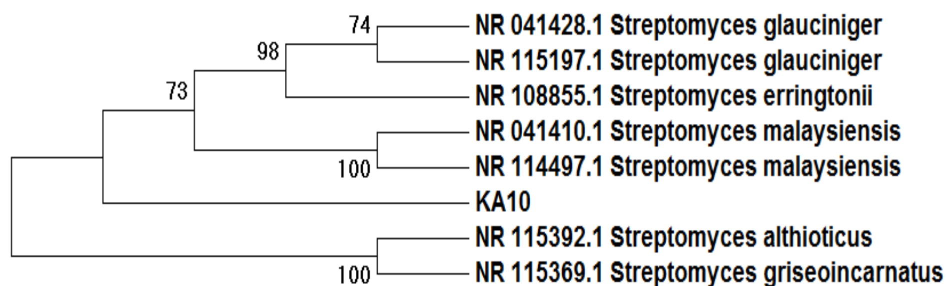
A): Phylogenetic tree of *Streptomyces* Isolate PA2



B): Phylogenetic tree of *Streptomyces* Isolate PA3



C): Phylogenetic tree of *Streptomyces* Isolate MA1



D): Phylogenetic tree of *Streptomyces* Isolate KA10

Figure 37: Phylogenetic tree of *Streptomyces* isolates (A: PA2, B: PA3, C: MA1, D: KA10)

Generally, the species which are generated separate clusters in the phylogenetic tree with bootstrap value (>50%) were considered to be discriminated strains successfully. The bootstrap values of the isolated strains were found to be different with each other. The top hit strain of *Streptomyces,* respectively while performing the nucleotide BLAST in NCBI also had similar result (Table 17) indicating that the strains isolated were of the groups mentioned in phylogenic tree analysis. Upon literature search most of these strains have been used for antibiotics production (Boudjeko *et. al.,* 2017).

Table 17: BLAST result of isolates (16S rRNA genomic DNA sequence alignment, A: PA2 strain, B: PA3 strain, C: MA1 strain, D: KA10 strain)

A) PA2 Strain

Description	Max score	Total Score	Query Score	E value	Identity	Accession
Streptomyces gardneri strain NBRC 3385 16S ribosomal RNA gene, partial sequence	465	465	95%	6.00E-132	81.73%	NR_041204.1
Streptomyces malaysiensis strain NBRC 16446 16S ribosomal RNA gene, partial sequence	465	465	93%	6.00E-132	82.18%	NR_041410.1
Streptomyces cameroonensis strain JJY4 16S ribosomal RNA, partial sequence	465	465	93%	6.00E-132	82.18%	NR_146825.1
Streptomyces solisilvae strain HNM0141 16S ribosomal RNA, partial sequence	460	460	93%	3.00E-130	82.02%	NR_158046.1
Streptomyces samsunensis strain M1463 16S ribosomal RNA gene, partial sequence	460	460	93%	3.00E-130	82.02%	NR_116165.1

B) PA3 strain

Description	Max score	Total Score	Query Score	E value	Identity	Accession
Streptomyces violaceolatus strain NBRC 13101 16S ribosomal RNA gene, partial sequence	1158	1158	95%	0	98.77%	NR_112370.1
Streptomyces humiferus strain NBRC 12244 16S ribosomal RNA gene, partial sequence	1158	1158	95%	0	98.77%	NR_112243.1
Streptomyces violaceolatus strain DSM 40438 16S ribosomal RNA gene, partial sequence	1158	1158	95%	0	98.77%	NR_027223.1
Streptomyces humiferus strain DSM 43030 16S ribosomal RNAgene, partial sequence	1158	1158	95%	0	98.77%	NR_025250.1
Streptomyces tricolor strain NBRC 15461 16S ribosomal RNA gene, partial sequence	1153	1153	95%	0	98.62%	NR_041189.1

C): MA1 Strain

Description	Max score	Total Score	Query Score	E value	Identity	Accession
Streptomyces coelicoflavus strain NBRC 15399 16S ribosomal RNA gene, partial sequence	1202	1202	99%	0	94.54%	NR_041175.1
Streptomycescoelicoflavus strain CSSP410 16S ribosomal RNA gene, partial sequence	1202	1202	99%	0	94.54%	NR_115371.1
Streptomyces fragilis strain NBRC 12862 16S ribosomal RNA gene, partial sequence	1180	1180	99%	0	94.03%	NR_112306.1
Streptomyces fragilis strain NRRL 2424 16S ribosomal RNA gene, partial sequence	1180	1180	99%	0	94.03%	NR_043381.1
Streptomyces flaveolus strain NRRL B-1334 16S ribosomal RNA gene, partial sequence	1175	1175	99%	0	93.77%	NR_116094.1

D): KA10 Strain

Description	Max score	Total Score	Query Score	E value	Identity	Accession
Streptomyces malaysiensis strain NBRC 16446 16S ribosomal RNA gene, partial sequence	500	500	65%	3.00E-142	80.79%	NR_041410.1
Streptomyces erringtonii strain I36 16S ribosomal RNA gene, partial sequence	496	496	65%	1.00E-140	80.61%	NR_108855.1
Streptomyces malaysiensis strain ATB-11 16S ribosomal RNA gene, partial sequence	494	494	65%	4.00E-140	80.43%	NR_114497.1
Streptomyces glauciniger strain NBRC 100913 16S ribosomal RNA gene, partial sequence	491	491	65%	1.00E-139	80.43%	NR_041428.1
Streptomyces glauciniger strain FXJ14 16S ribosomal RNA gene, partial sequence	491	491	65%	1.00E-139	80.43%	NR_115197.1

4.6 Resazurin Antimicrobial Assay of extracts produced by *S. coelicolor* strain M145 (SCP1-/SCP2-) and sequenced *Streptomyces* strain PA3

From the results of CADD analysis and isolated *Streptomyces* sps, it was assumed that a robust and time efficient protocol has to be devised since the plate AST was more time and resource consuming for testing a large number of *Streptomyces* extracts against a number of pathogens and there could be diffusion problem for some of the extracts because of agar in the media. Thus, alternatively fast and robust broth assay was designed to check the viability of the cells based on metabolism of resazurin to resorufin upon exposure to cellular redox mechanism. Hence, the resazurin assay developed by Drummond and Weigh in 2000 (Sarker *et. al*., 2007) has been modified for our convenience.

Salinity test was performed for the isolated strain PA3 to understand the optimum growth under different salt concentration since it is a pigmented strain. Salinity is considered as an important physiological parameter. It was found that optimum growth was observed when absorbance was measured at 600 nm incubating with 6% of sodium chloride (Figure 38). It has been reported that *Streptomyces* strains are able to tolerate from 4% to 13% salt concentration (Akond *et.al.*, 2016). The tiny amount of salts

enhances the proper growth of microorganisms while its concentration might produce an inhibitory effect for their growth by altering osmotic pressure and denaturing the proteins. In addition, higher salt tolerance was statistically associated with the "yellow" and "white"-spored *Streptomyces* (Akond *et.al.*, 2016). The tested strain was salt intolerable when the salt concentration was increased beyond 8%. Growth arrest was reported at salt concentration of 11%. The salt tolerance ability of the isolated strain suggests that it could have had ancestral relation with marine origin organism and marine organisms are looked for potential antibiotics production (Tortorella *et. al.*, 2018).

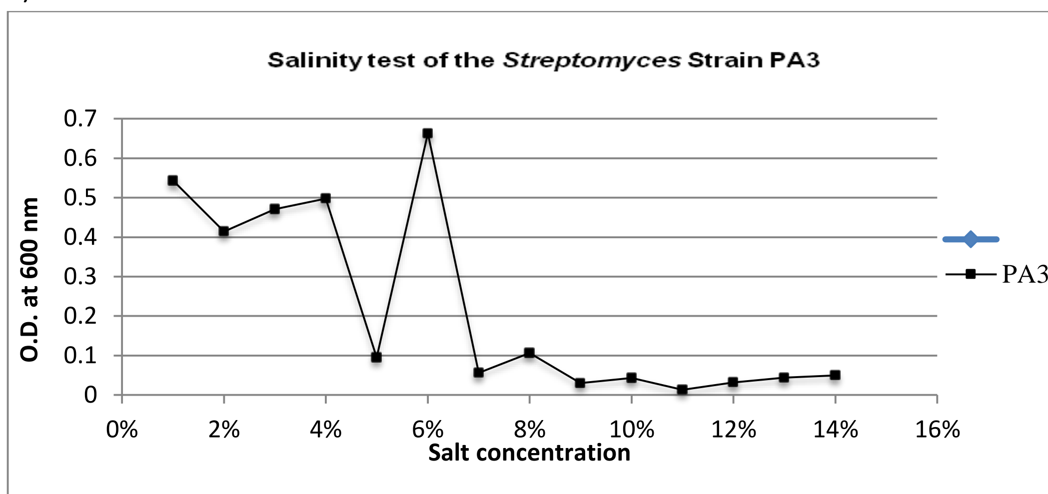


Figure 38: Salinity test performed for PA3 strain

Secondary metabolites are produced during idiophase that is the nutrient deprived condition and growth rate of bacteria decreases gradually. The remaining energy is utilized for the secondary metabolites production, however, secondary metabolism is greatly influenced by primary metabolism. The growth of both of these strains was measured at 600 nm after 8, 10 and 12 days of culture, respectively. Among the various carbon sources used, the growth of the both of the strains was found to be highest when co-incubated with glucose after 8, 10 and 12 days of incubation (data not shown). This might be reason that glucose is the simple and easily metabolizable carbon source for all living organisms that exert CCR effect.

Among the various lignin degradation products, growth of *S. coelicolor* and PA3 strain was found highest when co-incubated with furfuraldehyde and transferrulic acid with and without amino acids after 8 days, 10 days and 12 days respectively (data not shown). The structure of transferrulic acid is simple and easily metabolizable than others. But it was found that the growth gradually decreased with incubation time that signifies the *Streptomyces* went to stationary phase for the production of secondary metabolites.

Both of the strains exhibited the remarked ability to grow on almost all tested carbon sources. The growth rate was found to be increased after 10 days culture than that of 8 days culture. This might be due to degradation of complex carbon sources to simplex form and easily available to the *Streptomyces* for growth. These experiments clearly

indicated that different forms of reduced carbon or lignin degradation products could be used to explore for production of antimicrobial agent.

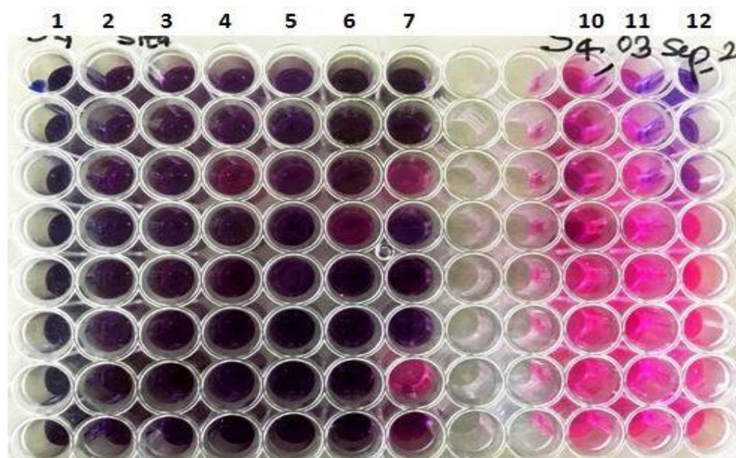


Figure 39: Resazurin antimicrobial assay of the extracts (lignin degradation products with and without mixture of two amino acids valine plus tryptophan) of *S. coelicolor* strain M145 (SCP^{1-}/SCP^{2-}) against *Sal4* where,

Column 1- Positive Controls (2 µl resazurin + 98 µl LB)
Columns 2, 3, 4, 5, 6 and 7 – Tests (48 µl test organism + 2 µl resazurin + 50 µl extract)
Column 10 – Negative Controls (98 µl Test organism + 2 µl Resazurin)
Column 11 –Tests with ampicillin (50 mg/ml in 1st well and 1:2 dilutions to respective wells)
Column 12- Tests with kanamycin (50 mg/ml in 1st well and 1:2 dilutions in the respective wells)

The growth culture was then tested for any antimicrobial agents present in culture broth. Modified resazurin assay was designed to test the antimicrobial properties of the extracts produced by *S. coelicolor* strain M145 (SCP^{1-}/SCP^{2-}) and sequenced *Streptomyces* strain PA3. The final volume in the well was adjusted 100 µl including resazurin, extract and test organism. The effects of ampicillin and kanamycin (second generation antibiotics) with different concentrations (50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.125 mg/ml, 1.56 mg/ml, 0.781 mg/ml and 0.39 mg/ml) were also tested against the test organisms in each batch of experiments. The positive antimicrobial extracts were confirmed by the retention of violet color of resazurin even after the incubation duration (Figure 39) because if the cells would have been viable they would have reduced resazurin to resorufin giving pink color. That indicates no cell growth while negative antimicrobial assay was confirmed by the change of culture to pink color resorufin or non-colored hydroresorufin due to the reduction (Riss *et. al.*, 2013b) after long time of incubation at 37 °C.

The antimicrobial potential of the extracts was determined by the absorbance readings at 551 nm. The absorbance readings close to the negative controls were considered as negative test extracts where as those with absorbance readings close to the positive controls were considered as positive test extracts.

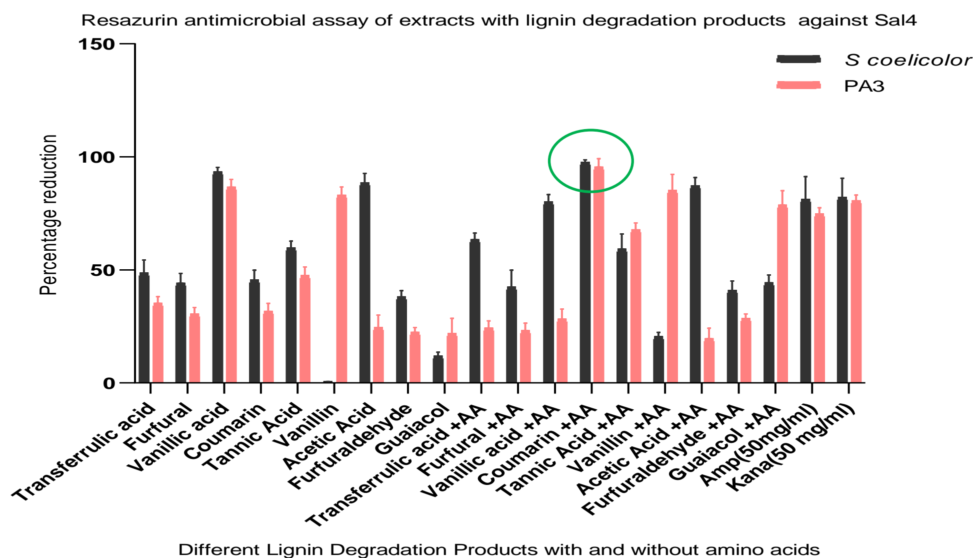


Figure 40: Graph of Resazurin antimicrobial assay of extracts with lignin degradation products against *Sal4*

The two *Streptomyces* strains (M145 ($SCP^{1-}/SCP^{2-}$and isolated *Streptomyces* strain PA3) when co-incubated with various lignin degradation products, the antimicrobial potential of the extract when co-incubated with coumarin and two amino acids (valine plus tryptophan) showed the highest percentage of the reduction when tested against *Sal4* (Figure 40) and MRSA, too (Percentage reduction graph against MRSA is not shown here). The percentage reduction against MRSA was found to be lesser than that of against *Sal*4. Similarly, vanillic acid only when supplied as reduced carbon source also seemed to induce antimicrobial agent development as the extract after incubation showed lethality toward MDR *Sal*4 but was less prominent than with the coumarin. From this experiment it was concluded that antimicrobial potential of the extracts decreases with the time duration of storage of the extracts at 4°C.

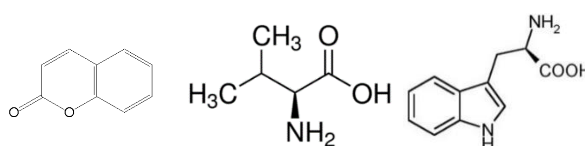


Figure 41: Structure of Coumarin, Valine and Tryptophan

The production of antibiotic property compound has been presumed to be due to the presence of cytochrome P450 system in *Streptomyces* sps., commonly referred as CYPome. Cytochrome P450 is the large heme-containing monooxygenase enzyme found in all living organisms except some (Danielson, 2002). The monooxygenases cytochrome P450s catalyze a reaction in which a single oxygen atom is transferred into a substrate. Therefore, the reaction can be summarized as:

$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$ where, RH indicates various organic compound substrates like alkanes and drugs. RH gets hydroxylated to become a pharmacologically active compound in the presence of NADPH (Nakamura *et.al.*, 2001).

It has been reported that the metabolism of benzene is complex that may yield glucuronide, hydroquinone and other sulfate conjugates of phenol, catechol and trans muconic acid by ring scission (Yardley-Jones *et.al.,* 1991) which is induced by Cytochrome P450 (Gut *et.al., 1996*). In addition, benzene also causes carcinogenicity and myelotoxicity by the production of oxidative metabolites.

The metabolism of Cytochrome P450 makes hydrophobic chemical compounds to water-soluble form (Rosic, 2009). It has been found that cytochrome P450s are involved in about 70-80% of drug metabolizing phase and also perform catalytic activity after biotransformation reaction. Thus, the *Streptomyces* sps. CYPome could play role in metabolizing the substrates provided during the incubation which eventually was converted to secondary metabolite as antibiotics.

This can be inferred because it has been found that some *Streptomyces* produces coumarin and quinolone type antibiotics, such as novobiocin, interfering with gyrase enzyme (Criswell, 2004). The ring structure of valine consists of two methyl groups which can act as electron donating groups and activate the electron ring by increasing the electron density on the ring through an inductive donating effect. From the literature review, it has been found that 25 mM of valine induces valine dehydrogenase of tylosin process in *S. fradiae* to induce secondary metabolism. But in our experiment, 5 mM of valine was only used. Thus, it can be presumed that even in this low concentration valine still could have donated the electrons that could have been involved in electron transfer system, benzene oxidation and synthesis of potential antibiotics that might have been catalyzed by cytochrome P450.

As our result suggested that the extract with coumarin and combination of two amino acids showed the highest percentage of inhibition, hence, coumarin (an aromatic benzopyrone) and its other derivatives could be used as a potential lead compounds in the drug discovery because of its anti microbial property and low toxicity in minimum concentration. Because it has been found that coumarin and its derivatives could be used as major scaffold for antituberculosis agents (Keri *et.al.,* 2015), anticancer agents (Kaur *et.al.,* 2015) and anti-neurodegenerative agents (Jameel *et.al.,* 2016). The side chain substitution in the coumarin has the ability to mimic different structures as the R4 and R5 position side chain that are fused to form a kinase inhibitor compounds (Torres *et.al.,* 2016) and the CADD mediated drug screening had also revealed some kinase inhibitors as potential antimicrobial agents.

In addition, based on the mechanism of Structure Activity Relationships (SAR), the biological activity of coumarin resembles to that of compounds showing the properties of kinase inhibitors (de Araújo *et. al.,* 2013). Thus, the computational result is supported by the result of wet lab inferring that kinase inhibitors and indole derivatives as promising drug scaffold. Both strains, (M145 (SCP^{1-}/SCP^{2-}) and *Streptomyces* strain PA3) are found indole negative suggesting that they don't produce indole. However, it is reported that 4 mM tryptophan acts as inducer for biosynthesis of ergot alkaloid by

dimethylallyl tryptophan synthetase (Krupinski *et.al*., 1976). Moreover, the indole group of tryptophan might have been the scaffold to make the antibiotics because the indole moiety acts as signaling molecule for the production of antimicrobials and as a parent compound having the ability to derive many biological compounds in making antimicrobial agent (Sravanthi and Manju, 2016).

Thus, it can be presumed that the coumarin might have reacted with indole group of tryptophan to give antimicrobial agent that is close to kinase inhibitors because the electron needed for oxidation of indole group of tryptophan could have been provided by valine to oxidize it that then might have reacted with the coumarin which could have been reduced to make the product with the oxidized indole group of tryptophan. This can be inferred because engineered human cytochrome P450 2A6 has been used to oxidize different indole derivatives and eventually produce indirubin to give potent kinase inhibitors (Gillam *et. al.,* 2000; Wu *et.al.,* 2005; Rosic NN, 2009).

Likewise, when different reduced carbon sources were used as carbon source for growth and induce CCR when co-incubating along with valine and tryptophan to prevent the use of these amino acids as carbon source so that these amino acids could be transported by respective transporter in the cytosol but instead of being used as building block they could be used as drug scaffold to make metabolites having antimicrobial potential. The extracts of the culture with cellulose incubated with the combination of amino acids with *S. coelicolor* M145 (SCP^{1-}/SCP^{2-}) and the extracts of the culture with pectin and two amino acids incubated with isolated *Streptomyces* strain PA3 showed the highest inhibitory effect against carbapenem and colistin resistant biofilm producing MDR *Salmonella* strain *Sal*4 (Figure 42).

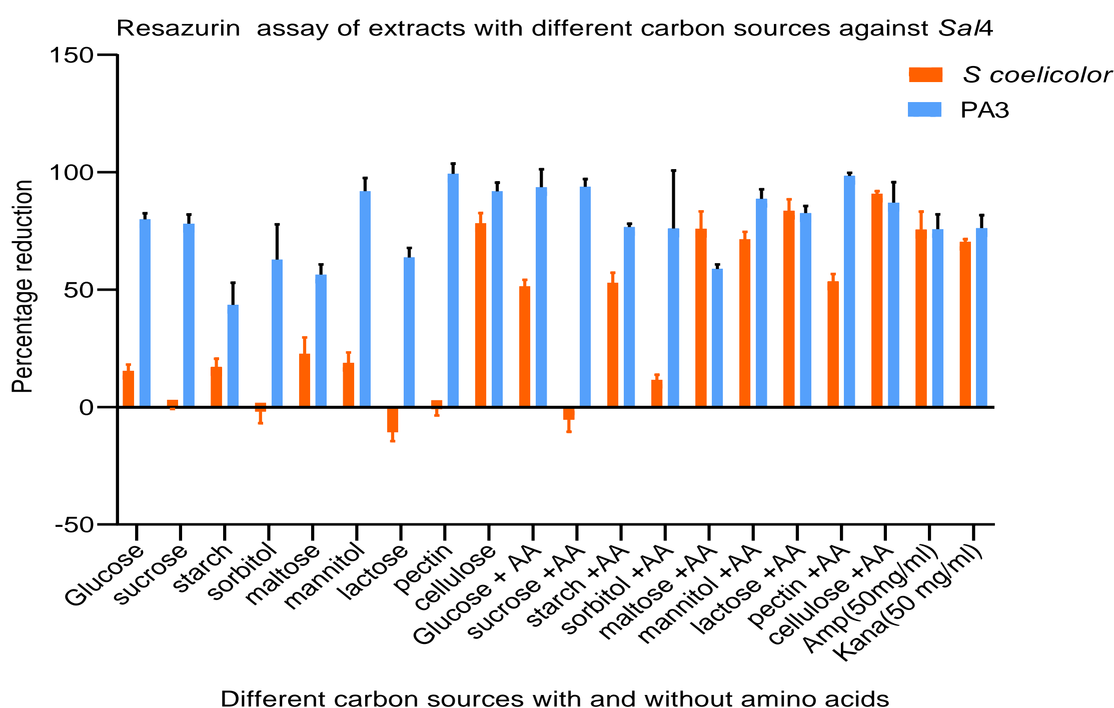


Figure 42: Graph of Resazurin antimicrobial assay of extracts with different carbon sources against *Sal4*

Similarly, both of these strains showed highest reduction by cellulose when co-incubated with two amino acids against MRSA (data not shown). As with the coumarin and two amino acids here also the two amino acids could have exhibited similar biochemical function and the metabolites of the reduced carbon source could have formed some compound with the indole group in giving antimicrobial compound.

The production of antimicrobial agents could have been because of induction of CYP enzymes as *Streptomyces* sps. have been found to metabolize various polymers like, lignin, keratin, pectin, hemicellulose and chitin to the simpler compounds and the extract of *S. flavovirens* was found to have induced expression of cytochrome P450 when phenanthrene was present in tryptone yeast extract broth (Sariaslani and Omer, 1992). This suggests that cytochrome P-450 helps in metabolizing the carbon sources. Similarly, Cytochrome P450 of *S. erythraea* causes β hydroxylation of 6-deoxyerythronolide (Sariaslani and Omer, 1992) that supports to understand the involvement of this cytochrome in the biosynthesis of various secondary metabolites.

Therefore, from the results of resazurin assay, it can be concluded that antimicrobial property depends upon the strain of *Streptomyces* as well as inducer for their production. It can also be analyzed that percentage reduction by crude extracts of the culture with different carbon sources is higher than that of lignin degradation products that might be due to simple structure of the used carbon sources. As far as to our knowledge, the tweaking of *S. coelicolor*, a well established and whole genome sequenced antibiotic producing strain, for the production of antibiotics by feeding with various lignin degradation substrates and different carbon sources is the first attempt in the world. This was feasible from the results of virtual screening to extrapolate possible chemical reactions that might lead to production of new antibiotics. Thus, it is suggested that the antimicrobial potential compound be purified from the culture extract and tested for its efficacy.

Thus, it can be concluded that antimicrobial compounds produced by *Streptomyces* have inhibitory effect towards to kill carbapenem and colistin resistant biofilm producing *Salmonella* pathogens. However, it is necessary to find the active components present in the crude extracts.

In addition, CADD revealed some compounds that could act against the CobA protein of *Salmonella* sps. Thus it was thought that the mechanism of inhibition has to be studied. Moreover, it was intriguing to know that CobA gene had long stretch of 5'-UTR in its mRNA. Since CobA protein uses SAM as the substrate to methylate corrin ring for corrin contraction during vitamin B12 biosynthesis and both SAM and vitamin B12 riboswitches have been reported, it was hypothesized that this long stretch of 5'-UTR of mRNA of cobA gene could potentially exhibit riboswitch mechanism, most probably SAM riboswitch S-box as observed for cysteine and methionine biosynthesizing genes (Ferla and Patrick, 2014).

Thus, to check the mechanism of action of our antimicrobial compounds and possible riboswitch mechanism the native promoter region of CobA gene along with the 5’-UTR consisting of *lac*Z gene as protein coding sequence was envisaged to study the promoter function and possible riboswitch through β-galactosidase reporter assay by cloning of promoter region with 5’-UTR followed by *lac*Z gene. This would give what length of promoter region is required for the expression of gene and also if the compound can exhibit riboswitch modulating effect if the β-galactosidase activity is inhibited in the strain with the construct but not with pUC19 plasmid only which expresses alpha complement of *Lac*Z protein that is devoid in *E. coli* DH5*α* strain used for cloning. Thus, gene cloning strategy has been devised.

4.7 Transformation of pUC19 in DH5α and construct (primer design)

For any cloning strategy choice of suitable vector is critical as it varies for the host specificity, copy number, multiple cloning sites (MCS) and restriction enzymes recognition sites in these multiple cloning sites. Cloning vector pUC19 was selected because it is high copy number plasmid with pBR322 based replication of origin ColE1 its relatively small size to allow cloning of long stretch of DNA, around 9 kb, has alpha compliment unit of *lac*Z gene that is not functional in cloning strain *E. coli* DH5α that facilitates to study as the control that the extract does not inhibit LacZ enzyme, this segment gets lost when cloned in MCS thus the study of reporter assay could be done with the intact *lac*Z gene, has several restriction enzyme recognition site at MCS. It is also easily propagated in *E. coli* strains.

At first, the vector was transformed into cloning host *E. coli* DH5α by heat shock method in chemically prepared competent *E. coli* DH5α cells. The transformed colonies were selected on the basis of Ampicillin resistance marker by plating on LBA Amp (50µg/ml) plates after overnight incubation at 37˚C. Due to the presence of the antibiotic Ampicillin, selective pressure was created and only those transformants expressing ampicillin resistance marker gene, grows on the plate. Thus, the colonies observed in the plate were thought to be transformants and special care was given not to allow satellite colonies to grow after the ampicillin has been digested for non-resistant untransformed cells to arise. Small satellite colonies were avoided when selecting the transformants.

4.7.1 Plasmid isolation from transformants and preliminary confirmation

Cloning vector pUC19 that was transformed into the host *E. coli* DH5α by heat-shock method was isolated and the plasmid was confirmed by restriction digestion with single endonuclease enzyme whose restriction site is present in the vector to validate the plasmid provided was pUC19.

Briefly, the colonies on LBA Amp plate were randomly selected and sub-cultured on LB broth supplemented with 50µg/ml ampicillin at 37°C at 200 rpm. Plasmid was extracted using alkali extraction method. After visualization of isolated pUC19 plasmid in 0.8% agarose gel electrophoresis and after confirming the presence of plasmid it was subjected to restriction digestion by *Bam* HI enzyme at 37˚C for 3 hours. The digested product was then run along with undigested plasmid during gel electrophoresis. Single band seen on digestion product approximately at 2.6 kb gave preliminary confirmation for the presence of the plasmid of interest.

4.7.2 Primer design

Primers were designed based on the nucleotide sequence derived from NCBI for *P. aeruginosa (*PA 96) by downloading whole genome sequence as the promoter region was to be cloned. Going through the restriction enzyme map of respective promoter regions from New England Biolab DNA cutter web tool, it was decided that the sub-cloning would be done between *Eco*RI (*GAATTC*); nucleotide position 284 and *Bam H*I (*GGATCC*); nucleotide position 263 restriction enzyme recognition sites of pUC19. Moreover, it was thought of making reporter assay the *lac*Z gene needed to be cloned downstream of the promoter and 5'-UTR region.

Restriction recognition site of *Bam*HI does not give ATG codon at appropriate place and if the *Bam*HI downstream of ribosome binding site (RBS) then additionally one more enzyme has to be used. Thus, *Spe*I site was created before the *Bam*HI site as this is rare enzyme and can be used for subsequent several cloning by link and lock method (McGoldrick *et. al.,* 2005) if two or more genes needed to be cloned. Apart from the reporter assay if the function of CobA protein would be required and if its function has relation with other proteins then cloning of two or more proteins under same promoter could be made easy with *Spe*I and *Xba*I mediated link and lock method as these sites would be lost on subsequent cloning. Thus, *Spe*I site was created in such a way that the distance between RBS and start codon ATG would be maintained even after cloning of *lac*Z gene. Hence the forward primer for *lac*Z amplification was designed with *Spe*I restriction recognition site just upstream of ATG start codon and reverse primer was designed with *Bam*HI downstream of TAA stop codon (Table 18).

The stability, dimer formation, self priming and hairpin structure formation was studied using different web based tools for optimal stable structure of primer (Mfold structure is shown in appendix section).

4.8 Optimization of PCR condition for *cob* A gene and *lac Z* gene

Certain numbers of the DNA sequences are required upstream of any promoters for them to function, however, the length of DNA sequences required for functioning of *Cob*A promoter of *P. aeruginosa* has not been experimentally validated yet. Hence, the three sets of forward primers were designed as 100 bp, 200 bp and 600 bp upstream of

the TATA box that represents -10 from transcription start site (TSS) as to be recognized as the place of promoter in order to identify the required DNA sequence for promoter functioning upstream of TATA box.

In addition, to check whether there is SAM riboswitch in the *Cob*A mRNA the reverse primer was designed in such a way that it had native ribosome binding site (RBS) and the restriction enzyme recognition site was inserted to allow cloning from ATG start codon of *lac*Z gene by introducing *Spe*I site with *ACTAGT* which upon digestion can ligate with the *lac*Z insert digested with restriction enzyme *Spe*I that recognizes *ACTAGT*. The flanking single strand DNA of both restriction digested products are complimentary to each other thus allows ligation. This was done because creating *Nde*I recognition site in reverse primer could have allowed cloning from ATG start codon as it recognizes *CATATG* region, but pUC19 plasmid has this restriction site already in it and for subsequent cloning it could have hindered and partial digestion only could have done which would result in low efficiency of cloning.

Cloning from start codon is complicated when use of *Nde*I site is difficult. However, *Xba*I site could have been created but this site is also present in the plasmid thus second cloning could have faced same fate. As there is no *Spe*I recognition site in the plasmid this site could be used in subsequent second cloning with *lac*Z gene insert. Upon ligation of SpeIvector or and the insert digested with *Xba*I would ligate but with removing any sites there in cloned vector allowing for future works in similar way.

The genomic DNA isolated from the ATCC strain of *E. coli* 25923 and *P. aeruginosa* 27853 were used as template for the PCR amplification of *lac*Z gene and *cob*A gene, respectively. The annealing temperatures were optimized on the basis of amplicon produced by gradient PCR method. Hence, standard annealing temperature were optimized as 60.6°C for *cob*A F100, 61.2°C for *cob*A F200, 62.9°C for *cob*A F600 and 46°C for *lac*Z gene (Table 18) and these conditions amplified respective amplicons (Figure 43). 2 % Dimethyl sulfoxide (DMSO) was used in our PCR mixture because it is an organosulfur compound with a high polarity and high dielectric constant which help to disrupt secondary structure formation in the DNA template as well as primer. The PCR product was purified using Foregene Kit to remove buffer and enzymes for subsequent experiments as the buffer would have impact to restriction digestion since different enzymes use different buffer.

Table 18: Optimized PCR conditions for (CobA F100), (Cob A F200), (CobA F600) and *Lac Z*

Steps	Temperature(°C)	Time	Cycle
Initial Denaturation	98	5 mins	1
Denaturation	95	1 min	
Annealing	46 (for Lac Z) 60.6 (for CobA F100) 61.2 (for CobA F200) 62.9 (for CobA F600)	1 min	30
Extension	72	3 mins 45 secs (for *Lac* Z) 2 mins 32 secs (for CobA F100) 2 mins 39 secs (for CobA F200) 3 mins 3 secs (for CobA F600)	
Final Extension	72	10 min	1
Hold	4	∞	

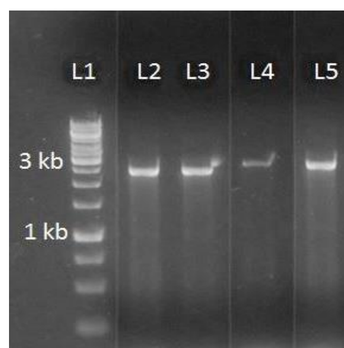


Figure 43: Bands of PCR products of inserts CobA and *Lac Z*) where, L1:1 kb ladder, L2:CobA F100 with 2522 bp amplicon size, L3:Cob A F200 with 2645 bp amplicon size, L4:CobA F600 with 3058 bp amplicon size, L5:Lac Z with 3075 bp amplicon size on 1% agarose by their respective primer

4.9 Restriction digestion of insert and pUC19 vector

The double restriction digestion of the purified insertCobA F100 and vector by same sets of restriction enzyme *Eco*RI and *Bam* HI gave the digested products of 2.5 kb and 2.6 kb respectively on 0.8% low melting agarose. Other high copy pBR322 origin plasmids could have been used whose size is bigger but upon cloning of promoter and the reporter gene the plasmid size would become big that might impact gene expression thus the small size plasmid was taken.

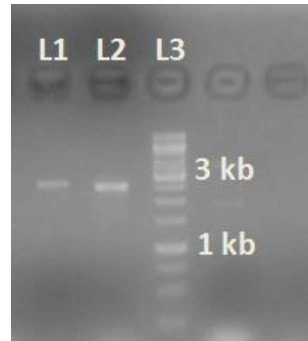


Figure 44: Restriction digested products of insert and vector where, L1: pUC19 vector with 2665 bp, L2 : CobA F100 with 2501 bp, L3: 1 kb Ladder

Moreover, this plasmid has higher number than other similar origin plasmid. Though cloning of insert that is close to or bigger than the vector is difficult but careful calculation of vector and insert DNA intensities would support in cloning albeit less efficient. The difference between sequence of restriction sites of two enzymes in the vector is only about 21 bp, so the small fragment of digested products was not visualized after gel electrophoresis. After excising of the corresponding bands from gel, both products were purified by low melting gel extraction kit. This helps to prevent the effect of ligation reaction between insert and vector due to presence of metal ions in buffer, unused dNTPs, excess primers used in PCR, restriction enzymes used in restriction digestion. After purification, the concentration of the insert and vector were quantified (Figure 44).

4.10 Ligation and transformation

The ligation reaction was carried out for purified insert (CobA F100) and vector by using T4 DNA ligase enzyme. The enzyme forms the covalent phospho-di-ester bond between nick of double strand DNA. Since the flanking region of DNA digested with these cohesive end generating restriction enzymes are complementary to insert and vector DNA they make double strand and the nick present would be ligated by T4 DNA ligase making phosphate-di-ester bond similar to the phosphate backbone of DNA. The ligation mixture contained approximately 3:1 molar ratio of insert and vector. The DNA ligase enzyme is found to have optimal activity at 25°C but overnight incubation was done at 16 °C. After incubation, whole of the ligation mixture was directly subjected to transformation into fresh *E. coli* DH5α competent cells by heat shock method as described. The ampicillin resistant transformants were selected after plating on LBA-Amp (50 µg/ml) plate (Figure 45) as newly cloned vector plasmid will have ampicillin resistant gene intact as the marker gene.

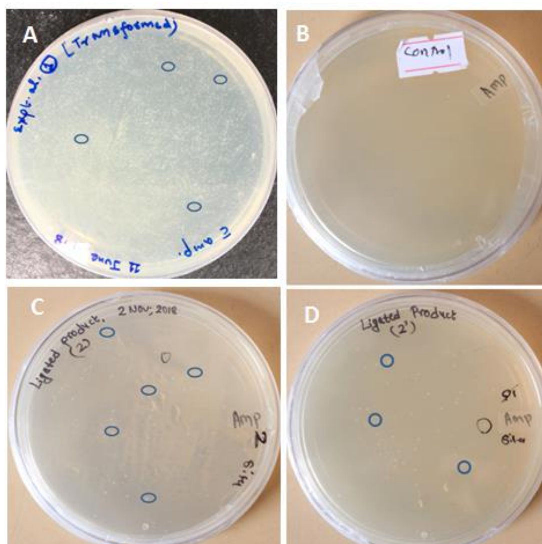


Figure 45: Transformation of *E. coli* DH5α competent cells. A; positive control (pUC19), B; Negative control, C and D; with ligation mixture (pUC19 + CobA F100) as experiment. Transformed colonies are enclosed in circle.

4.11 Screening of transformants

The transformants in ampicillin plates were randomly selected to confirm the presence of clones. Further confirmation of sub cloning was done by the single restriction digestion of isolated transformed plasmid and PCR using CobA F100 primer set. Although sequencing is done to confirm that the cloned DNA is exact the same as that has been reported in database but as this was preliminary experiment to devise a protocol the clones were not sent for sequencing.

Screening by Restriction digestion

The randomly picked clones were grown overnight for plasmid extraction in LB media with ampicillin. The plasmid was extracted as mentioned and upon running in 0.8 % agarose gel electrophoresis and visualized under Gel doc system gave clear bands indicating presence of plasmid DNA (Figure 46).

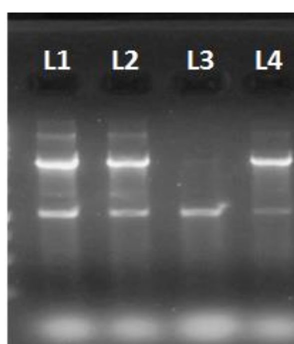


Figure 46: Gel Electrophoresis of plasmid extracted from transformants. L1, L2 are plasmid from Transformant colonies C1 and C2 respectively, L3 and L4 are plasmid from Transformant colonies D1 and D2 respectively.

The plasmids thus extracted were subjected to screening by single restriction digestion. An endonuclease enzymes used were *Bam*HI and *Spe*I that would yield the digestion

product of size approximately 5.2 kb. After visualization in 0.8% agarose gel electrophoresis, two prospective cloned plasmid constructs gave expected digestion product size and hence it was confirmed to be our required construct and it was named as **"pAG101"** (pUC19 +CobA F100) onwards. The digestion with *Bam*HI and *Spe*I enzyme (Figure 47) confirmed that the cloned DNA was the one that we had amplified and used as insert for sub-cloning because the plasmid does not have this restriction enzyme recognition site.

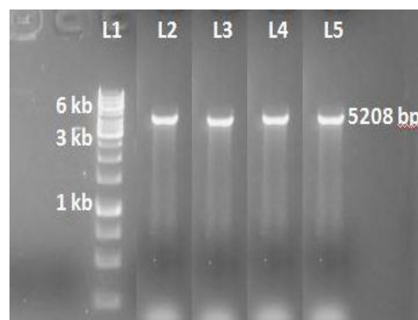


Figure 47: Agarose gel electrophoresis (0.8%) of Single digested plasmid by *Bam HI* and *Spe I*. L1- 1 kb DNA ladder, L2- Digested C1 plasmid by *BamH*I, L3- Digested D1 plasmid by *Bam*HI, L4- Digested C1 plasmid by *Spe* I and L5- Digested D1 plasmid by *Spe*I

Preliminary Screening by PCR

The plasmids isolated from putative transformed colonies were used as template for PCR amplification using CobA F100 specific primers. This PCR gives preliminary screening of transformation if ligated giving the construct and gives amplicon size of 2522 bp similar to the amplicon size. The PCR product gave amplicon size corresponding to the insert size (Figure 48) indicating that the DNA has been sub-cloned between *Eco*RI and *Bam*HI restriction recognition sites present in pUC19 vector.

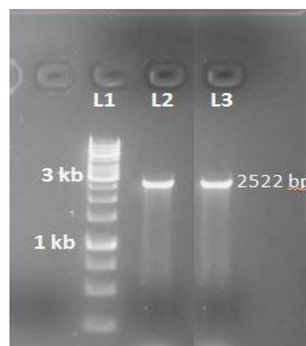


Figure 48 : Preliminary screening of transformation after ligation by PCR where, L1: 1kb ladder, L2: PCR product from C1, L3: PCR product from D1 .

4.12 Restriction digestion of *Lac Z* gene (second insert) and "pAG101"

The double restriction digestion of the purified insert *Lac Z* and **"pAG101"** vector by same sets of restriction enzyme *Bam*HI and *Spe*I gave the digested products of 3 kb and 5 kb respectively on 0.8% low melting agarose (Figure 49). The difference between

sequence of restriction sites of two enzymes is less, so the small fragment of digested products was not visualized after gel electrophoresis. After excising of the bands from gel, both products were purified by low melting gel extraction kit.

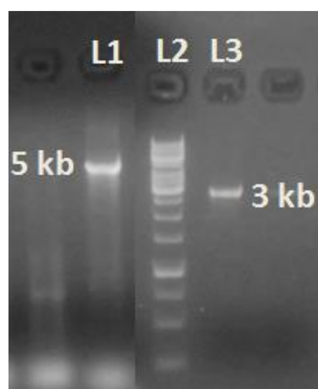


Figure 49 : Restriction digested products of insert *Lac Z* and vector **"pAG101"** where, L1 : **"pAG101"** vector with 5000 bp, L2 : 1 kb Ladder, L3: *Lac Z* with 3000 bp

4.13 Ligation and transformation for second insert

The ligation was carried out for purified insert consisting coding sequence of entire *lac*Z and newly developed vector **"pAG101"** by using T4 DNA ligase enzyme as mentioned above. After incubation, transformation was done as above into fresh *E. coli* DH5α competent cell. The ampicillin resistant transformants were selected after plating on LBA-Amp (50µg/ml) plate as second cloned vector plasmid will have ampicillin resistant gene intact as the marker gene. The insertion of *lac Z* gene under the promoter of *cob*A with around 100 nucleotides upstream to study whether the promoter is active or not by the function of this protein to confirm whether it will be expressed under this promoter. The same cloning mechanism could be used for developing bicistronic construct.

The main purpose of this cloning is to elucidate the promoter function. In addition, this cloning is to study the native promoter and the 5'-UTR region is affected by the antimicrobial substance produced by the *Streptomyces* sps. isolated and also the standard *S. coelicolor* that was tweaked with different substrates by assaying *LacZ* function. Furthermore, the compounds derived from CADD could also be tested once the assay protocol is optimized. If the protocol could be optimized then it would new assay system to look for SAM analogues as riboswitch modulators that can have impact in vitamin B12 biosynthetic during corrin ring contraction. This will be new avenue for tackling pathogens as drugs against vitamin B12 biosynthesis is not yet common. Although bacteria have transporters for this vitamin but due to its bulk size the bacteria have to use tremendous energy to transport impacting its cell growth.

5 SUMMARY

The rapid increase in global antibiotic resistance demands the speedy development of novel antibiotic to solve this problem. Taking this into account, cost and time effective computational approaches were applied to find putative drug candidates. From the virtual screening, two kinase inhibitors N-[3-[4-[4-(trifluoromethyl) benzoyl] piperazine-1-carbonyl] phenyl] pyridine-3-carboxamide (ZINC000040086879) and 1-(4-phenyl piperazin-1-yl)-2-[4-[2-(trifluoromethyl) benzoyl] piperazin-1-yl] ethanone (ZINC000026610689) and one indole derivative (1R,4R)-4- (1H-indol-3-yl methyl)-1-methyl-2,4- dihydro-1H- pyrazino [5,4-b] quinazoline-3,6-dione *(*ZINC15219763_uff_E=554.59) were screened out as drug candidates against Uroporphyrinogen III methyl transferase of *Salmonella* sps. which are to be further verified *in vitro.* These leads suggested that the compounds with pyridine and indole has some impact. Thus, this lead molecule structure was taken to develop antibiotics from *Streptomyces* sps.

Streptomycetes is the ideal antibiotics producers thus were explored in this study to find noble strains and potential secondary metabolites against MDR pathogens of WHO prioritized list. The screening and production of antimicrobials through traditional method did not reveal much antibiotic potential and was modified by co-incubating various lignin degradation phenolic products as substrate and to enhance the antibiotics production into the culture media. The crude non concentrated antimicrobial extracts of four sequenced *Streptomyces* strains (PA2, PA3, MA1, KA10) showed the promising inhibition effect against carbapenem and colistin resistant biofilm producing *Salmonella* sps when coincubated with 5 mM indole and tannic acid. Further expansion of antimicrobial assay was performed by fast and robust high throughput resazurin antimicrobial assay for standard *S. coelicolor* strain and newly isolated pigment producing PA3 strain. Out of various lignin degradation products, coumarin with addition of valine plus tryptophan (5 mM) showed the highest percentage reduction against *Salmonella* sps and MRSA. Likewise, among the various carbohydrates sources used, pectin and cellulose were found to have highest percentage inhibition against those two bacteria. It is hypothesized that the metabolized indole generated from tryptophan is activated by cytochrome of *Streptomyces* for the production of antibiotics.

In addition, it is now necessary to explore the mechanism of action of newly produced antibiotics from *Streptomyces* strains. Hence, unknown riboswitch mechanism of 5'- UTR of gene was tried to observe via promoter cloning of essential gene CobA by β-galactosidase reporter gene assay. But the complete experiment was not performed due to time and resource constraints hence should be verified and expanded further.

Thus, it is presumed that the simultaneous work of computational biology, tweaking of strain and promoter cloning of essential gene could become handy and effective for the development of lead candidates and to explore their mechanism of action against MDR pathogens.

6 CONCLUSION

The increased antimicrobial resistance problems in the poultry and clinically isolated pathogens have rendered all the developed antibiotics ineffective. This demands the development of new and potent antibiotics to prevent the world from apocalyptic condition.

From the present study, with computational approaches to find out new drug candidates revealed potent inhibitor against *Salmonella* sps. It was hypothesized that one of the screened indole derivatives could be putative competitive inhibitors for SAM binding pocket and also might act as kinase inhibitors. This leads to tweaking of isolated *Streptomyces* with modification in culture conditions. Four isolates were found to be active against MDR *Salmonella.* The antibiotics producer standard *Streptomyces* strain *S. coelicolor* and isolated strain PA3 were further used to produce antibiotics by modifying the media component with incorporation of various lignin degradation products and carbon sources and found to give good results. The produced antibiotics by these strains were thought to act upon methyl transferases so were planned for exploring the mechanism of action against pathogen by riboswitch inhibition mechanism and β-galactosidase reporter assay should be performed further for the validation of promoter cloning of essential gene CobA.

RECOMMENDATIONS

Thus, it is recommended that the putative lead molecules be tested *in vitro* for AST and further pursued as potential lead molecule to develop potential drugs. Respective reporter gene assay could be studied and riboswitch structure could also be investigated. The lead molecules could be taken for animal testing and toxicity testing.

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8 APPENDIX

8.1 Reaction Mixture for PCR

Components (stock concentration)	Volume (working concentration)
Template(100ng/µl)	2 µl (more than 100 ng)
Forward primer (10pM/µl or 10µM)	2 µl (1 µM)
Reverse primer (10pM/µl or 10µM)	2 µl (1 µM)
Master Mix (2X)	10 µl (1X)
NFW	4 µl (To adjust final volume)
Total	20 µl

8.2 Resazurin Assay for lignin Degradation Product (*S. coelicolor* against *Sal4)*

	1	2	4	6	10	11	12
A	2.71	1.442	2.264	2.738	0.299	2.658	2.586
B	2.754	1.537	1.205	3.017	0.202	2.966	2.982
C	2.855	1.347	0.484	0.683	0.204	2.905	2.56
D	2.649	1.506	1.864	2.513	0.198	1.985	2.681
E	2.787	2.712	2.269	1.213	0.209	1.336	2.752
F	2.812	1.507	1.508	1.315	0.196	0.567	2.841
G	2.627	2.821	1.915	1.588	0.202	0.37	2.315
H	2.961	0.22	2.289	1.735	0.208	0.37	1.466

8.3 Resazurin Assay for Carbon sources (*S. coelicolor* against *Sal4)*

	1	3	5	7	10	11	12
A	2.487	1.429	2.114	1.796	1.02	0.045	2.8
B	1.956	1.262	1.831	1.607	1.088	0.051	2.725
C	2.359	1.407	1.155	1.286	1.064	0.038	2.733
D	2.227	1.191	1.773	1.838	1.037	0.044	2.615
E	2.369	1.422	1.398	1.917	1.071	0.041	2.724
F	2.418	1.457	2.091	2.566	1.723	0.042	2.715
G	2.319	1.164	2.043	2.213	1.546	0.04	2.701
H	2.262	1.229	2.184	2.205	1.625	0.033	2.777

8.4 Use of lignin sources for resazurin assay

	2	3	4	5	6	7
A	-	-	Acetic acid	Acetic acid	Coumarin+aa	Coumarin+aa
B	-	-	Furfuraldehye	Furfuraldehye	Tannic acid +aa	Tannic acid +aa
C	Furfural	Furfural	Guaiacol	Guaiacol	Vanillin+aa	Vanillin+aa
D	Transferrulic acid	Transferrulic acid	-	-	Acetic acid +aa	Acetic acid +aa
E	Vanillic acid	Vanillic acid	-	-	Furfuraldehyde+aa	Furfuraldehyde+aa
F	Coumarin	Coumarin	Furfural+aa	Furfural+aa	Guaiacol+aa	Guaiacol+aa
G	Tannic acid	Tannic acid	Transferrulic acid +aa	Transferrulic acid +aa	Furfural+aa	Furfural+aa
H	Vanillin	Vanillin	Vanillic acid +aa	Vanillic acid +aa	Guaiacol+aa	-

8.5 Use of Carbon Sources for resazurin assay

	3	4	5	6	7
A	Glucose	Glucose	Cellulose	Cellulose	Pectin+aa
B	Sucrose	Sucrose	Glucose+aa	Glucose+aa	Pectin +aa
C	Starch	Starch	Sucrose+aa	Sucrose+aa	Glucose +aa
D	Sorbitol	Sorbitol	Starch+aa	Starch+aa	Sucrose+aa
E	Maltose	Maltose	Sorbitol+aa	Sorbitol+aa	Starch+aa
F	Mannitol	Mannitol	Maltose+aa	Maltose+aa	Maltose+aa
G	Lactose	Lactose	Mannitol+aa	Mannitol+aa	Cellulose+aa
H	Pectin	Pectin	Lactose +aa	Lactose +aa	Cellulose+aa

8.6 Growth media for *Streptomyces*

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

8.7 Primer Sequences

1. Sequence of Forward and Reverse primer of Universal 16S rRNA gene

Forward Primer: 5' – AGAGTTTGATCMTGGCTTCAG-3'

Reverse Primer: 5'- CGGTTACCTTGTTACGACTT-3'

2. Sequence of Forward and reverse primer for *Salmonella* specific 16S rRNA gene

Forward Primer: 5'- CGG ACG GGT GAG TAA TGT CT -3'

Reverse Primer: 5'- GTT AGC CGG TGC TTC TTC TG – 3'

3. Sequence of Forward and reverse primer for *mcr*-1 gene

Forward Primer: 5'- CGG TCA GTC CGT TTG TTC -3'

Reverse Primer: 5' –CTT GGT CGG TCT GTA GGG – 3'

4. Sequence of Forward and reverse primer for *bla* NDM gene

Forward Primer: 5'- AAT GCT GAA TAA AAG GAA AAC T -3'

Reverse Primer: 5' –GGC AGA TTG GGG GTG A – 3'

5. Sequence of Forward and reverse primer for *Streptomyces* Specific PCR (Set I)
Forward Primer: 5'-ACAAGCCCTGGAAACGGGGT-3'
Reverse Primer: 5' –CA CACCAGGAATTCCGATCT– 3'
6. Sequence of Forward and reverse primer for *Streptomyces* Specific PCR (Set II)
Forward Primer: 5'-ACAAGCCCTGGAAACGGGGT-3'
Reverse Primer: 5' –ACGTGTGCAGCCCAAGACA– 3'

8.8 Reagents Preparation

Alkaline Lysis Solution I

50 mM Glucose

25 mMTris-Cl (pH 8.0)

10 mM EDTA (pH 8.0)

The solution was sterilized by autoclaving and stored at 4°C.

Alkaline Lysis Solution II (Freshly prepared)

0.2 N NaOH

1 % (w/v) SDS

Alkaline Lysis Solution III (per 100 ml)

5 M Potassium Acetate	60.0 ml (Final concentration in 100 ml is 3 M)
Glacial Acetic Acid	11.5 ml
Autoclaved Distilled Water	28.5 ml

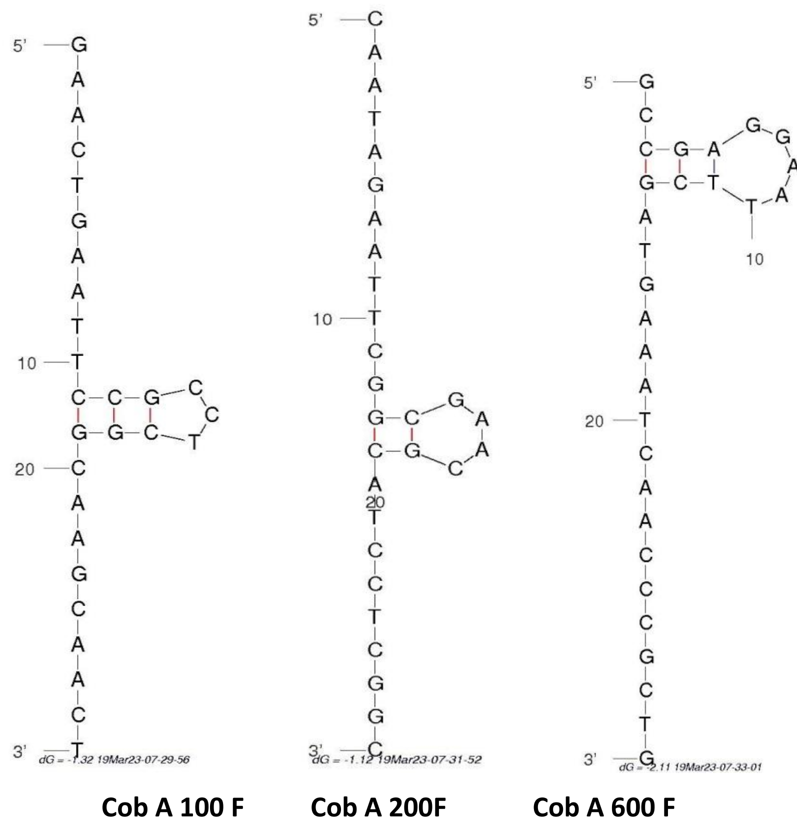
8.9 Chromatogram Figure of isolated *Streptomyces*

Reverse chromatogram of PA2

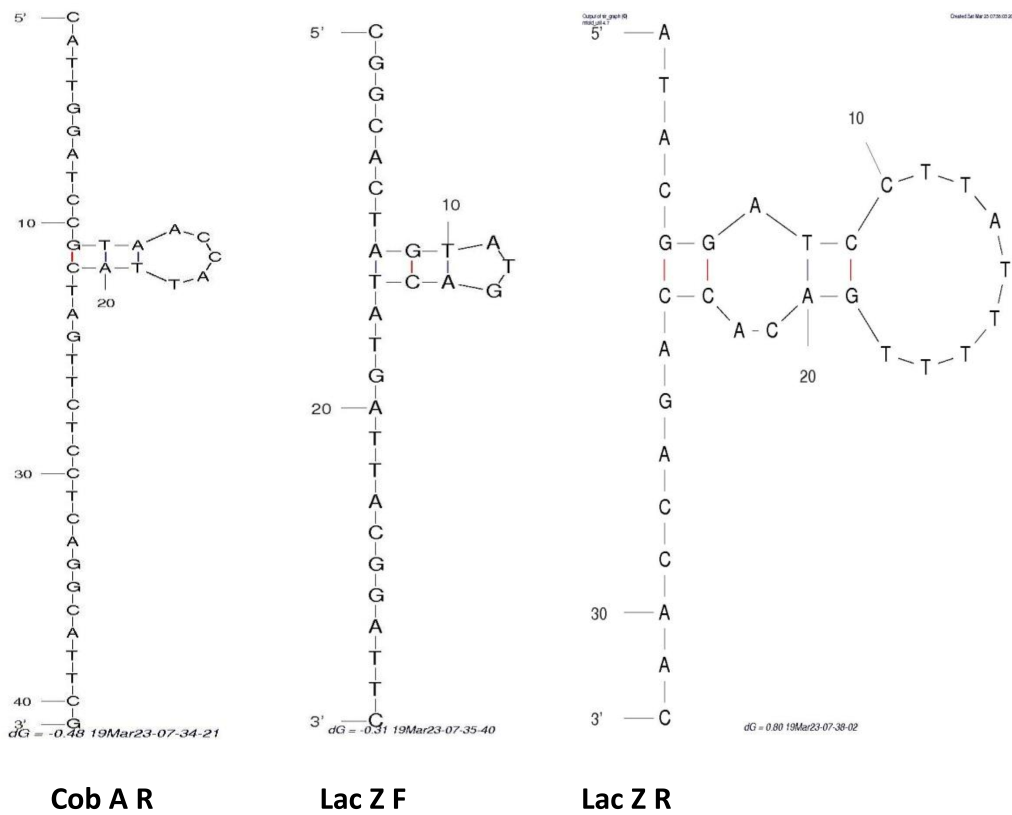
Forward chromatogram of PA3

Reverse chromatogram of KA10

8.10 M fold Structure of designed Primer of Cob A and Lac Z gene



Cob A 100 F **Cob A 200F** **Cob A 600 F**



Cob A R **Lac Z F** **Lac Z R**

8.11 Biochemical tests of MDR Bacteria

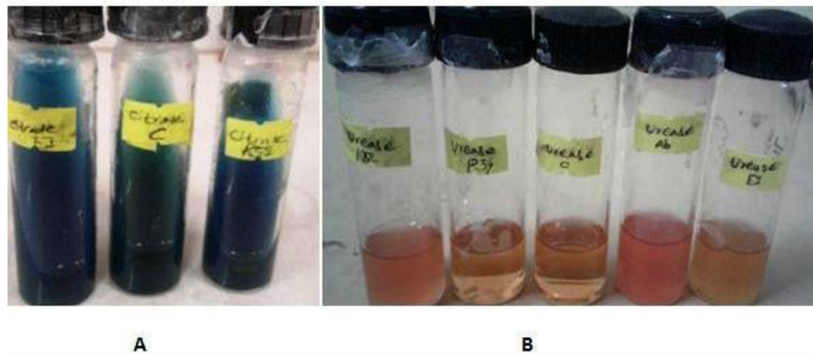


Figure : Citrate and urease test of MDR pathogens

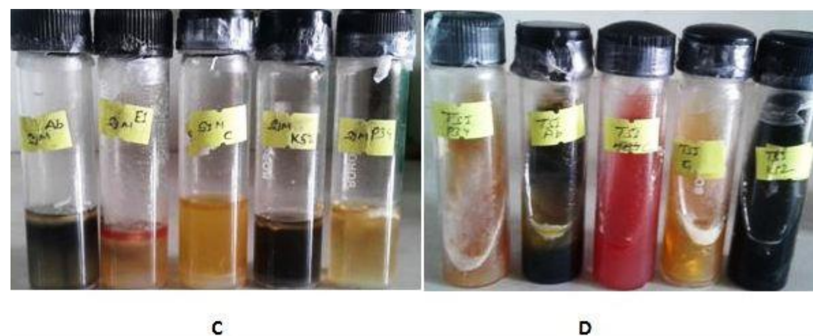
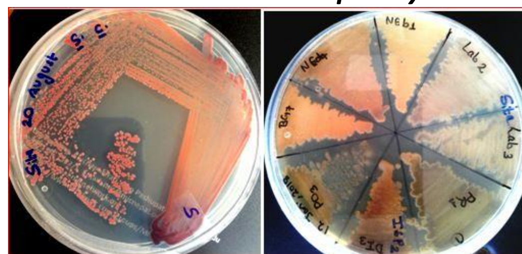
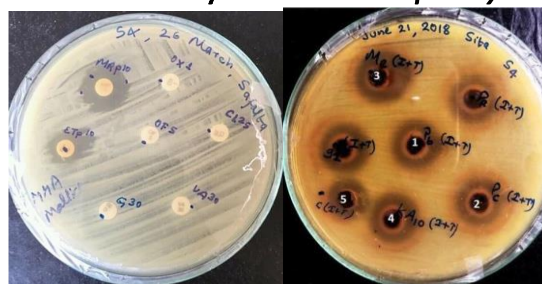


Figure: SIM and TSIA tests of MDR pathogens

8.12 Single pure colony Isolation of Putative *Streptomyces*



8.13 Comparative antimicrobial test by extract of *Streptomyces* and antibiotics discs



8.14 Biochemical Tests of putative *Streptomyces*

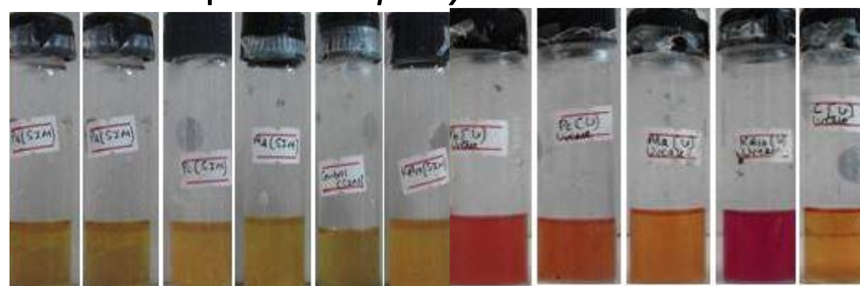


Figure: Indole and urease tests of putative *Streptomyces*

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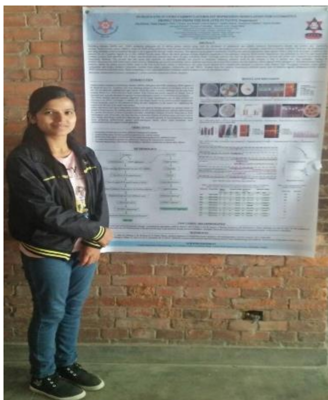

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