Geomicrobiological Investigation and Environmental Implications of Bacterial Isolates of Kashmir Caves, Pakistan.

By

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This work is submitted as a dissertation in partial fulfillment for the award of the degree of

Doctor of Philosophy
In
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Dedicated to My Parents and Son
“Abdullah”

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List of Acronym/abbreviations (alphabetically)

Abbreviations	Full Name
%	Percentage
°C	Degree celsius
µl	Microlitre
A	Adenosine
AMP	Ampicillin
ANalyzerMn	Manganese
ATP	Adenosine-5'-triphosphate
BLAST	Basic Local Alignment Search Tool
BLAST X	BLAST search using a translated nucleotide query
Ca	Calcium
CHL	Chloramphenicol
CTAB	Cetyltrimethylammonium Bromide
DNA	Deoxyribonucleic acid
e.g.,	Exempli gratia, for example
et al.	et alii/alia, and others
Fe	Iron
Fig.	Figure
FTIR	Fourier Transform Infra-Red spectroscopy
GBL	Guano bottom layer
GML	Guano middle layer
GTL	Guano top layer
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid LBB
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid LBB
LDPE	Low density polyethylene
MCO	Multi Copper Oxidase
MEGAN 5	MEtaGenome
MOB	Manganese Oxidizing Bacteria
NaCl	Sodium Chloride
NAL	Nalidixic acid
NCBI	National Center for Biotechnology Information
OD	Optical Density
PCR	Polymerase Chain Reaction
pH	Power of Hydrogen
RDP	Ribosomal Database Project
rRNA	Ribosomal ribonucleic acid
SEM	Scanning Electron Microscopy
SOD	Superoxide Dismutase
TBE	Tris base, boric acid EDTA
UV-Vis	Ultra Violet Visible
VOC	Volatile Organic Compounds
X-RD	X-ray Powder Diffraction
Zn	Zinc

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In the Name of ***Allah****, the Most Beneficent, the Most Merciful, who has taught (the writing) by the pen and Peace Be Upon Prophet* ***MUHAMMAD*** *about whom He says in The QURAN "And verily, for you (O MUHAMMAD PBUH) will be an endless reward. And verily, you (O MUHAMMAD PBUH) are on an exalted (standard of) character.*

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Syed Umair Ullah Jamil

Abstract

Much of the current microbial research has focused on biotechnological applications of bacterial strains isolated from soil and water. There exists limited data on potential of bacteria isolated from extreme environments like caves. Our understanding regarding extreme habitats of this earth are still limited and there is a need to explore them for better understanding of their role in natural regulation of earth's resources.

Geological formations are closely associated with microbiological processes. Microorganisms of all types play an important role in formation and evolution of various geological features of the earth. In the current study, an attempt was made to illustrate geochemical and microbiological properties Bat guano from a karstic limestone cave in Khyber Pathunkhwa Province of Pakistan. Analysis was carried out for 3 layers of bat guano pile (top, middle and bottom). Geochemical profiling for various minerals and heavy metals was carried out using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS). Nutrient composition including total organic carbon, total nitrogen, phosphorus and potassium was evaluated using C-N analyzer. Culturable bacterial diversity was established by culturing bacterial species from each layer on Nutrient Agar plates. DNA extraction was done for sequencing of 16S ribosomal RNA gene analysis. In this study, we report antimicrobial compounds producing bacterial strain isolated from Bats guano of a limestone cave. Screening of bacterial strains was carried out from top, middle and bottom layer of the guano sample. Out of 20 isolated strains, 3 showed antimicrobial activity against all tested ATTC strain of common bacterial (both gram positive and gram negative) and fungal species. 16s RNA gene sequencing of the strain showing maximum activity revealed the isolated strain as *Bacillus subtilis.* The current study was aimed to investigate occurrence of multidrug resistance in bacterial species inside Kashmir cave of province Khyber Pakhtunkhwa, Pakistan. A total of 14 bacterial strains were isolated from 3 different layers of bat guano samples of the cave and screened for their potential to resist 6 commonly used antibiotic classes namely, aminoglycosides, beta lactams, carbapenems, fluroquinolones, macrolides, and glycopeptides. The occurrence of antibiotic resistance genes namely*, bla-CTXM*, *bla-NDM*, *Gyr A* and *vanA* gene was screened through

polymerase chain reaction by amplifying the DNA. This study focused on evaluation of biodegradation potential of *Alcaligenes faecalis* bacterial strain, isolated from cave bat guano samples. The guano sample was collected from deep end of the cave and screening for LDPE bacteria was carried out from three layers of the bat guano. GTL 3 strain was selected for the experimentation based on maximum growth shown during the screening process. Biodegradation was carried out in MSM medium amended separately with glucose, Tween 80 and $CaCO_3$. Assessment of biodegradation process was done on the basis of growth of the bacterial strain (OD 620nm), weight loss percentage of LDPE pieces, mechanical characterization including young's modulus, tensile strength, tear strength and elongation at break at every 10^{th} of the experiment till 90^{th} day. Lipase production of GTL 3 strain was also evaluated at every 10^{th} day for 90 days using NPP as substrate. Chemical changes and change in functional groups were checked using Fourier Transform Infra Red spectroscopy at the end of the experiment. Physical changes were observed using Scanning Electron Microscopy at the end of the experiment.

ICP-MS showed presence of various minerals including heavy metals in different layer of bat guano. The mineral concentration was high in fresh guano samples (top layer) and decreased as we move towards lower layer (old deposits). Carbon-Nitrogen Analysis (CN Analyzer) of Guano showed top layers to be rich in organic carbon, total nitrogen, potassium and phosprus content that decreased in amount we moved from top layer to the bottom layer. Phylogenetic analysis of Bat guano revealed 64.28% bacteria belonged to phylum *firmicutes*, 35.70% to *proteobacteria*, and 7.10% to phylum *bacteroides*. Optimum production of antimicrobial compounds took place in TSB medium after 72h of incubation at 30˚C. FTIR of crude extract showed chemical similarity with bacitracin. GCMS analysis of the crude extract revealed a range of antimicrobial and anticancer compounds. Almost all the isolates showed resistance to more than 1 of the tested antibiotic classes. *Firmicutes* demonstrated high resistance against carbapenems, while *Proteobacteria* and *Bacteroides* were more resistant against the β lactam and aminoglycoside class of drugs. 78.5% of the strains were positive for *bla-CTXM* gene, 64.2% for *bla-NDM*, 42.8% for *Gyr A* and 35.70% for *vanA* gene. The results of the study revealed an initial decrease in in OD that stabilized with increasing time of incubation. Maximum growth (OD 1.9) was observed in culture with added $CaCO_3$ in it. Weight loss % increased continuously throughout the experiment with maximum loss of 6.9% was observed by the end of the experiment in $CaCO_3$ medium. Maximum decrease in Young's modulus was from 385.423Mpa to 316.964Mpa, tensile strength

from 15.6Mpa to 2.62Mpa, tear strength from 126.486 N/mm to 65.581 N/mm and elongation at break from 17.98mm to 1.98mm in $CaCO_3$ amended medium by end of experiment. Lipase production increased in the initial days of the experiment, with a maximum production of 18.4 U/mL at day 30 and decreased continuously form hereafter. FTIR showed peak formation at 2345cm^{-1}, 3493cm^{-1}, 726cm^{-1},1145cm^{-1}, 1875cm^{-1}, 2250cm^{-1}, and 3560cm^{-1} suggests oxidative breakdown taking with the polyethylene polymer. SEM images revealed clear signs of physical damage, erosion, abrasion of LDPE pieces. Bacterial adherence and biofilm formation by GTL 3 on LDPE surface was also observed in SEM images.

This study is a first comprehensive research on biodiversity assessment and environmental implications of bacterial isolates of bat guano samples of Kashmir cave, Pakistan. It can be concluded form this research that the guano samples contained various essential and non essential elements and diverse bacterial flora. The isolates were capable of producing a variety of bioactive compounds, resist multiple commonly used drugs (MDR) and efficiently biodegrade polyethylene polymer.

The future work should focus on metagenomic studies to document unculturable biodiversity of bat guano. Metabolomics studies should be conducted to decipher hidden metabolic range of these organisms. Studies on biogeochemical cycles should be carried to determine niche of bacteria isolates of bat guano in cave ecosystem.

Chapter 1

Introduction and Literature Review

Caves are formed by natural geobiological process and are mostly large underground enclosed place mostly within a mountain or inside the sea. Since they are subsurface ecosystem, complete darkness prevails inside the caves. Based on the geography, there are 2 types of caves, the caves that develop in seas and those develop on land. Caves formed inside sea rocks are usually shorter in dimensions ranging from five to fifty meters and may exceed to maximum of 300 meters (Burcham, 2009). Due to constant minimal pressure and decreased oxygen quantity inside caves, the environment is hostile specially for larger animals, including human beings. Large cave systems are many miles long and have an air circulation system of their own. Most large caves experience wind speeds in excess of 80 miles per hour inside them (Barton & Northup, 2007).

Scientists start studying caves in 17th century but later, but it was not until the 19th century, when the researcher started value caves for their environment significance and they become a topic of interest for other basic and applied sciences, like biology, geography, chemistry and archeology. Exploring caves is difficult owing to darkness, unpredictable terrains with no pre-indications (Crane & Fletcher, 2015). Environmental conditions inside caves consist of constant low temperature, high humidity, with scarce availability and slightly acidic pH (Biswas, 2010a). Temperature inside cave may rise to a maximum value of 10^{o}C, the humidity in deep parts of the cave is almost 100%. It was a popular opinion among scientists that living organisms cannot live inside caves due to complete darkness, nutrients limitation, acidic condition, and presence of hydrogen sulfide gas. The opinion was soon changed when the first reports of life from the caves came to light (Engel, 2010).

Speleology is the scientific study of cave, and studying cave with a biological perspective is known as biospeleology (Onac & Forti, 2011). The study of caves includes various aspects of the cave like the physical features, structure of the cave, history of the rock or the mountain, life forms it contains and the process of its formation. The process of cave formation is called speleogenesis. Speleology is an interconnected branch of sciences with research inputs from chemistry, biology, geology, physics, meteorology and cartography. Edouard-Alfred Martel (1859-1938),

French geologist, was the first who studied the modern speleology and is considered as the father of modern speleology. He founded an association called Societe de Speleologie in 1895 that was the very first association for scientifically study caves. To take caves to general public, caving has been made a sport to attract public interest and impart awareness. Most of the basic speleological fieldwork is carried out by sport cavers. Different countries have given different name for the study caves. Like in United States, it is known as spelunking while in Canada and other close areas, potholing is the term used (Engel, 2010).

Caves have remained under humans use for multiple purposes in early era including usage as temporary shelter during harsh environmental conditions, celebration of religious or non religious rituals, hiding wealth and other valuable items, important source of minerals, paleolithic artworks and painting etc.

1.1 Cave genesis

The process through which caves come into existence is called speleogenesis. Speloegenesis of caves takes place through a multidimensional array of geological processes including wide range of redox chemical reactions, dissolution of soluble rocks, physical and mechanical weathering, tectonic and subsurface forces, erosion and corrosion by water, melting of ice or glacier, mineralization by microorganisms, external or internal pressure, atmospheric influences and by excavation. Dissolutional caves with the soluble rocks are formed by water flowing on the surface and through the surface of limestone rocks. The process of dissolution can be epigenic (starts from top-down) or hypogenic (starts from bottom-up process). Pseudokarst is a term used for all the dissolutional caves that are formed in basalt or sandstone and not within limestones (Lace et al., 2013). Caves formed by erosional processes are mainly formed by mechanical scrubbing action or by contact wave action rather than by dissolution of the rock mineral itself. Dissolution caves can change their type and transform` into erosion caves as the time passes. Sea caves are usually developed by erosional processes along sea cliffs whereas anchialine caves are formed via dissolution process along its sides. Lava tube caves, on the other hand, are different than dissolution caves as they are formed from cooled crust around the flowing lava.

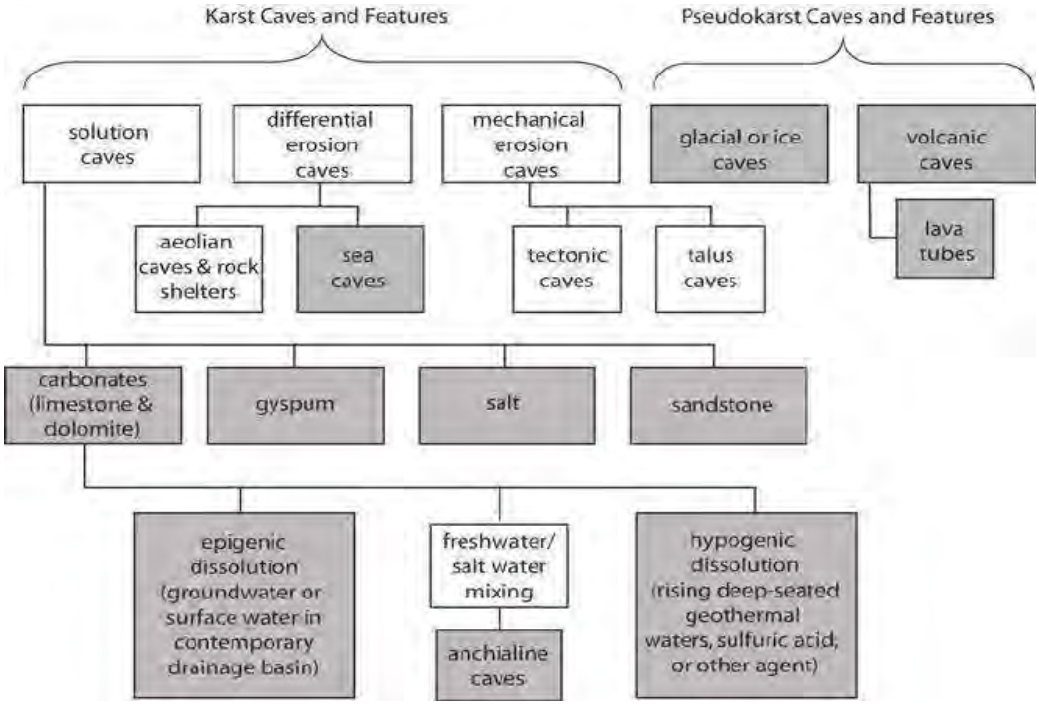


Figure. 1.1Flow diagram illustrating formation processes of different types of caves (White & Culver, 2011).

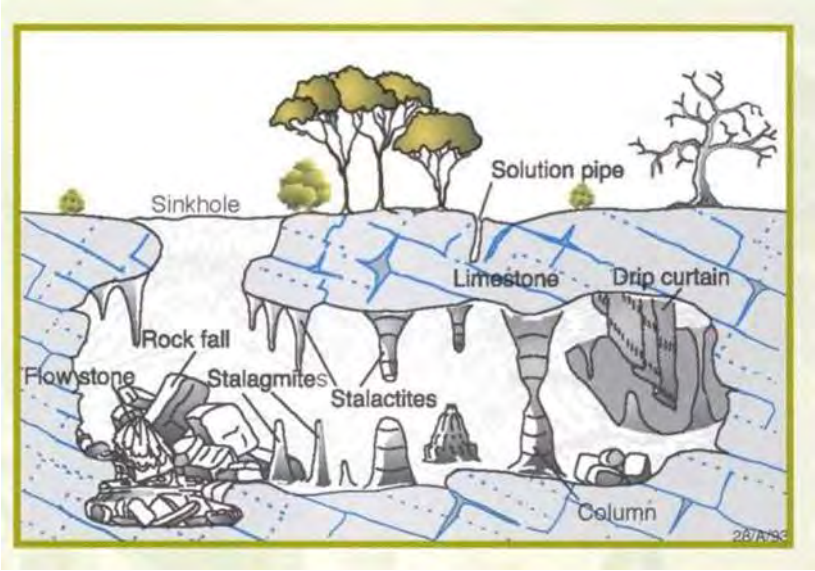


Figure 1.2. Processes involved in Formation of Solutional cave (Gutierrez et al., 2008).

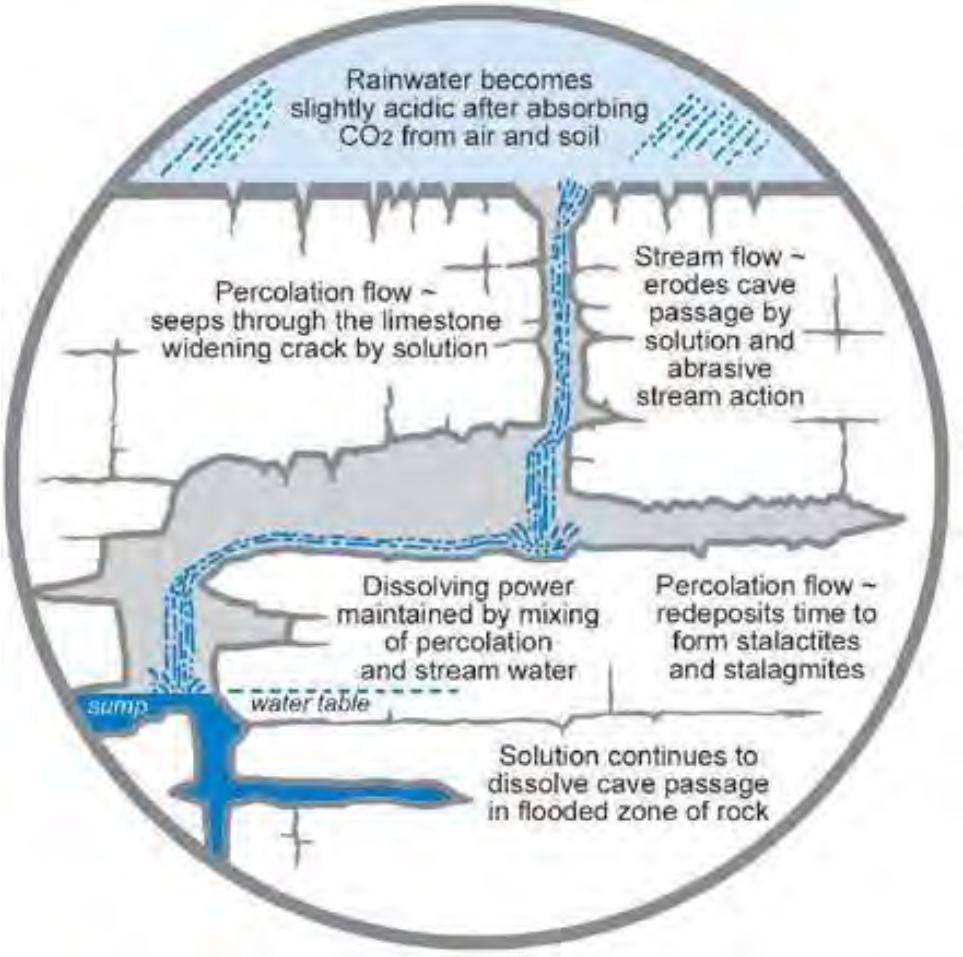


Figure. 1.3. processes involved in formation of a limestone cave (Ford & Williams, 2007).

The most common caves that we see are formed through dissolution of minerals by water. Rock solubilization is one of the fundamental processes in initiation of cave formation but is not powerful enough to continue speloegenesis for prolonged time periods. Climatic parameters influence the strength and pace of mineral dissolution bywater. Speleogenesis occurs at a much rapid pace in humid and warm conditions and vice versa.

1.2 Caves and microbial life

Limestone caves are the most common type of caves formed throughout the world followed by basaltic caves. Caves invariably are oligotrophic (low nutrients) ecosystems constant cold temperature, absence of light and high relative humidity,

yet they harbor rich microbial life within them. Thetypical amount of bacteria in cave biome is 10^6 cells/g sample of rock (Barton & Jurado, 2007). Autotrophic bacteria are concentrated along the entrance zone of cave where biologically active light is available. These bacteria can also be found deep inside the cave due to incandescent light mounted by public/visitors/tourists. Absence of bioavailable light beyond the entrance zone in cave the primary production by microbes through the process of photoautotrophy further down is almost negligible. In these zones, the prominent process for fixation of carbon and autotrophy is chemolithoautotrophy, predominantly carried out by cave microorganisms. Since the light is not available, microorganism carry out autotrophy in such environmental conditions by utilizing hydrogen, nitrogen or any other inorganic compound. They are also capable of reducing metals such as manganese and iron, on the cave rocks and walls in order to obtain energy (Gadd, 2010). The allochtonous material source that connects caves with external environment is drip water that leaks through cracks and crevices within the rocks, or the organic particulate matter get transported inside the cave through air currents. Other sources of organic carbon inside the cave can include roots of the plants, passing mammals (excreta and remnants) and anthropogenic activities. This organic matter helps heterotrophic bacteria to thrive in cave environments. Many physical and chemical factors including nutrient availability, pH, oxygen concentrations, concentrations of various metals and substrate susceptibility to for biofilms can positively or negatively influence microbial communities inside the cave. (Engel, 2010). Harsh environmental conditions as described above make caves a habitat for extremophilic microorganism. Absence of natural light prevents photosynthesis inside caves resulting in severe nutrient deficiency for resident microbial communities (Barton & Jurado, 2007). The chemoautotrophs are the primary producers in aphotic zones of the caves ecosystems where they generate food in caves by fixing carbon. Fungi, archaea an d bacteria are omnipresent in areas of a cave ecosystems including rock surfaces, sediments and cave soil and water (Engel, 2010). By utilization of 16s rRNA sequencing identification methods, a variety of bacterial phyla have been reported inside cave ecosystems (Ortiz et al., 2013). Among them, the most abundant taxa on cave walls are *proteobacteria*, followed by *Actinobacteria* and *Acidobacteria* (Cuezva et al., 2012). When compared with

surfaces of the cave rocks, bacterial diversity of cave soil and sediment is more diverse and rich (Ortiz et al., 2013). Cave microbiota varies significantly due to variations in microhabitats within the cave. In a single cave, different bacterial diversity was observed on different rocks, possibly due to the changes in geochemistry of rocks. Access to nutrients availability and minor variations in physical conditions may impact the bacterial populations inside a cave. Microbes and organic matter could be transported into caves by air flow, water seepage, and may be by entrance of human and animals into caves (Pernthaler et al, 2013).

1.3 Applications of Cavernicoles

The cave microbes have been successfully tested for many industrial applications. By identifying the microbes involved in precipitating protective coating on calcite minerals, cavernicoles have been used for the preservation of ancient testimonials and sculpture. Cave environment is nutrients limited in which the microbes compete for nutrients and fight for survival. This survival pressure forces cavernicoles to produce metabolites against other microbes. Therefore, these microbes can be used the production of novel antimicrobial compounds. Besides production of valuable antimicrobial compounds, these microbes also produce antifungal and anticancer compounds which are the basic need of today medical science. Cavernicoles play a pivotal role in the process of biomineralization inside cave ecosystems. These microbes precipitate calcium carbonate five times more than outside microbes. The rate of manganese oxidation by cave microbes is more significant than other environment microbes. Cave microorganism are known to degrade organic polymers as well. This makes these organisms a potential tool in the field of bioremediation and environmental biotechnology. The cave microbes are also a possible good source of beneficial compounds like extremozymes (Bajaj & Singh, 2015), biosurfactants (Thavasi & Banat, 2014), antitumor (Cheeptham et al., 2017), exopolysaccharides (Lepidi et al., 2007), radiation-protective drugs (Lin & Xu, 2020). Other applications of these microbes are they take part in biospeleogenesis and biocementation.

1.4 Kashmir Cave, Pakistan

Kashmir smast (cave) (‘smast’ in local language means cave), Nanser, Buner, Khyber Pakhtunkhwa (GPS coordinates 34o25’42.12”N 72o13’10.82”E). The cave is 188 m long, with average height and width ~28 m and ~25 m, respectively. The Kashmir smast

is a series of natural limestone caves (probably of marine origin), most part is of stalagmite, located in the Babozai Mountains in between Mardan and Buner in Northern Pakistan. According to study on a rare series of bronze coins and artifacts found in the region, the caves and their adjacent valley probably comprised a sovereign kingdom in Gandhara which maintained at least partial independence for almost 500 years, from 4th century AD to the 9th century AD. It is a limestone cave, with an internal temperature around 10°C, having pH of 5 and the internal surface of cave was muddy due to dripping water from the top. The only source of water was drip water. The cave has been the subject to a series of microbiological studies on cave soil and soil water. This PhD work is continuation of this series and focuses on the bat guano samples found inside the cave.

The bacterial strains isolated from the soil of this cave are known to produce antibiotics, antioxidants, degrade organic polymers, biomineralize Ca, Mn and other inorganic chemicals, and produce industrially important enzymes (Zada et al., 2021).

1.5. Aim of the Research

The aim of this research was to determine bacterial diversity of cave bat guano and evaluate their potential applications in applied and environmental microbiology.

1.5. Objectives of the Research

The objectives of this PhD research were,

1. To carry out Geochemical investigations and culturable biodiversity assessment of bacterial isolates from bat guano samples.
2. To Isolate antimicrobial active compounds producing bacterial strain from bat guano of the cave.
3. To conduct studies on multidrug resistant bacterial strains isolated from bat guano samples of the cave.
4. To conduct studies on biodegradation of Polyethylene from bacterial strains isolated form bat guano

1.6. Review of Literature

Caves are geological settings formed as result of natural hollowing within a rock. Caves are considered as extreme ecosystems that are not conducive for life because of severe abiotic conditions. On the hands, caves are also considered to host ecological niche for highly specialized organisms (Mammola, 2019). Among many types of caves, karstic caves are the most common type that are formed within limestone rocks or formed by lava within basaltic rocks. Having less than 2mg of total organic carbon per liter of soil, caves are oligotrophic in nature. Adding to this, low levels of biologically active light, constant low temperatures and high humidity make living conditions more extreme. The maximum temperature inside a cave may rise to 10°C with 100% relative humidity. The scientists for a long time, were of an opinion that caves have no indigenous life form (Crane & Fletcher, 2020). Despite the non-favorable environmental conditions, average number of microorganisms per gram of rock sample in cave is 10^6 (Barton & Jurado, 2007). The study of caves stated somewhere between 17^{th} century, but it was in the 19^{th} century when scientists recognized caves not only a subject of interest for geologist, but also for other sciences, like archeology, chemistry and biology (Zada, 2017).

The formation of caves is termed as speleogenesis and biological studies conducted in caves is known as biospeleology (Richards, 1971). The best cave systems are documented for countries where caving is considered as a sport or a hobby. For long, visiting caves was considered as an extreme sport or an adventure, and one would think nothing lives here, but biologists and microbiologists are busy discovering wealth of knowledge from this dark, cold and humid place. A scientific revolution is now underway in how we view caves (Gabriel & Northup, 2013).

1.6.1. Types of Caves

Caves can be classified into various types based on the type of rock they are formed in, the speleogenic process and the history of the cave. Caves are most commonly formed in limestone rocks (calcareous) and basaltic rocks (lava tubes). Rocks can also be formed inside other rocks like granite, talus and sandstone, but their range and number is very limited (Palmer, 1991). Within a limestone, caves can both be formed, on terrestrial and aquatic ecosystems. (Onac & Forti, 2011). Caves are easily formed

within limestone rocks because of their solubility in weak acids, that is generally present in all underground waters (Onac & Forti, 2011). The cracks and fissures are formed slowly, over the span of thousands of years due to the dissolutional process. The cave may get overflown with when the groundwater level rises, as the water enters inside the cave through these cracks. This is why we see ponds of water inside deep limestone caves (Cheeptham, 2013).

1.6.2 Solutional cave

These caves are formed in soluble rocks like limestone, chalk, dolomite, salt beds, and gypsum. The caves are mostly formed in terrestrial ecosystems. The largest cave of the world is a solutional cave (Limbert et al., 2016). The solutional caves are formed when the soluble rocks are dissolved with weak carbonic acid, naturally present in rain and ground water or by weak organic acids produced by a number of microorganisms (Chikkanna & Ghosh, 2018). $CaCO_3$ precipitation is a major chemical reaction that occurs inside a limestone solutional cave. Formation of characteristic structures of a limestone cave, like stalactites, stalagmites, soda straws and columns are all formed as a result of calcium carbonate precipitation (FAULKNER, 2022). Speleothems (stalactite and stalagmite) are the secondary mineral deposits found inside a solutional cave.

1.6.3. Lave caves

Also known of lava tube caves, are still not completely understood by scientists. Lava tubes are usually formed in shield volcanoes, dispensing lava away from the vent. The caves are formed when the volcano goes extinct and the secondary chamber, the lava tube, now becomes a cave. (Grimes, 2002). The basaltic lava deposits differ in viscosity, heat and gaseous content. The thick pahoehoe lava flows slowly, cracking the crust and making gaps and crevices. The size of the lava tube cave may vary for very small finger sized tunnel to more than 6000 m and diameter of 17m (Jay, 2005).

1.6.4. Ice Cave

An ice cave is characterized by having a considerable amount of ice inside it and at least one portion of cave with constant subzero temperature. Theses caves are commonly known as glaciers and are present abundantly on the poles of the earth and

on high mountainous ranges (Bella, 2007). The surface of the rocks in these caves are thermally insulated, hence the surface temperature remains fairly constant (Spötl et al., 2022).

1.7. Zonation of Caves

Caves are highly zonal ecosystems and are generally divided in five zones, each defined on the basis of physical characteristics and have different community of organisms (Bonacci, 2009). The zones are named as These are the entrance, twilight, transition, deep, and stagnant air zones (Howarth & Medeiros, 2020). The entrance zone is the zone where cave is linked with its external environment. It is the area that links the surface and subsurface of the earth. The twilight area, as the name indicates, is the extent within the cave where external light diminishes, and this is how far plants and other photosynthetic organisms can grow. The transition zone is characterized by prevalence of total darkness, but the physical environment, including humidity level, airflow and transpiration rates, has not been stabilized yet. In the deep cave zone, the humidity remains constantly high, air remains still, and the soil and rocks remain moist. The last is the stagnant air zones, that is not a necessary zone present in all caves, is the deepest segment of the cave where air exchange is extremely restricted, resulting in stagnation of the air (Frączek et al., 2019). The zones may vary considerably from cave to cave, nevertheless, the zonation provides valuable information to study cave ecology, microbial ecology and evolution of cave species.

1.8. Sources of Energy Inside a Cave

Availability of energy sources within a cave is critical and often scarce but is not altogether absent. Organic carbon, that serves as fuel for heterotrophic mode of nutrition may enter the cave in many ways. Caves linked with hydrological systems with running water inside the cave brings animal and plant debris inside the cave that is consumed by heterotrophic macro and microorganisms (Birdwell & Engel, 2009). Caves of arid environments may not have running water inside them, still the eater enter theses caves through cracks and crevices as drip water and carries little but very important carbon compounds (Marques et al., 2019). Organic carbon as particulate matter can find itself entering the cave through circulating air, specially in caves with more than one entrances (Datta et al., 2022). Visitors can bring carbon inside the cave

with them. The visitors may include worms, animals, and bats (Scherer et al., 2022). Another way through which energy may be generated inside a cave is chemolithoautotrophy (Chiciudean et al., 2022). Reduction of iron and manganese may be a vital process in caves that generate fixed carbon for the consumer community (Jones & Northup, 2021). Microorganisms in caves can also utilize cave gases such as hydrogen, hydrogen sulfide and carbon monoxide to produce energy. Reduced iron may also fuel microbial metabolism, but to a limited amount (Talà et al., 2021).

1.9. The Cave Environment

A number of physical and chemical variables influence the cave environment. Caves are habitat for a diverse group of life form and everyone of it exhibit a different mechanism of adaptation to survive inside cave environment (Barton & Northup, 2007; N. M. Lee et al., 2012). In contrary to entrance, cave environmental conditions deep inside the cave are more constant and stable (in terms of temperature variations and nutrient availability) (Nicolosi et al., 2021). Life supporting factors such as light and available organic carbon is mostly concentrated near the entrance of the cave, the conditions become more oligotrophic as we move deep inside it (Reboleira et al., 2020). Based on geographical conditions, caves may have abundant supply of water or no water at all. Caves that have direct hydrological link from ground water may face floods, whereas caves formed in desert environments face prolonged dry periods. This variability in water supply effects distribution of life form within the caves as well (Peyraube et al., 2021). Based on the type of rock where the cave is formed, the cave environment may have variable pH and redox conditions. The rock might be made up of variety of reduced chemicals, thus creating different redox gradients at different places within the same rock. These redox-variable conditions are critical for development of complex biological systems, that plays vital role in cave biogeochemical processes (N. M. Lee et al., 2012). The study of caves thus holds importance in making us understand the dynamic biodiversity and biological processes taking place inside extreme environmental conditions.

1.10 Cave and Surface Environments

Caves are different from surface environments. On the obvious note, caves differ from surface environments in the amount of light they receive, presence of energy producing

compounds, and the stability of physical environmental parameters (Ferreira et al., 2021). Beyond the twilight zone, the caves are completely dark, strongly influencing the microbial communities and ruling out photosynthesis as primary source of energy (Pešić at al., 2019). The lack of light also has probably influenced the loss UV resistance observed in cave bacteria (Northup at al., 2009). The temperature inside cave is close to annual mean temperature of the surface over the cave. In temperate regions, the warm air from the outside moves inside the cave bringing in moisture and vice versa occurs in winter. In this way, the air in transition and deep cave zones circulates seasonally (Mattey et al., 2016).

1.11 Life Inside Caves

Viruses, bacteria (containing cyanobacteria), fungus, algae, protists, plants, and animals have mostly been discovered within caves (Zgonik et al., 2021). The organisms can both be mobile or sessile, can form mutualistic, parasitic and predatory ecological relationships (L. Ma et al., 2021). A cave may contain resident organisms that are permanently inside any cave from a long period of time or the non resident organisms, also known as the accidental species (Romero, 2011). The accidental species may be transported inside cave with winds, sediment flow, spores or water. Various forms of adaptations and modifications in response towards the cavern settings are carried out by organisms living in the caves. A good example of these adaptation are microbial species that have metabolically acclimatized themselves to such a severe chemolithoautotrophic lifestyle where they have to live off inorganic substances found in rocks and groundwater, like metals, Sulphur, as well as methane (Engel, 2010). Troglomorphy is a combination of qualities exhibited by a wide variety of microorganisms that are obligatory acclimated to the subterranean, whether in terrestrial (troglobionts) or aquatic (sytgobionts) settings. Such obligate subsurface microorganisms have restricted dispersal opportunities, which might limit genetic lineages/populations to localized, and possibly regional, hydro stratigraphic zones (Sendra et al., 2021).

1.12. Bacterial Communities Inside a Cave

Bacteria belonging to a wide range of taxonomic segments are inhabitants of the cave ecosystems. *Proteobacteria*, *Firmicutes*, *Actinobacteria*, or *Acidobacteria* are some of

the bacterial phyla most commonly reported from inside cave systems (Zhu et al., 2019). Among the mentioned bacterial phyla, *Proteobacteria* are the most numerous followed by *firmicutes* and *actinobacteria* (Dalfes at al., 2021). Cyanobacteria and photoautotrophic bacteria are usually reported from illuminated areas of the cave near the entrance zone (Krett at al., 2022). *Pseudomonas, Alcaligenes* and *Bacillus* bacteria are frequently reported heterotrophic bacteria in a cave and are responsible to degrade organic matter present inside a cave in the form and shape of insects, animal manures, and foreign waste (Baskaran et al., 2020). Although heterotrophy maintains biogeochemical systems inside a cave ecosystem, it becomes a serious concern in caves with Paleolithic artwork and ancient paintings. Nutrients availability and disturbance also affect the bacterial diversity inside a cave. Sulfidic caves environments are largely inhabited by chemotrophic organisms forming a thick filamentous microbial mat. These microbes are capable to oxidize sulfur for the energy requirement (Spilde at al., 2006). Presence of *β-proteobacteria* have been reported in cave waters in several studies (Shabarova et al., 2013). According to the latest SILVA database release 132, presence of *Betaproteobacteriales*, *Pseudomonadales*, *Pseudonocardiales*, and *Bacillales* were the dominant bacterial communities based on the average abundances in the cave water samples (Northup at al., 2021).

Table 1.1 Microorganisms isolated from different caves around the world.

Caves	Country	Microbes	Proponents/ Year
Nullabor Caves	Australia	*α, β, γ and δ-Proteobacteria* and novel microorganisms	Holmes *et al*. 2001
Altamira Cave	Spain	*Proteobacteria, Plantomycetales, Cytophagal/Flexibacter/Bacteroides, Acidobacterium*, Actinobacteria and green-sulfur bacteria	Schabereiter-Gurtner *et al*. 2002 Cuezva et al, 2009
Reed Flute	Guilin, Guangxi, China	*Knoellia sinensis* and *K. subterranea (Actinobacteria)*	Groth *et al*. 2002
Lower Kane Cave	Wyoming, USA	*ε-Proteobacteria*	Engel *et al*. 2003
Lechuguilla Spider	Carlsbad Cavern NationalPark New Mexico, USA	*α, β and γ-Proteobacteria, Enterobacteriaceae, Xanthomonas, Bacillus/Coliform* group and *Lactobacillaceae*	Northup *et al*. 2003
Llonin La Garma	Asturias, Northern Spain	*Proteobacteria, Actinobacteria*, Gram-positive bacteria	Schabereiter-Gurtner *et al*. 2004
Kartchner Caverns	Arizona, USA	*Bacillus, Brevibacillus, Rhizobium, Sphingomonas, Staphylococcus, Pseudomonas*, and other uncultured*β-Proteobacteria*	Ikner *et al*. 2006

Proteobacteria are a universal group of bacteria that is abundantly present in caves. They are mostly present in the water dripping for the walls and roof of the cave as well as found aggregated on the rock surface (Jurado et al., 2022). Presence of these bacteria is also reported in basaltic caves (Hathaway et al., 2014). They are most dominant group of organisms reported in very extreme environments, for example, sulfurous cave (Galdenzi et al., 2010). Microorganisms belonging to *Actinobacteria* type are Gram-positive bacteria with a wide range of metabolic potential, DNA rich in GC base pairs and predominantly present in soils. Within caves, they are often found colonizing the rock walls, as well as geological formations like as stalactites and stalagmites (Pellegrini at al., 2022). Actinobacteria were the most numerous bacteria reported in Altamira caves (Cuezva et al., 2012). They are also reported to have impacts on cave paintings by producing organic and inorganic metabolic byproducts (Rölleke at al., 2004). Microorganisms of *Bacteroidetes* type are phenotypically diverse. They can aerobic or facultatively anaerobic, are mostly chemoorganotrophs and often produce pigments like carotenoids and/or flexirubin which confer yellow or orange colony coloration. Bacteria belonging to this group are psychrophiles, mesophiles and thermophiles. Although *Bacteroidetes* microorganisms are often found in caves, knowledge about their functional role in these ecosystems is limited (Zada et al., 2021). It is believed that they are involved in fermentation process and circulation of metals inside caves (Ikner et al., 2007b). *Bacteroidetes* were the most numerous bacterial phylum reported in Carter Saltpeter cave (S. K. Carmichael et al., 2013). Bacteria that are in *Firmicutes* are often found in caves and other ecosystems where they are characterized by heterotrophic and chemolithoautotrophic growth. *Firmicutes* are most often found in microbial populations inhabiting the surface of rock walls and sediments in caves (Lange-Enyedi et al., 2022). These bacteria possess a wide metabolic range enabling them to grow on various organic compounds. They also exhibit enhanced resistance to stress triggered by low amount of nutrients and dehydration in comparison to *Proteobacteria*. *Firmicutes* were the dominant bacterial phylum in the phototrophic biofilm formed in the Cave of Bats (49.5% of 16S rRNA sequences) with low to medium tourist visitation (Urzì, De Leo, Bruno, & Albertano, 2010). Another commonly found bacterial phylum in caves is the *Chloroflexi* phylum, formerly known as “green non-sulfur bacteria”. It is a systematic group with a small number of cultivated representatives, but with rapidly accumulating sequences identified in

metagenomic studies. A small number (14% of the sequences) of this phylum was reported by metagenomics in gray water mats, Lower Kane Cave (Engel, 2010).

1.13. Production of Bioactive Compounds by Cave Bacteria

Oligotrophic, stressed environment forces bacterial species inhabiting caves to produce unique bioactive compounds for their survival. Cave bacteria have been reported to produce ethanol that is used as biofuel, and extremozymes that are successfully employed in industry as ecofriendly, robust alternatives (Banerjee, Jha, & Joshi, 2019b). These microbes also have the ability to produce novel antibiotics and anticancer metabolites (Silva, Jayasingha, Senanayake, Dandeniya, & Munasinghe, 2021). Bacterial strains isolated from different caves have been reported to produce biosurfactant (Schultz & Rosado, 2020), antitumor compounds (dos Santos et al., 2021) and exopolysaccharides (Thakur at al., 2020). Production of new antimicrobial compounds from extreme environments is now becoming the new trend in the field of microbiology and biotechnology. There has been an overwhelming gap of 30 years since discovery of new antibiotic class and resistance against the existing classes is developing at a rapid pace. Recent studies have shown caves to be new avenue for discovery of novel antibiotics (Pawlowski et al., 2016).

1.14. Bat Guano and Antimicrobial Resistance

Antimicrobial resistance is the monster of the modern world. Scientists are looking for the clues if resistance has travelled into pristine environments of our earth, like the caves. Antimicrobial resistance (AMR) is not only a threat to the public health but also for the wildlife (Greig et al., 2015). Bats are among the lesser appreciated and less explored groups of mammals that acts as reservoirs of antimicrobial resistance. Their unique way of living, lifespan longevity and repeating cycles of torpidity and hibernation contribute in the long-term and long lived persistence of microbes in a stable conditions and then they distribute these resistant organisms and genes to far off ecosystems (Cláudio et al., 2018). Many studies have reported mammals harboring MDR bacteria in their system, pointing towards possibility of them being reservoirs for antibiotic resistant bacteria and ARGs (Coleman at al., 2021). A bacteria can acquire antibiotic resistance from any environmental medium, be it soil, water or a cave. Resistant bacteria their ARGs to environmental bacteria through horizontal gene

transfer. This shows that development of antibiotic resistance does not require the organism to be exposed to antibiotics first (Suzuki at al., 2020).

1.15. Cave Bacteria and Biodegradation of Organic Polymers

Degradation of plastics is carried out by organisms that are chemoheterotrophs. Caves are inhabited by a variety of bacterial species belonging to this category, that are surviving on the organic residues available inside caves (KoilRaj et al., 2012). Biodeterioration of wall paintings by bacteria is widely documented. Bacteria living in caves are capable of extracting organic residues from these paintings as source of carbon, that is already scarcely available (He et al., 2021). Extensive reports research data is available on the microbial colonization on the paleolithic paintings in the Lascaux Cave in France (Alonso et al., 2019). A diverse variety of culturable chemohetertrophs are also reported in caves and catacombs by De Leo at al., (Urzì at al., 2012). Several studies have reported cave bacterial species capable of producing lignocellulose-degrading enzymes and proteolytic enzymes, making them potent biodegraders of organic polymers. In our previous study, we have documented biodegradation of polyethylene by bacterial strains isolated from soil of Kashmir cave, Pakistan (Jamil et al., 2017). According to one study, Isolation of bacterial species capable of biodegrading high and low density polyethylene has been isolated from cave environment (Tabassum et al., 2022). Psychrophilic enzymes possessed by cold adapted microbial strains, such as those found in caves, have higher flexibility to due to dissimilarity in the amino acid composition from their mesophilic homologues that results in weak protein interactions (Bajaj & Singh, 2015). Capability of cave microbes to degrade complex aromatic compounds, such as enzothiazole and benezenesulfonic acid, used in plastic manufacturing, to extract growth nutrients is reported by (Barton, 2006).

1.16 Caves in Pakistan

Like other countries Pakistan has many caves which are still unexplored biospeleologically. Pakistan Cave Research and Caving Federation is a national body established by Mr. Hayatullah Khan Durrani who also represented Pakistan in Union of International Speleology (UIS), and British Caving Federation (BCA).

Table. 1.2. List of caves in Pakistan

Cave Name	**Length (km)**	**Location**
Pir Ghaib Gharr cave	>1.20	Balochistan
Kashmir smast (cave)	0.188	Mardan KPK
Gondrani cave	Unknown	Bela, Balochistan
Bhaggar cave	Unknown	Azad Kashmir, Pakistan
Juniper Shaft cave	Unknown	Balochistan
Mughagull Ghara cave	Unknown	Balochistan
Mughall saa cave	Unknown	Balochistan
Mangocher cave	Unknown	Balochistan

Chapter 2

Geochemistry and Culturable Bacterial Diversity of Speleothem and Bat Guano samples from Kashmir Cave, Pakistan

Abstract

Geological formations are closely associated with microbiological processes. Microorganisms of all types play an important role in formation and evolution of various geological features of the earth. In the current study, an attempt was made to illustrate geochemical and microbiological properties Bat guano from a karstic limestone cave in Khyber Pathunkhwa Province of Pakistan. Analysis was carried out for 3 layers of bat guano pile (top, middle and bottom). Geochemical profiling for various minerals and heavy metals was carried out using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS). Nutrient composition including total organic carbon, total nitrogen, phosphorus and potassium was evaluated using C-N analyzer. Culturable bacterial diversity was established by culturing bacterial species from each layer on Nutrient Agar plates. DNA extraction was done for sequencing of 16S ribosomal RNA gene analysis. ICP-MS showed presence of various minerals including heavy metals in different layer of bat guano. The mineral concentration was high in fresh guano samples (top layer) and decreased as we move towards lower layer (old deposits). Carbon-Nitrogen Analysis (CN Analyzer) of Guano showed top layers to be rich in organic carbon, total nitrogen, potassium and phosprus content that decreased in amount we moved from top layer to the bottom layer. Phylogenetic analysis of Bat guano revealed 64.28% bacteria belonged to phylum *firmicutes*, 35.70% to *proteobacteria*, and 7.10% to phylum *bacteroides*. This is the first study on bat guano diversity in Kashmir cave, Pakistan.

2.1 Introduction

Recent interest in the role of microbial processes in biogeochemical cycles and the largely unexplored subsurface diversity has spurred research on the geomicrobiology of deep marine and terrestrial environments. Although, in the last 20 years, numerous studies have started to investigate microbial biodiversity in a wide range of habitats, the physiological and biochemical features of these communities are still awaiting exploration (Ikner et al., 2007a). Caves provide a window into the subsurface and are a prime habitat for investigating subsurface microbial life (Rusznyák et al., 2012). A number of studies have investigated microbial biofilms on cave surfaces (Barton et al., 2014; Wu et al., 2015). Microbial diversity has been studied in the Lechuguilla Cave in New Mexico (Northup et al., 2003), the sulfidic Frasassi Cave system in Italy, the Movile Cave in Romania (Chen et al., 2009), the nitrate/nitritedominated Nullarbor Cave in Australia (Holmes et al., 2001) and karstic caves of Herrenberg Germany (Rusznyák et al., 2012).

In caves throughout the world, bats in sufficient populations produce copious amounts of faecal droppings (guano) reported at rates up to 10 cm/year (Hutchinson, 1950). Under favourable conditions, guano can accumulate in deposits many meters thick. These deposits have been used to examine palaeoenvironments through stable isotope analyses and palynology (Wurster, Munksgaard, Zwart, & Bird, 2015). Organic matter decomposition usually proceeds rapidly, with the organic content in guano deposit being largely lost within a few cm depth (Bird et al., 2007). Although, high organic carbon contents (>40 %) can persist over 2 m of depth under certain environmental conditions (Wurster, McFarlane, & Bird, 2007), loss of organic carbon is associated with a reduction of nitrogen and sulphur, with concomitant increases through residual accumulation in elements such as aluminium, potassium, and iron (Shahack-Gross, Berna, Karkanas, & Weiner, 2004). The geochemistry of cave guano may also be unique. There are a few reports that have suggested that cave guano is particularly enriched in transition metals compared with local soils (Bird et al., 2007). High abundances of Cd, Pb, Cu, and Zn in water were also associated with a bat guano source (Cuculić, Cukrov, Kwokal, & Mlakar, 2011). Both phosphate and sulphate minerals are commonly associated in guano rich environments such as in caves with large populations of bats. These minerals form as a result of the interaction of guano derived

solutions with the cave bedrock or with secondary (chemical or detrital) cave deposits (Giurgiu & Tămaş, 2013). High abundances of many transition metals is found in the cave guano samples. For example Pb in Batu guano, located in Peninsular Malaysia, averages well over 200 mg/kg compared to 30 mg/kg for anthropogenic soils from mine tailings. (Wurster et al., 2015). A further potential use of bat and bird guano is in contaminant monitoring. Faecal matter has been suggested to be a good biomonitor of heavy metal contamination, however, much of the work is focused on marine and coastal systems (Wang et al., 2008).

This study investigates the mineralogy, biogeochemistry and culturable bacterial diversity of bat guano samples from Kashmir Cave, Pakistan. The cave is situated with in a steep mountainous range isolated from general public and large animals. The study is first of its kind from Pakistan to document geomicrobiology and geochemistry of guano.

2.2 Material and Methods

2.2.1 Sampling Site and Sample Collection

Guano samples were collected from Kashmir Cave, Khyber Pakhtunkwa Province of Pakistan (GPS coordinates 34°25'42.12"N and 72°13'10.82"E). The cave is 188 m long, with average height and width ~28 m and ~25 m, respectively (figure 1.1). It is a limestone cave, with internal temperature around 10°C and the internal surface of cave was muddy due to dripping of water from the top. The only source of water was drip water. A pile of fresh guano sample was collected from ground surface of the cave in sterile zipper bags under aseptic conditions. The sample was collected from the dark end of the cave about to minimize influence of external environment. This cave is located far away from human access so human intervention is negligible. The samples were then brought to the laboratory in an ice box and stored at 4°C for further processing.

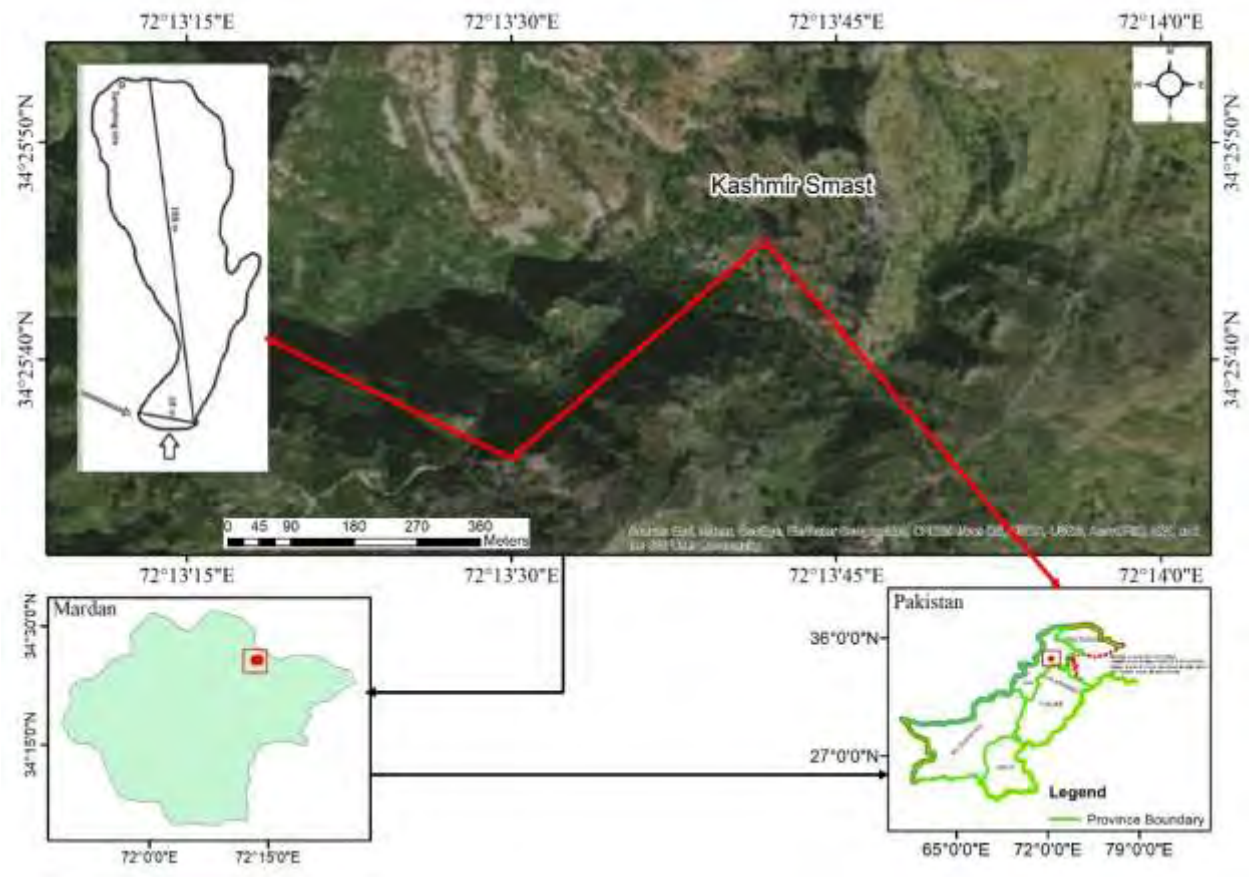


Figure 2.1 Kashmir cave, sample collection site in KPK province, Pakistan

2.2.2 Geochemical Analysis of Guano

The collected samples were analyzed for quantification of various elements, including trace and heavy metals. For metal analysis, one gram of sample was added to 10ml deionized water and shaken briefly. The samples were digested using aqua regia at 100°C and analyzed using Inductively coupled plasma mass spectrophotometer (ICP-MS) (PQ3S; Thermo Electron, United Kingdom) (Rusznyák et al., 2012). For the estimation of Carbon and Nitrogen in guano samples, the samples were freeze dried, finely ground to 200 mesh size (<75 mm), homogenized and analyzed using a C-N analyzer (Vario Max; Elementary Analysensysteme GmbH, Germany). For each test triplicate samples were analyzed.

2.2.3 Culturable Bacterial Diversity of Guano Samples

Culturable bacterial diversity of guano samples was carried out by isolating bacterial strains from 3 layers of guano (top, middle and bottom) on Nutrient Agar plates. 5g of

guano sample was suspended in 10ml of autoclaved normal saline and shaken vigorously. 1ml of this solution was serially diluted 5 times and 100µl of the final solution was spread over nutrient agar plates and incubated for 72hrs.

2.2.4 Molecular identification of the selected isolates

A total of 14 bacterial strains were isolated from guano sample (Table 1) and were sequenced through 16S ribosomal RNA gene sequencing. The isolated DNA was sent to Macrogen, South Korea for sequencing.

Table2.1: Isolated strains for DNA sequencing

S.No	Strain ID
1	GBL1
2	GML1
3	GML2
4	GML3
5	GML4
6	GML5
7	GTL2
8	GTL3
9	GTL4
10	GTL5
11	GTL6
12	GTL7
13	GTL8
14	GTL9

2.2.5 DNA Extraction of the isolated strains

For extraction of DNA, bacterial colonies were refreshed on Nutrient Agar plates and incubated at 30°C for 24h. pure colonies thus obtained were transferred into nutrient broth medium and re-incubated at 30°C for 24h. 2ml of this medium was collected and shifted to sterilized and labelled Eppendorf tubes. The culture was then centrifuged at 10000rpm for 10mins. Supernatant was discarded and pallets were used for further steps. The pallets were suspended in 1mL TE buffer. 8 µL lysozyme (G-positive), 5µL of proteinase K (Thermo Scientific), and 30µL of 10% SDS was added to the reaction mixture. The Eppendorf were again incubated at 37°C for 1h inside water bath. Following incubation, 100µL of CTAB buffer and 80µL of 5M NaCl was added to the mixture and placed in water bath for 10min at 65°C. The next involved addition of 500µL of phenol:choloroform:isoamyl alcohol followed by centrifugation at10000 rpm

for 20min. two distinct layers formed at the end this step, the upper layer was collected and shifted to another Eppendorf with addition of 500µL of PCI. After loading with PCI, centrifugation was carried out on parameters described in previous step. Once again, upper layer of the two layers was collected and 500µL of Isopropanol and 300µL of 3M sodium acetate was added to the mixture, followed by incubation for 15min at room temperature. After completion of incubation, the mixture was centrifuged at 10000rpm for 5min. The supernatant was discarded, and the pellet was washed 200µL of 70% ethanol three times. Once the washing was done, the ethanol was removed and pallet was resuspended in TE buffer. The mixture was finally run in gel electrophoresis (1% agarose gel) for visualization of DNA. The extracted DNA was stored at -20°C until sent to Macrogen for sequencing.

2.2.6 Phylogenetic analysis

Phylogenetic analysis was performed through ClustalW program implemented in MEGA X software (Thompson et al., 1994). The similar sequences were downloaded from NCBI. All sequences were aligned and the phylogenetic tree was constructed using Neighbor Joining method in MEGA X bootstrap analysis, 1000 replicate was performed for the significance of the generated tree.

2.3 Results

2.3.1 Geochemical Analysis of Guano

Geochemical analysis of guano showed presence of various metallic and nonmetallic elements (Table 2). Geochemical of bat guano shows high concentrations of total organic carbon (%) in top layer which subsequently decreases with deoth in the bottom layer. Similar trend has been observed for total nitrogen (%), total phosphorus (%) and total potassium (%) content. Small quantities of calcium (35.8 mg/kg) and magnesium (12.6 mg/kg) are also detected along with trace heavy metals.

Table 2.2. Geochemical analysis of different layers of Bat guano

Minerals	**Concentration**		
	Top Layer	Middle Layer	Bottom Layer

Total Organic Carbon (%)	92.8	71.2	49.7
Total N (%)	13.7	8.9	4.3
Total P (%)	4.9	2.1	1.6
Total K (%)	1.5	1.2	1.0
Ca (mg/kg)	35.8	2.21	2.09
Mg (mg/kg)	12.6	0.27	0.19
Fe (mg/kg)	4.8	3.2	2.9
Cu (mg/kg)	1.5	0.9	0.5
Co (mg/kg)	0.006	0.006	0.004
Zn (mg/kg)	0.085	0.020	0.015
Pb (mg/g)	0.015	0.013	0.013

2.3.2 Culturable Bacterial Diversity of Guano Samples

16s ribosomal RNA molecular identification was performed on 14 isolated strains from Bat guano sample. Based on genomic data, it was revealed that 64.28% (n=9) belonged to phylum *firmicutes*, 35.70% (n=4) belonged to phylum *proteobacteria* and one strain (n=1) belonged to phylum *bacteroides* making a percentage of 7.10%. The phylum wise analysis of the strains carried out after blast sequence matching is shown in table 3.

Table 2.3 Phylum wise detailed analysis of the isolated strains

Phylum	**ID**	**Matched specie**	**Identity%**
	GBL1	*Bacillus simplex*	98.81
	GML1	*Sporosarcina koreensis*	98.45

Firmicutes	GML2	*Bacillus mobilis* strain	99.05
	GML5	*Bacillus haynesii* strain	99.04
	GTL2	*Bacillus depressus* strain	97.18
	GTL4	*Bacillus Subtilis* strain	99.18
	GTL5	*Oceanobacillus chungangensis* strain	94.7
	GTL7	*Bacillus aerophilus strain*	99.24
	GTL 9	*Solibacillus silvestris* strain	97.26
Proteobacteria	GML4	*Brevundimonas diminuta*	99.31
	GTL3	*Alcaligenes faecalis* Strain	97.26
	GTL6	*Brevundimonas diminuta*	99.05
	GTL8	*Alcaligenes faecalis* strain	96.94
Bacteriods	GML3	*Sphingobacterium yamdrokense* strain	96.43

2.4 Discussion

The current research was conducted to evaluate mineralogical studies and biodiversity assessment of bat guano samples isolated from Kashmir cave, Pakistan. It is the first report of its kind for this cave. Mineralogical studies were conducted using ICP-MS and C-N analyzer. The results showed high amounts of carbon and nitrogen in Bat guano with significant amounts of phosphorus and potassium. These elements serve as source of carbon as well as source of micronutrients, essential for cave dwelling microorganisms. In our study, total carbon in top layer was 92.8 percent that decreased

with depth of the sample. Similarly, 13.7 percent of Nitrogen, 4.9 percent phosphorus and 1.5 percent potassium were detected. The concentrations of the nutrients were more in top layer and decreased as the depth increase, showing their fast utilization by cave organisms. Similar finding have been reported by Sridhar et al., while investigating manure quality of bat guano in Assaigoli village (Sreepada at al., 2006). Palita et al., reported high carbon contents and high N:P in bat guano samples while studying their application in fertilizers (Panda at al., 2021). Our samples contained trace amounts of heavy metals in it as well. The probable source of these metals is drip water that enter the caves through crevices with the cave structure. Presences of heavy metals inside caves have been reported in a study focusing upon fresh bat guano in Eastern Mediterranean karstic cave sites (Shahack-Gross at al., 2021). Presence of metals, specially iron is in bat guano is also reported in iron ore caves, Brazilian Amazonia (BERNARD et al., 2022). Guano samples of clastic sediment caves showed varied amount of metals specially Zn, Pb and Co (Baldantoni at al., 2022). Our results showed a decreasing trend of mineral concentration as the depth of the guano sample increased. A similar decreasing trend of minerals and metal with depth of guano samples is reported by Putral et al., while studying composition of Guano from Solek Cave, West Sumatera (Putra, Rifai, & Wurster, 2019). Calcium concentration was very high in our samples. Formation of calcium as calcium phosphate corresponds to the early stage of guano breakdown from the reaction of sulfuric and phosphoric acids with limestone stone caves. The results are in accordance with the studies performed on European caves (Audra et al., 2019).

Bacterial isolates form the current research belong to 3 phyla namely, *firmicutes*, *proteobacteria* and *bacteroides*. According to a study, guano produced by the frugivorous bat *Rousettus leschenaultii* from a cave in India, the isolated bacteria belonged to four different phyla accounting for 37 genera (Banskar, Bhute, Suryavanshi, Punekar, & Shouche, 2016). Contrary to our results, studies on Dupnisa cave, Turkey, the bacterial diversity was dominated by *proteobacteria*, followed by *bacteroids* and *firmicutes*. Presence of *proteobacteria* in caves is mostly associated with drip water and dissolved rocks and these bacteria can easily be found in bat guano when the water mixes with it (Tok et al., 2021). Presence of *Bacillus sp* in bat guano found in caves is almost universal. Many studies have reported presence of bacteria belonging

to this genera in guano samples of the caves. A study conducted in Hampoeil cave reported isolation of no of *Bacillus* sp capable of producing a variety of bioactive compounds (Ranjbaran at al., 2019). *Lactobacillus* bacteria having probiotic activities has been reported to be present in bat guano samples of Er-rachidia, Morocco (Sakoui et al., 2022). Another ubiquitously found bacterial genera in caves and bat guano is *Alcaligenes faecalis.* The bacterium is found in our study of Kashmir cave as well. This bacterium is known to possess unique metabolic pathways capbale of degrading wide range of organic polymers (Tabassum et al., 2022). *Alcaligenes* sp capbale of degrading paints is reported by Ravikumar et al., (Karigar at al, 2012)isolated from We could not find much biodiversity in the bottom layer of the guano samples. The probable reason may be poor organic content, lower oxygen levels and high pH conditions prevailing in the older deposits of guano (Ferreira, 2019).

2.5 Conclusion

It can be concluded from this study that bat guano from Kashmir cave, Pakistan contains a variety of essential and non-essential elements in varying concentrations, playing a vital role in nutrient channeling inside the cave. The bacterial diversity shows presence of distinct bacterial flora with prominent members in it that could put to potential biotechnological applications.

Chapter 3

Isolation of Antimicrobial Active Compounds producing *Bacillus subtilis* Strain from Bat Guano of Kashmir Cave, Pakistan

Abstract

In this study, we report antimicrobial compounds producing bacterial strain isolated from Bats guano of a limestone cave. Screening of bacterial strains was carried out from top, middle and bottom layer of the guano sample. Out of 20 isolated strains, 3 showed antimicrobial activity against all tested ATTC strain of common bacterial (both gram positive and gram negative) and fungal species. 16s RNA gene sequencing of the strain showing maximum activity revealed the isolated strain as *Bacillus subtilis.* Optimum production of antimicrobial compounds took place in TSB medium after 72h of incubation at 30˚C. FTIR of crude extract showed chemical similarity with bacitracin. GCMS analysis of the crude extract revealed a range of antimicrobial and anticancer compounds. It is concluded that *Bacillus subtilis* strain isolated from Bat Guano is a potential source of novel antimicrobial compounds of broad-spectrum nature and have inhibitory effects against potential pathogens.

3.1. Introduction

Microbial resistance towards antimicrobial compounds has clearly established itself as a global threat to public health. Presently, various strategies are continuously tried and validated to curb this ever increasing menace. Among other possible sources of novel antimicrobial compounds, special focus has been laid in exploration of hidden treasures with in extreme environmental conditions. Bioprospecting for such novel bioactive compounds from cave started 30years ago and still it is under exploration (Ko & Lee, 2017). Environments, both marine (Dharmaraj, 2010; Romano et al., 2017) and terrestrial (Javoreková at al., 2018; Devi & Rutledge, 2017) are constantly being looked for discovery of novel antimicrobial compounds to fight against growing threat of antibiotic resistance against commonly used drugs. Caves have also been a potential source of novel biomolecules with biotechnological applications (Zada et al., 2016). Caves represent a set of environmental conditions that is not very conducive towards life such as limited nutrient availability, low temperatures, variable levels of bioavailable light, high humidity, limited or no connection to the surface, low air flow and poor pressure conditions (Joshi at al., 2019a). These highly oligotrophic and extreme conditions offer severe competitive environment for microorganisms and their survival depends upon wining this competition. The antimicrobial byproducts thus produced by microorganisms in a cave environment are secondary metabolites produced as a mechanism of survival (Lee at al., 2019). Since potential for novel drug discovery inside a cave is very high, studies of bioactivity in caves is witnessing an uphill trend with very promising results (Marques et al., 2018). Antimicrobial activity of compounds secreted by microorganisms isolated from caves have been reported against both ATCC and clinical pathogenic strains of Gram-positive and Gram-negative bacteria. Caves not only harbor already well known taxa that demonstrate high ability to produce specialized bioactive compounds, they present an opportunity to look into previously unknown strains that can serve as a new avenue for novel antimicrobial compounds discovery. (Ouhdouch et al., 2019). Quality and quantity of antimicrobial compound production at lab and at commercial scale is greatly affected by growth culture conditions. Recovery in lab of bacteria capable of bioactive compound production have been reported on heterotrophic, auxotrophic, and enriched media.

(Ghosh et al., 2017). The bacterial species of Genus *Bacillus* is widely reported to produce antibiotic of peptide nature through ribosomal and non-ribosomal approaches. Bat guano is the major source of energy inside a cave (POULSON, 2000). Among other means, Bat guano serves as an important medium transporting microorganisms in and out of the cave (Mulec, 2008). Since bats are not confined with in cave environments, they get access to many micro and macro nutrients that are not abundantly present inside the cave. Nitrogen, that is otherwise limited inside cave, is abundantly present in bat guano which can be utilized by microbes for incorporation into antimicrobial compound synthesis.

Pakistan is a country with diverse nature of flora and fauna. Currently very little is investigated about caves diversity in Pakistan, specifically in term of microbiology. Like the rest of the world, Pakistan is also suffering from emergence of antibiotic resistance and spread of antibiotic resistant genes in the environment. Finding new sources for production of novel antibiotics is as much important for Pakistan as it is for the rest of the world. Currently, very little research is being carried out to explore caves as source of antibiotics in Pakistan. Considering this, the key purpose of the study was to identify and characterize bioactive compounds produced by bacterial strain isolated from bat guano from Kashmir Cave, Pakistan. The current study is the pioneer research to document bacterial strain capable of producing antibiotics effective against different pathogenic groups of bacteria.

3.2. Materials and Methods

3.2.1. Sampling and Site Description

Kashmir cave is in Khyber Pakhtunkhwa province of Pakistan (GPS coordinates 34°25’42.12”N, 72°13’10.82”E). The cave is a limestone cave residing within a mountainous terrain that restricts human intervention. The average length, height and width of the cave is 188 m, 28 m and 25 m, respectively and average temperature of 10°C (Figure 1). Huge piles of Bat Guano were observed inside the cave. Samples of fresh and old guano were collected aseptically in sterilized zipper bags. Samples were collected from top (10cm), middle (20cm) and bottom layers (30cm) of guano pile, representing different time frames of guano accumulation inside the cave. The samples were then brought to the Applied Environmental and Geomicrobiology laboratory at

Quaid-i- Azam University Islamabad in an ice box and stored at 4°C for further processing.

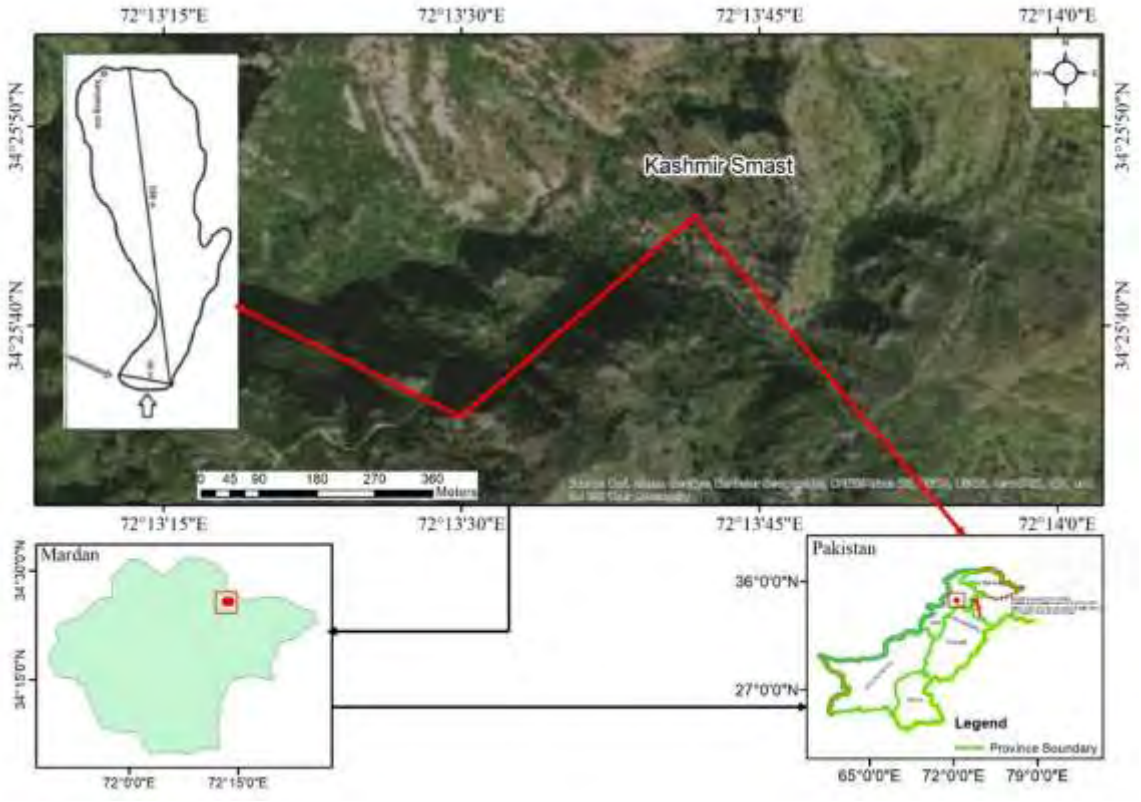


Figure 3.1 Map of the study area, Kashmir Cave, Pakistan.

3.2.2. Microbiological Studies

3.2.2.1. Total Viable Cell Count

Autoclaved laboratory grade Nutrient agar (Sigma-Aldrich) media was utilized for the isolation of bacterial strains from the three layers of bat guano sample designated by names as Guano top layer (GTL), Guano middle layer (GML) and Guano bottom layer (GBL). 2g of bat guano sample from each layer was added into 20ml of autoclaved distilled water and was shaken until a uniform mixture was obtained. The sample was serially diluted (10^5) and spread over agar plates and incubated for 24h at 30°C.

3.2.2.2. Screening for Antimicrobial Compounds producing Bacterial Strains

The ATCC strains of common pathogens were grown in sterile Mueller-Hinton agar (MHA) media plates. Point inoculation was done against ATCC strain of *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumonia* (ATCC 700603), *Staphylococcus epidermidis* (ATCC 14990) and *Candida albicans* (ATCC 10231). Lawn formation was carried by adjusting the density to 0.5 McFarland standard. Incubation was done for 72h at temperature 30°C. All the isolated strains were screened for their potential to produce antibacterial compounds by checking the formation of clear zone of inhibition around test bacterial colony (Paun, Lavin, Chifiriuc, & Purcarea, 2021). Bacterial isolate GTL4 produced maximum zone of inhibition, hence was purified and stored at 4°C for further experimentations.

3.2.2.3. Molecular Identification through Bacterial DNA extraction

The recovered bacterial strains of the study were identified though 16S rRNA sequencing. For this purpose, the genomic DNA was extracted through Gene JET Genomic DNA Purification Kit (Thermo Scientific K0721) according to manufacturer's protocol. The DNA sample was commercially sequences from Macrogen Korea. The obtained sequences were analyzed and trimmed accordingly to obtain good quality sequences. The study isolates were identified by BLAST search in GenBank NCBI. The 16S rRNA sequences were also used for phylogenetic analysis and phylogenetic tree construction. Identical sequences were obtained from NCBI for each isolate and phylogenetic tree was constructed through Neighbor Joining method in MEGA4.0.

3.2.3. Process optimization for Antibiotic Production

Attempts were carried out to investigate the maximum antibacterial compounds production by GTL4 bacterial isolate by optimizing parameters such as type of media, temperature, incubation time and pH (Zhang et al., 2008). The bacterial strain was cultured in different media such as Tryptic Soy Broth (TSB), Nutrient Broth (NB), Luria-Bertani broth (LB) and Zobell Marine Broth (ZMB). Zone inhibition assay was carried out at temperature ranging from 25°C, 30°C and 35°C, while the pH values were adjusted to 5, 7 and 9. Samples were taken out after every 24h to determine optimum time of incubation (Singh et al., 2017).

3.2.3.1. Fermentation and Extraction of produced Antimicrobial Compound

Submerged fermentation of GTL4 strain in 500ml TSB media was carried for antibacterial compounds production. The process was carried out at 30°C for 72h at 150rpm with continuous shaking. At the end of incubation, contents of the flasks were centrifuged at 1000rpm, at 4°C for 15 minutes. Pellet was discarded and cell free supernatant was then subjected for solvent extraction. Different organic solvents such as chloroform, butanol, ethyl acetate, n-hexane in equal volume while combination of chloroform: ethyl acetate (1:1 v/v) and chloroform: n-hexane (1:1 v/v) were used for extraction of antibacterial compounds in a separating funnel. The extract of organic solvent was evaporated and then dissolved in dimethyl sulfa oxide (DMSO). Antibacterial activity of each extract was tested against the selected ATCC strains.

3.2.4. Fourier Transform Infrared spectroscopy (FTIR)

The infrared spectrum (IR) of crude extract in N- hexane, chloroform, and N-hexane: chloroform (1:1 v/v) were analyzed in the range of 400-4000 cm^{-1} on FT-IR spectrophotometer (Shimadzu FTIR-8400 S, Kyoto, Japan). FTIR spectrum of Bacitracin was used as standard for comparison.

3.2.5. Gas Chromatography-Mass Spectrometry (GCMS)

The gas chromatography-Mass-spectroscopy technique was used to characterize the chemical moiety of the study strain GTL4 crude extract. The experiment was proceeded by dissolving 10 mg of the crude extract in 1 mL of solvent ethyl acetate. The specification of the column used for analysis was 0.25 mm x 25 m in GC 17A, Japan GC-MS. For the stationary phase 5% phenyl poly siloxane and helium (3 ml/mm) as a carrier gas, the flow rate was maintained at 0.4 ml/minutes. To identify the chemical composition of the crude-extract a temperature gradient program is implemented to remove the organic solvent by evaporation. A gradually increase in the temperature from 70°C at an increase of 10°C /minute to a final 250°C. The crude extract sample was injected into the GC-MS at 250°C on the 18^{th} minute. The obtain peaks were used for characterization and identification.

3.3. Results

3.3.1. Microbiological Studies

3.3.1.1. Total viable cell count

A total of 20 bacterial strains were isolated from various layers (Top, Middle and Bottom layer) and sub cultured on nutrient agar plates in order to obtain pure cultures based upon difference in morphological characteristics.

3.3.1.2. Screening of Antibiotic producing bacterial strains

The antimicrobial activity of isolated strains were tested for all the isolates (table 1). Among them GTL3, GTL4 and GTL8 were most effective against the given ATCC strains. The strain GTL4 was selected for further studies.

Table 3.1. Antimicrobial activity of isolated strains against test organisms (zone of inhibition in mm).

Codes name	***K. pneumonia***	***S. aureus***	***E. coli***	***C. albicans***	***S. epidermidis***	***P. aeruginosa***
GTL1	_	_	_	_	_	_
GTL2	_	_	_	_	_	_
GTL3	21	28	19	24	26	30
GTL4	25	31	21	27	36	29
GTL5	_	_	12	_	_	8
GTL6	_	_	_	_	_	_
GTL7	_	14	_	_	11	_
GTL8	17	21	18	23	15	23
GTL9	_	_	_	_	_	_
GML1	_	7	_	_	_	_
GML2	12	_	_	_	_	_
GML3	_	11	_	_	15	10
GML4	_	9	13	_	_	_
GML5	_	_	_	_	_	_
GBL1	_	10	6	_	_	_
GBL2	_	-	_	_	_	5
GBL3	_	-	12	_	_	_
GBL4	_	14	21	_	_	_

GBL5	_	-	_	_	_	_
GBL6	_	-	8	_	_	_

3.3.1.3. Molecular identification through bacterial DNA extraction

The sequences of 16S rRNA were deposited for accession number in online database NCBI. The study isolate GTL4 is identified by BLAST search in NCBI and was identical to *Bacillus subtilis* (GenBank accession number OK491089) (Figure 2).

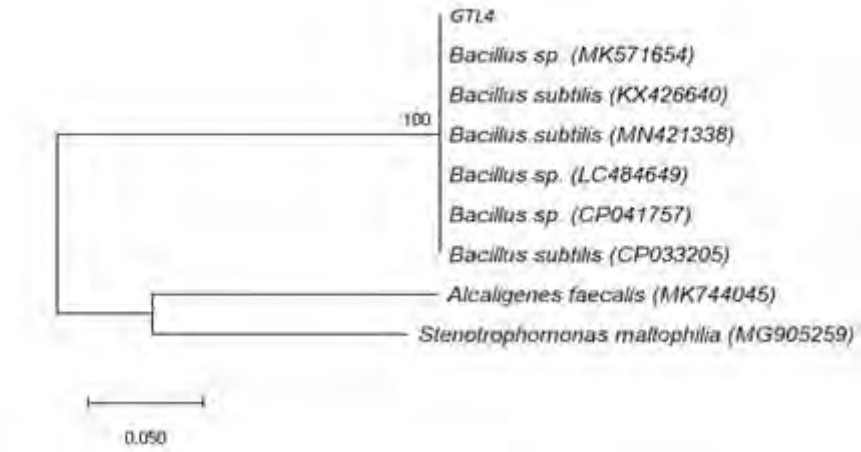


Figure 3.2 Phylogenetic Tree of isolate GTL4

3.3.2. Process optimization for production of antimicrobial compounds by Bacillus subtilis GTL4

Maximum antimicrobial activity was found in Tryptic soy agar (TSB) medium after 72h of incubation with zone of inhibition of 17, 15.1 and 14mm against *Klebsiella pneumonia*, *Escherichia coli* and *Staphylococcus aureus* respectively. Maximum production of antimicrobial compounds was observed at temperature 30°C with zone of inhibition of 19mm, 17mm and 16.3mm against *Staphylococcus aureus, Klebsiella pneumonia* and *Escherichia* respectively. Similarly, the strain showed maximum zone at pH 7 at incubation period of 72hrs (Fig. 3 a-d).

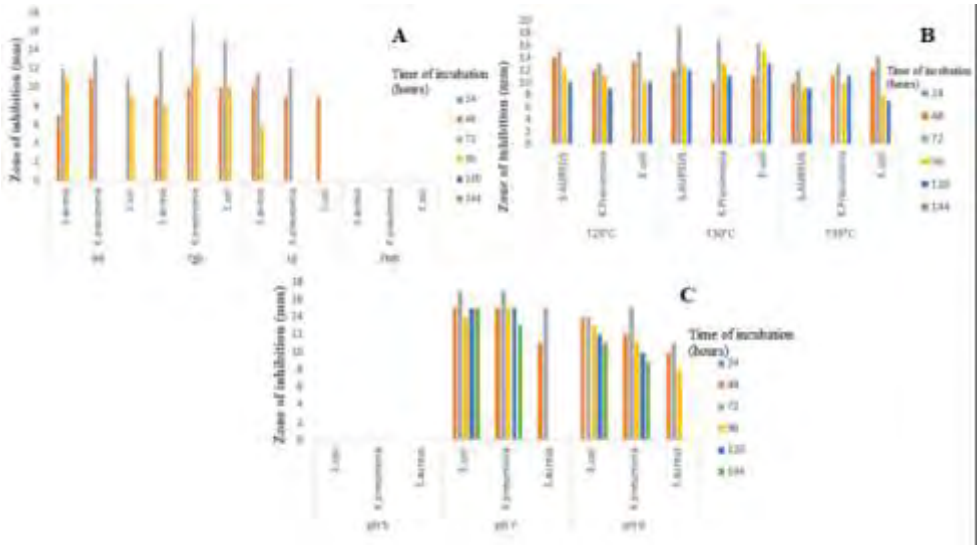


Figure 3.3 Effect of (a) type of culture medium, (b) temperature, and (c) pH on production of antimicrobial compounds by GTL 4 strain

3.3.2.1 Extraction of crude antibacterial compounds

In this study, various organic solvents were tested for their potential to extract antimicrobial compounds from fermentation mixture. Maximum extraction was achieved using N-Hexane:Choloform mixture used in 1:1 (v/v). The results of antimicrobial activity of extracted compound by different organic solvents is shown in figure 4.

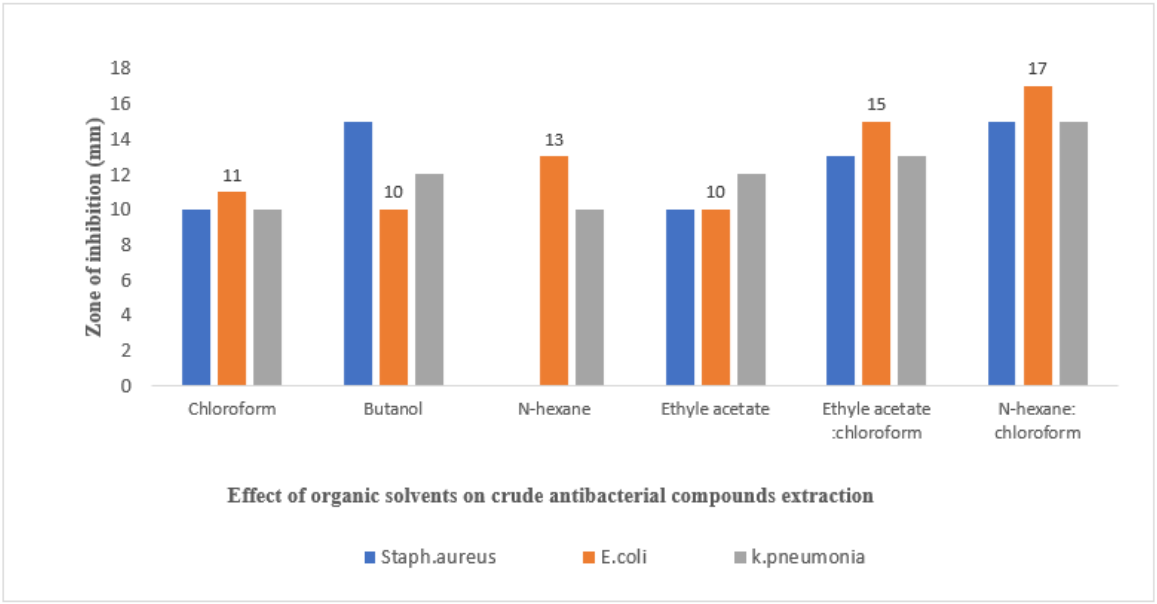


Figure 3.4 Effect of organic solvents on crude antibacterial compounds extraction by GTL4

3.3.3. FTIR spectroscopy of partial purified crude extract

The dried extract having antagonistic antibacterial activity was analyzed by FTIR spectroscopy. The range and the absorbance spectrum indicates presence of multiple organic compounds illustrating various peaks at 3432.87, 2996.14, 1658.96, 1436.09, 1018.53, 952.15nm depicting presence of functional groups of N-H, CH_3, CH_2 and CH, C=C, αCH_2, O-C, =C-H and $=CH_2$, C-Br and C-Cl respectively (Table 2 and Figure 5).

Table 3.2 FTIR Spectrum of crude extract of antimicrobial compound extracted in different solvents.

Range (cm^{-1})	**Frequency (cm^{-1})**	**Intensity**	**Vibration type**	**Functional group**
3400-3500	3432.87	weak	stretch	N-H (amines)
2850-3000	2996.14	strong	stretch	CH3, CH_2 and CH (alkanes)
1600 and 1500	1658.96	variable	stretch	C=C (alkenes)
1400-1450	1436.09	strong	bending	αCH_2 (aldehydes and ketones)
1000-1300	1018.53	strong	stretch	O-C (carboxylic acid and derivatives)
880-995	952.15	strong	stretch	=C-H and $=CH_2$ (alkenes)
500-600	595.78	strong	stretch	C-Br (alkyl halide)
600-800	667.79	strong	stretch	C-Cl (alkyl halide)

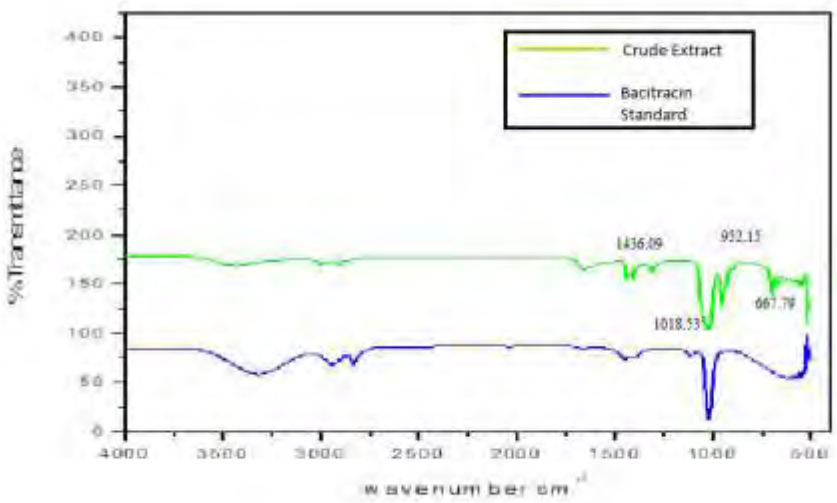
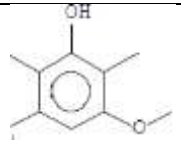
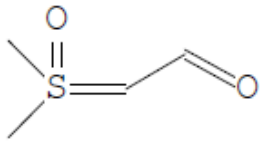
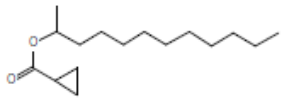
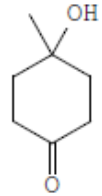


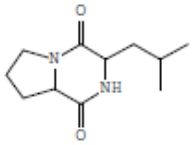
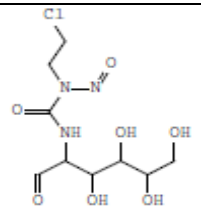
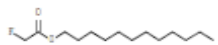
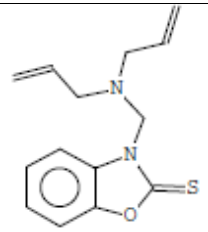
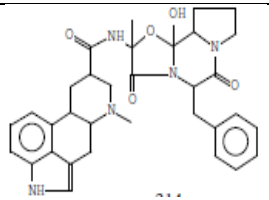
Figure 3.5 Comparison of FTIR spectra of control (Bacitracin) and the antibacterial compound produced by *B. Subtilis* GTL4 extracted in different organic solvents.

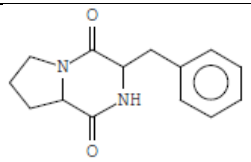
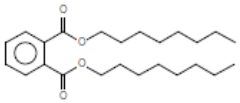
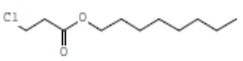
3.3.4. Gas Chromatography Mass Spectrometry (GC-MS)

The crude extract compounds were identified by performing GC analysis of the extract and compared with the constituents of the NIST library. A total of 13 different organic compounds were observed (Table 3.2). GC-MS Spectrogram of the crude extract is shown in Figure 3.6.

Table 3.3. GCMS profile of the crude extract

S NO	Retention Time (min)	Compound	Molecular Formula	Molecular Weight	Structure	Bioactivity	Reference
1.	14.525	3-Methoxy-2,5,6-trimethylphenol	$C_{10}H_{14}O_2$	166		volatile compounds (VOCs) that inhibit the growth and spore germination of *Fusarium oxysporum* f.sp.	(Ajilogba & Babalola, 2019)
2.	15.267	Dimethylsulfoxonium formyl methylide	$C_4H_8O_2S$	120		Cytotoxic effects on cyanobacteria and bacteria	(Zaki et al., 2022)
3.	15.933	1-Methylundecyl cyclopropane carboxylate	$C_{16}H_{30}O_2$	254		Antifungal, antiviral, antiHIV, anticancer, antitumour, antimycobacteria	(Grace et al., 2011)
4.	16.917	4-Hydroxy-4-methyl cyclohexanone	$C_7H_{12}O_2$	128		varying extents antimicrobial activity	(Gein et al., 2010)

5.	17.658	Pyrrolo[1,2-a] pyrazine-1,4-dione	$C_{11}H_{18}N_2O_2$	210		antibiotics, anti-tumor, antifungal, anti-inflammatory and cholesterol reducing drugs	(Law et al., 2020)
6.	18.367	Chlorozotocin	$C_9H_{16}ClN_3O_7$	313		Antineoplastic actions	(RANI & KAPOOR, 2019)
7.	20.900	Fluoroacetic acid	$C_{14}H_{27}FO_2$	246		a metabolic poison	(Lemaire et al., 2008)
8.	24.950	Benzoxazol	$C_{14}H_{16}N_2OS$	260		broad spectrum of activity against Gram-positive and Gram-negative bacteria and fungi	(Grela et al., 2014)
9.	27.150	Dihydroergotamine	$C_{33}H_{37}N_5O_5$	583		antibacterial property against clinical pathogens	(Shanthakumar et al., 2015)

10.	27.858	Pyrrolo[1,2-a]pyrazine-1,4-dione	$C_{14}H_{16}N_2O_2$	244		antibiotics, anti-tumor, antifungal, anti-inflammatory and cholesterol reducing drugs	(Law et al., 2020)
11.	30.108	Di-n-octyl phthalate	$C_{24}H_{38}O_4$	390		Antifungal and antibacterial properties	(Singh et al., 2018)
12.	35.917	3-Chloropropionic acid	$C_{11}H_{21}ClO_2$	220		Antibacterial and cytotoxic effects	(Jaradat et al., 2021)
13.	48.775	1-Undecene	$C_{11}H_{22}$	154		strong antifungal activity	(Lee et al., 2019)

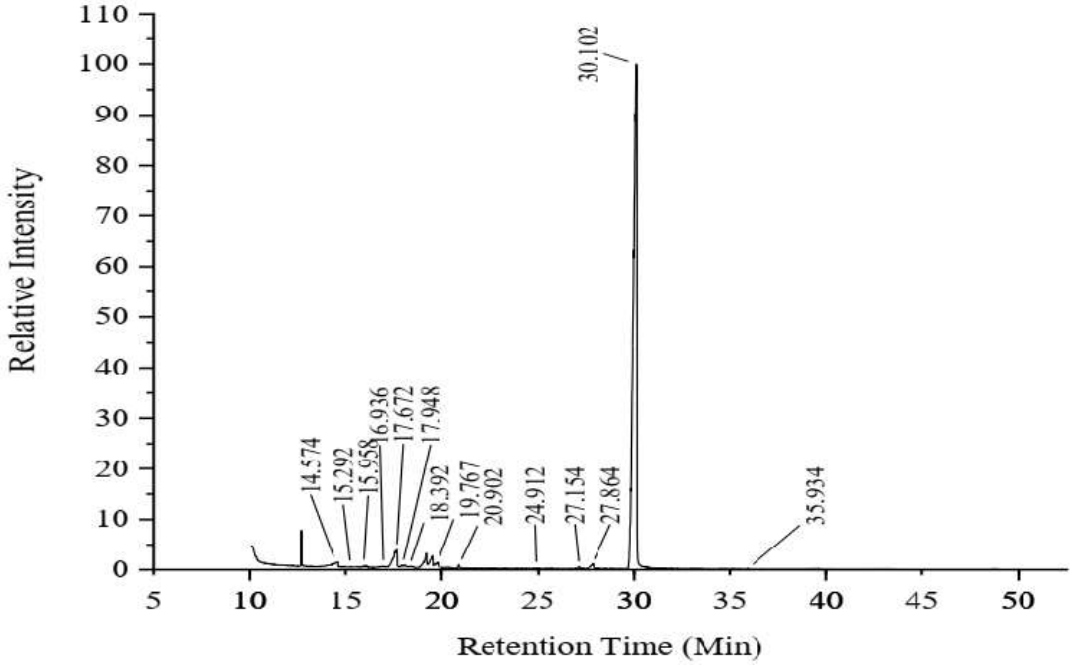


Figure 3.6 GCMS Spectrogram of crude extract.

3.3.5. Discussion

Various classes of antibitics have been discovered to treat bacterial infections, howover overtime resistance against commonly used antibiotics has been evolved. New intrest to discover novel products from novel habitats comes when the discovery of these compounds cease and treatement become difficult due to resitance mechanism. Despite having extremely starved environmental conditions, caves contain diverse and often distinctive microbial communities (Barton & Northup, 2007). In the absence of photoautotrophic organisms, cave consumers need to find other allochthonus food resources, which are normally scarce. Karstic caves are one of many extreme habitats that are still underexplored in terms of the biocapacity of resident bacteria to produce valuable biologically active compounds (Vasileva-Tonkova et al., 2013). Bacterial communities found in caves are reportedly grouped into nine different genera, namely *Proteobacteria, Acidobacteria, Planctomycetes, Chloroflexi, Bacteroidetes, Gemmatimonadetes, Nitrospirae, Actinobacteria* and *Firmicutes* (Saiz-Jimenez et al., 2008). Proteobacteria are the dominant of all bacterial genera in cave. In the current study, the antibiotic producing strain was identified as *Bacillus subtilis.* Presence of

gram positive bacteria like *Bacillus*, *Arthrobacter* and *Micrococcus* is also reported in Magura Cave, Bulgaria (Tomova et al., 2013). Studies performed by Zada et al., have reported bacitracin as a major polypeptide antibiotic produced by *Bacillus licheniformis* and *Bacillus subtilis* isolated from soil samples of Kashmir cave, Pakistan, against *M. luteus* as a test organism (Zada et al., 2016). A study conducted by Lisboa et al., has revealed a bacteriocin-like substance produced by *Bacillus amyloliquefaciens*, which inhibited pathogenic and food-spoilage bacteria (*L. monocytogenes, Bacillus cereus, Serratia marcescens,* and *Pasteurella haemolytica*) (Brandelli et al., 2006). *B. subtilis* isolated from soil have been reported to produce novel RLID 12.1 antimicrobial peptide that showed a broad-spectrum antimicrobial activity (Ramachandran, Chalasani, Lal, & Roy, 2014). *Bacillus* sp LM7 isolated from the korean traditional fermented soybean food, produced an antimicrobial lipopeptides inhibiting the growth of Gram positive bacteria (*L. monocytogenes and B. cereus*), but it could not inhibit lactic acid bacteria (*Lactobacillus plantarum* and *Lactococcus lactis*) (Kim et al., 2004). External factors such as temperature, pH and incubation are reported to have a direct impact on an organisms capability to produce antimicrobial compounds (Caracciolo et al., 2018; Burgess et al., 1999). The optimum temperature for antimicrobial compound production in this study was observed at 30°C. Similar findings have been reported for production of bacitracin by *B. licheniformis* (Eldewany et al., 2011). We found that our selected organism *Bacillus subtilis,* showed optimum activity at pH 7. The results are in accordance with a study in which maximum antimicrobial activity was observed at pH range of 6-8 by *Bacillus sp.* (oglu Gulahmadov et al., 2006). In the current study, FTIR spectrum of the crude extract showed peak formations at 3432.87cm^{-1}, 2996.14 cm^{-1}, 1658.96 cm^{-1}, 1436.09 cm^{-1}, 1018.53 cm^{-1}, 952.15 cm^{-1}, 595.78 cm^{-1} and 667.79 cm^{-1}. Absorbance peaks in similar ranges are documented in various studies suggesting the antimicrobial compound to be closely associated with bacitracin (Kong & Yu, 2007). GC-MS of the crude extract obtained in this study revealed presence of 13 different compound (Table 4). All the identified compounds have antimicrobial, insecticidal or toxic effects. Pyrrolo [1,2-a]pyrazine-1,4-dione,hexahydro as known for various therapeutic applications and has been used as antibiotics, anti-tumor, antifungal, anti-inflammatory and cholesterol reducing drugs. It is an antioxidative agent present in microorganisms present in extreme environments (Ser et al., 2015). Pyrrole has also been the ability to inhibit HIV-1 viruses and DNA polymerases and

protein kinase activity (Pettus et al., 2016). Bacterial species isolated form marine environment has been reported of producing cyclopropane containing fatty acids which inhibit growth of range of bacteria and fungi (Amiri Moghaddam et al., 2018). Cyclohexanone rings are precursors to many antibiotics and are produced both naturally and artificially synthesized. Chlorozotocin is reported to have anticancer and antineoplastic effects (RANI & KAPOOR, 2019). Fluoroacetic acid is toxic in nature and is being used in many formulations of herbicides, pesticides and fungicide (Krawczyk et al., 2017). Benzoxazole and related heterocycles (benzimidazole and benzothiazoles) have shown different pharmacological activities such as gram-positive antibacterial agents, antibiotics, antiparasitic, anti-inflammatory, elastase inhibitors, anti-stress, ulcer and anti-cancer agents, antiviral and anti-parkinson properties (Padalkar et al., 2016). A study conducted on antimicrobial potential of Dihydroergotamine has reported the compound to be active against clinical isolates of *S. enterica, S. flexneri, E. coli and K. pneumoniae* (Shanthakumar et al., 2015). Di-n-octyl phthalate produced by fungal specie in Nigeria was effective against a wide range of gram positive and gram negative bacteria (Awala et al., 2017). 1- Undecene is a precursor molecule for synthesis of variety of antimicrobial and antibacterial compounds (Sugiyama et al., 2016).

3.3.5. Conclusions

It can be concluded on the basis of results of the current study that *Bacillus subtilis* strain GTL-4, isolated from cave bat guano is a potential source of antimicrobial and antioxidant compounds. Maximum production of bioactive compounds took place at 30°C, 7 pH and 72hrs of incubation period. FTIR and GC-MS results indicate production of wide variety of bioactive organic compounds by the isolated strain. Further research should focus on isolation of more organisms from cave and other extreme environments in order to evaluate their potential to produce novel antibiotics to combat ever increasing threat of antibiotic resistance in the environment.

Chapter 4

Studies on Multidrug Resistant Bacteria Isolated from Kashmir Cave, Pakistan

Abstract

Multidrug resistance among microorganisms is wreaking havoc in the modern world. Caves are considered as pristine, less explored, and minimally anthropogenically influenced ecosystems, still antibiotic resistance at remarkable levels has been reported from them. The existence of antibiotic resistance in such isolated environments is not a good news for public health. The current study was aimed to investigate occurrence of multidrug resistance in bacterial species inside Kashmir cave of province Khyber Pakhtunkhwa, Pakistan. A total of 14 bacterial strains were isolated from 3 different layers of bat guano samples of the cave and screened for their potential to resist 6 commonly used antibiotic classes namely, aminoglycosides, beta lactams, carbapenems, fluroquinolones, macrolides, and glycopeptides. Among the isolates 64.28% belonged to phylum *Firmicutes*, *Proteobacteria* (35.70%), and *Bacteroides* (7.10%) phylum. Almost all the isolates showed resistance to more than 1 of the tested antibiotic classes. *Firmicutes* demonstrated high resistance against carbapenems, while *Proteobacteria* and *Bacteroides* were more resistant against the β lactam and aminoglycoside class of drugs. The occurrence of antibiotic resistance genes namely, *bla-CTXM*, *bla-NDM*, *Gyr A* and *vanA* gene was screened through polymerase chain reaction by amplifying the DNA. 78.5% of the strains were positive for *bla-CTXM* gene, 64.2% for *bla-NDM*, 42.8% for *Gyr A* and 35.70% for *vanA* gene. The overall results present an alarming situation of presence of multidrug resistance inside cave ecosystems and calls for strict surveillance and monitoring to avoid dissemination of resistant genes in clinical settings.

4.1 Introduction

Caves are natural geological formations that are formed mostly by dissolution processes, mechanical weathering, volcanic activity, or even glacial ice melting, creating gaps and empty spaces within the rocks (De Waele & Gutiérrez, 2022). Presence of low biologically active light, constant low temperatures, scarcity of nutrients and high humidity makes caves an extreme environment for all form of living organisms (Schabereiter-Gurtner et al., 2004). Being nutrient deficient, caves are oligotrophic in nature with less than 2mg/kg of total organic carbon, yet they can harbor microbial biomass of 106 cells/g of soil, on average (Barton & Jurado, 2007). Surprisingly, cave settings demonstrate a tremendous variety of bacterial diversity along with the existence of organisms belonging to domain archaea. (Zada et al., 2021). The bacterial diversity within the caves depends upon many factors, one of which is access to tourists. Further, each niche inside a cave is dominated by a separate group of bacterial species. For instance, *Proteobacteria* is mostly associated with rocks inside the caves and abundantly present in caves that are frequently visited by tourists. *Firmicutes*, on the other hand are mostly chemoorganotrophs that utilize carbohydrate fermentation as an energy source. They are mostly found in sediments of the caves. *Bacteroides* are usually the third most abundant type of bacteria in caves preceded by *Proteobacteria* and *Firmicutes* (Tomczyk-Żak & Zielenkiewicz, 2016). It is suggested that bacteria belonging to this group are involved in fermentation process and circulation of metals within the cave ecosystem. They are known to establish extensive biofilms coating Manganese and other metal precipitates (Bräuer et al., 2013).

Extensive use of antimicrobial agents, not only in human health system, but in veterinary and agriculture practices has resulted in drastic increase in antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) all over the world. The universal presence of such organisms is circulating these genes in ecosystems that are not even exposed to these chemical, giving rise to global antimicrobial resistance (AMR) (Chen et al., 2018). Scientists are now trying to investigate if AMR has travelled into pristine ecosystems, such as a cave. The topic is still less explored and there are only few studies that report presence of AMR and ARGs in cave bacteria (Turrini et al., 2020). Caves connect with external environment through water (drip water), winds, and wildlife that goes in and out of a cave. Among wildlife animals, the less recognized animal which

act as reservoirs for most of the ARB are bats. Bats are unique members of class mammalia in their ability to fly long distances and migratory patterns (Kuzmin et al., 2011). They receive great importance in infectious disease studies because of their association with emerging diseases being vector of many zoonotic viral pathogens, such as Ebola and Corona viruses (Moratelli & Calisher, 2015). In addition to presence of various clinically important viruses, bats are known carriers of bacterial pathogens as well (Konieczna et al., 2007). On a worldwide scale, approximately half of all known bat species spend part of their lives in caves (Hughes et al., 2022).

Since prevalence of AMR in caves is an unexplored research area, especially in Pakistan, this study has been designed to isolate bacterial species form bat guano of Kashmir cave, Pakistan. The study focuses on antibiotic susceptibility of the isolated strains against 6 classes of commonly used antibiotics in Pakistan followed by gene mapping of resistant genes present in the isolates.

4.2 Materials and Methods

4.2.1 Sample processing

The bacterial strains used in this study were isolated from 3 different layers of bat guano sample, collected from Kashmir cave, KPK, Pakistan. The strains were grown on nutrient agar plates using serial dilution method after incubation at 30°C for 24h.

4.2.2 Preservation of isolated bacteria

Isolated bacterial strains were preserved for future usage. For this purpose, 50% glycerol solution was prepared by mixing 50mL of autoclaved glycerol with 50mL autoclaved distilled water. The freshly prepared bacterial culture was added to 700µL of LB broth using inoculation loop and was left for overnight incubation at 30°C. 300µL glycerol was added to this culture, vortex for 10seconds and preserved at -20°C.

4.2.3 Screening of isolated bacteria based on antibiotic susceptibility test

The isolated bacterial strains were phenotypically screened for antibiotic susceptibility following Kirby-Bauer agar disc diffusion method. A total of 12 commonly prescribed antibiotics belonging to 6 different classes were used for this purpose. All the antibiotic discs were provided by OXOID, UK. The details of the antibiotics used are shown in Table 4.1.

Table 4.1 Classes of Antibiotics used in screening process

Aminoglycosides		
Antibiotics	**Abbreviation**	**Dose (µg/disc)**
Amikacin	AMK	30
Gentamicin	CN	10
Tobramycin	TOB	3O
Carbapenem		
Imipenem	IPM	1O
Ertapenem	ETP	10
Fluoroquinolones		
Levofloxacin	LEV	5
Ciprofloxacin	CIP	5
Ofloxacin	OFX	5
Glycopeptides		
Vancomycin	VA	5
Macrolides		
Erythromycin	E	15
β-lactam		
Ceftazidime	CAZ	10
Carbenicillin	CAR	100

4.2.4 Kirby-Bauer disc diffusion method

All the isolated bacterial strains were tested for their antibiotic susceptibility. For this purpose, individual bacterial strain was suspended in 2mL sterile normal saline solution using inoculating loop. The turbidity of the solution was maintained by comparing it with 0.5 McFarland standard solution. The turbidity is maintained by adding more saline solution if it needs to be decreased and by adding more bacterial colonies if the solution seems to be very dilute. Bacterial lawn was prepared using this mixture. Cotton swab was dipped in the solution as spread over MH agar plates unless all of the mixture is transferred to the plate. To maintain homogeneity, the plate was rotated at 60° three times. Antibiotic discs of specific dose (table 4.1) were dispensed on the plates using sterilized forceps. The plates were incubated at 30°C for 24h. Antibiotic susceptibility was assessed by measuring zone of inhibition (Jomehzadeh et al., 2021).

4.2.5 DNA profiling for Multidrug resistant genes

DNA of the bacterial strains was extracted and amplified to monitor the presence of selected multidrug resistant genes. Visualization of DNA amplicons was done by agarose gel electrophoresis.

4.2.6 Gel electrophoresis of the DNA

The suspension formed was microwaved for 30seconds for complete dissolution of the mixture. The mixture was let to cool for 15min before addition of 4µL ethidium bromide. The mixture was then transferred to gel tray and comb was inserted to create wells. The solidified gel was shifted to gel tank filled with TBE buffer (1X). 3µL of extracted DNA sample was mixed with 2µL of DNA loading dye (6X) (Thermo Scientific) and loaded into wells. 1kbp DNA ladder was also loaded for comparison. The gel tank was provided with power supply of 110v and 400mA and was run for 30minutes. The extracted DNA was visualized under UV-transilluminator and Gel doc system (Cheng & Jiang, 2006).

4.2.7 Polymerase chain reaction

PCR of selected antibiotic resistant genes was carried out to determine their occurrence in the isolated bacterial strains. For this purpose, primers were acquired through Thermo Scientific and were diluted by preparing 10picomolar stock solution. Further dilution was carried out by adding 90µL of distilled water in 10µL of stock solution. This dilution was then used for PCR reaction. To initiate the process, 5X derman green master mix, selected primers, nuclease free water and DNA samples were mixed in PCR tube. The mixture was inserted in the thermocycler machine to obtained copies of DNA segments. Primers used in this study are detailed in table 4.2. 40 cycles of thermocycler were run, and the PCR product was visualized on 2% agarose gel by electrophoresis.

Table 4.2 List of primers used for isolation of selected resistant genes.

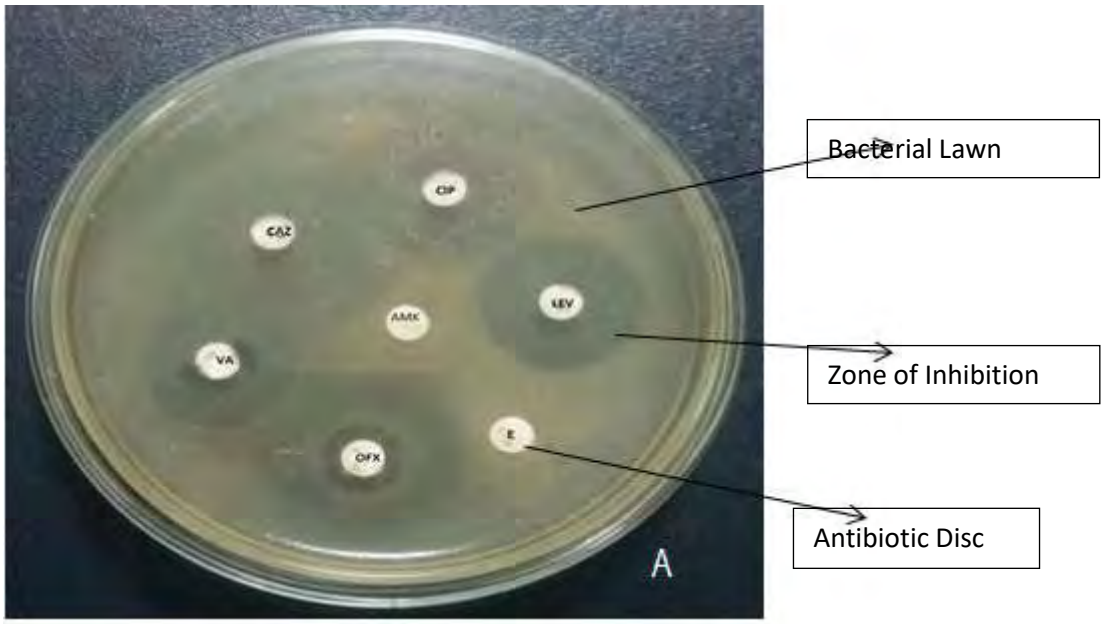
Target Gene	**Primer sequence**	**Product size**	**References**
blaCTX-M	P1C: 5'TTAATTCGTCTCTTCCAGA 3'P2D: 5' CAGCGCTTTTGCCGTCTAAG 3'	1000bp	(Gniadkows ki *et al*.,1998b)
GyrA	F 5'-AAATCTGCCCGTGTCGTTGGT- 3' R 5'-GCCATACCTACGGCGATACC-3'	344bp	(Park *et al*.,2011)
vanA	F: 5' TCTGCAATAGAGATAGCCGC 3' R: 5' GGAGTAGCTATCCCAGCATT 3	377bp	(Zischka *et al*., 2015)
*bla*NDM	F: 5' CGCCATCCCTGACGATCAAA3' R: 5'CTGAGCACCGCATTAGCCG3'	214bp	(Tan *et al*, 2018)

4.3 Results

4.3.1 Antibiotic Susceptibility profile

A total of 14 isolated strains were tested using Kirby-Bauer disc diffusion method to assess their antibiotic susceptibility following CL SI guideline (Bakr at al., 2019).

Figure 4.1 (A and B) shows selected images of antibiotic susceptibility testing of bacterial strains.



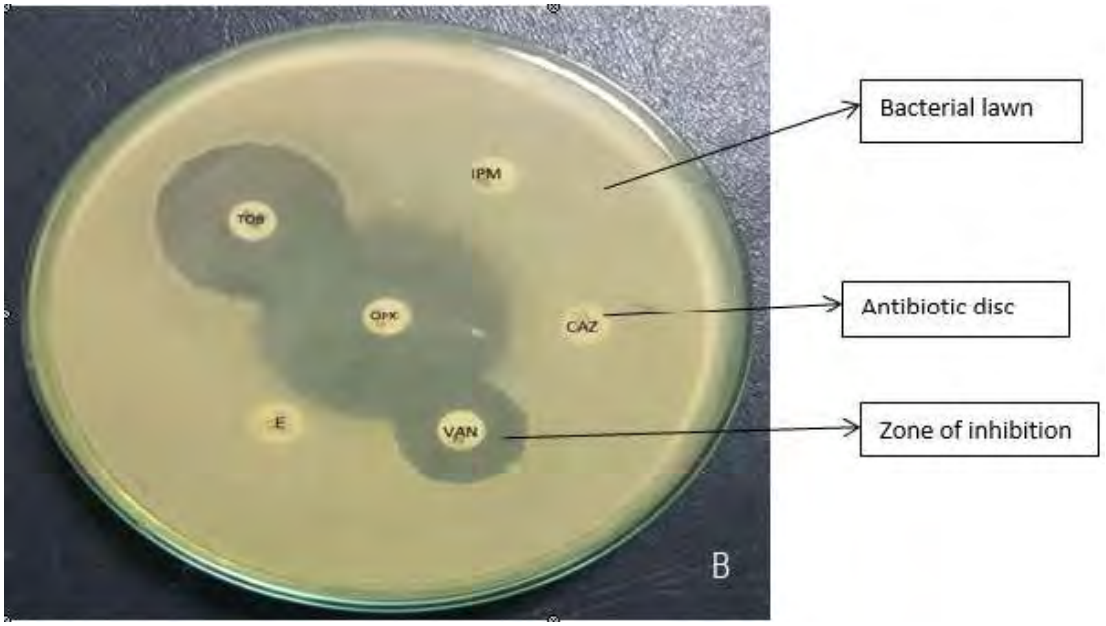


Figure 4.1 (A) Antibiotic susceptibility test for GTL6 strain (B) Antibiotic susceptibility test for GTL4 strain

Among the classes of antibiotics used, highest resistance was observed against glycopeptides, followed by β-lactams and least resistance was observed against Fluoroquinolones. Antibiotic susceptibility profile of all the strains is elaborated in table 4.3.

Table 4.3 Antibiotic susceptibility profile of all the isolated strains. S=Susceptible, I=intermediate, R=Resistant

S#	STRAIN	AMK	TOB	CN	IPM	ETP	LEV	CIP	OX	VA	E	CAZ	CAR
1	GBL1	R	S	I	R	R	R	I	S	I	R	R	R
2	GML1	R	R	S	R	R	R	R	I	R	R	R	S
3	GML2	R	S	R	R	R	I	R	S	R	S	R	I
4	GML3	R	S	R	R	I	R	I	S	R	R	R	I
5	GML4	R	S	R	I	S	R	R	I	S	R	R	S
6	GML5	I	R	S	I	I	S	I	S	R	R	I	R
7	GTL2	I	S	I	R	R	I	S	S	S	I	R	S
8	GTL3	R	R	I	R	I	R	S	S	R	S	R	R
9	GTL4	S	S	I	R	R	S	I	S	I	S	R	I
10	GTL5	S	R	R	S	R	R	R	R	R	R	R	I
11	GTL6	R	I	R	I	R	R	S	R	R	R	I	R
12	GTL7	R	S	S	R	I	I	R	S	R	I	R	R
13	GTL8	R	I	S	R	S	I	R	I	I	S	R	R

14	GTL9	I	I	R	R	S	R	R	S	R	I	R	I

All the isolates showed resistance against more than one antibiotic, so they are all termed as multidrug resistant (MDR) bacteria. The majority of the strains were capable of resisting glycopeptides (64%). Glycopeptides are followed by β-lactams (60%) and then carbapenems (57.10%). Minimum resistance was observed for fluroquinolones (33.3%). The results are shown in figure 4.2.

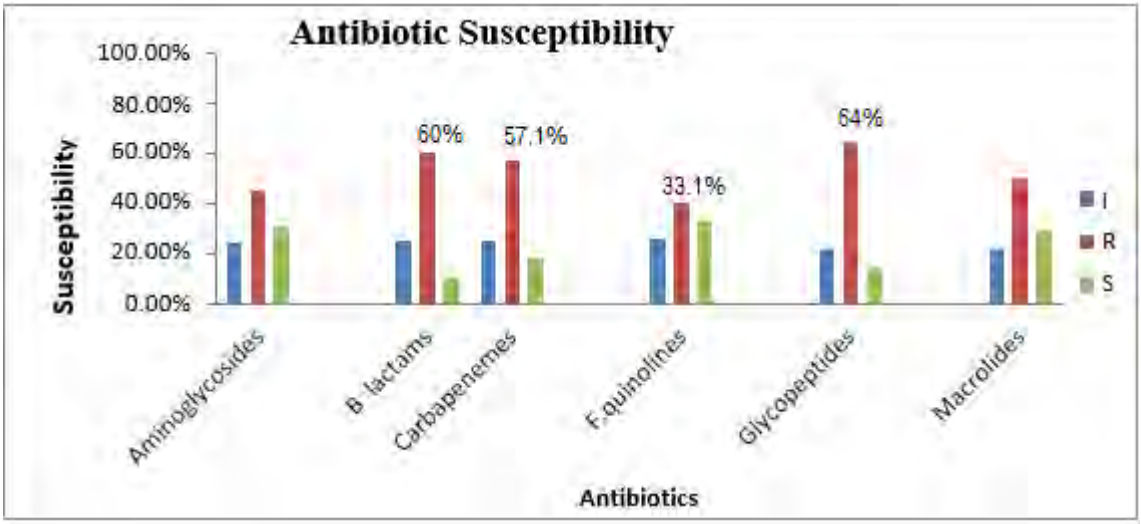


Figure 4.2 Prevalence of resistance against tested classes of Antibiotics

4.3.2 Phylum wise distribution of antibiotic resistance

When resistance was studied phylum wise, gram positive *firmicutes* showed maximum resistance against carbapenems (72.20%) whereas showed minimum resistance against fluroquinolones (37.03%). The data is presented in table 4.4 and figure 4.3.

Table 4.4 Antibiotic susceptibility profile for *firmicutes*

Strain	AMK	TOB	CN	IPM	ETP	LEV	CIP	OFX	CAZ	CAR
GBL1	R	S	I	R	R	R	I	S	R	R
GML1	R	R	S	R	R	R	R	I	R	S
GML2	R	S	R	R	R	I	R	S	R	I
GML5	I	R	S	I	I	S	I	S	I	R
GTL2	I	S	I	R	R	I	S	S	R	S

GTL4	R	S	S	R	R	S	I	S	R	I
GTL5	I	R	S	S	R	R	R	R	R	I
GTL7	R	S	S	R	I	I	R	S	R	R
GTL9	I	I	R	R	S	R	R	S	R	I

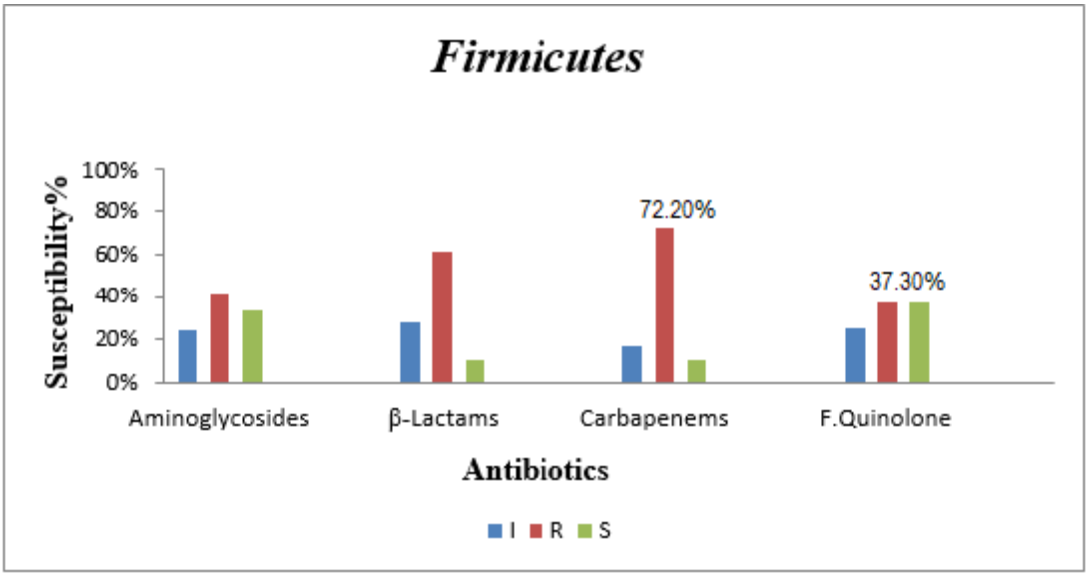


Figure 4.3 Antibiotic susceptibility study for *firmicutes*

Gram negative *proteobacteria* showed maximum resistance to the β-lactams class of antibiotics. 87.50% of the *proteobacteria* were able to resist this class. Intermediate resistance was observed against aminoglycosides (58.30%) while minimum resistance was recorded against carbapenems (37.50%) (table 4.5 and figure 4.4).

Table 4.5 Antibiotic susceptibility profile for *proteobacteria*

Strains	AMK	TOB	CN	IPM	ETP	LEV	CIP	OFX	CAZ	CAR
GML4	R	S	R	I	S	R	R	I	R	S
GTL3	R	R	I	R	I	R	S	S	R	R
GTL6	R	I	R	I	R	R	S	R	I	R
GTL8	R	I	S	R	S	R	R	S	R	R

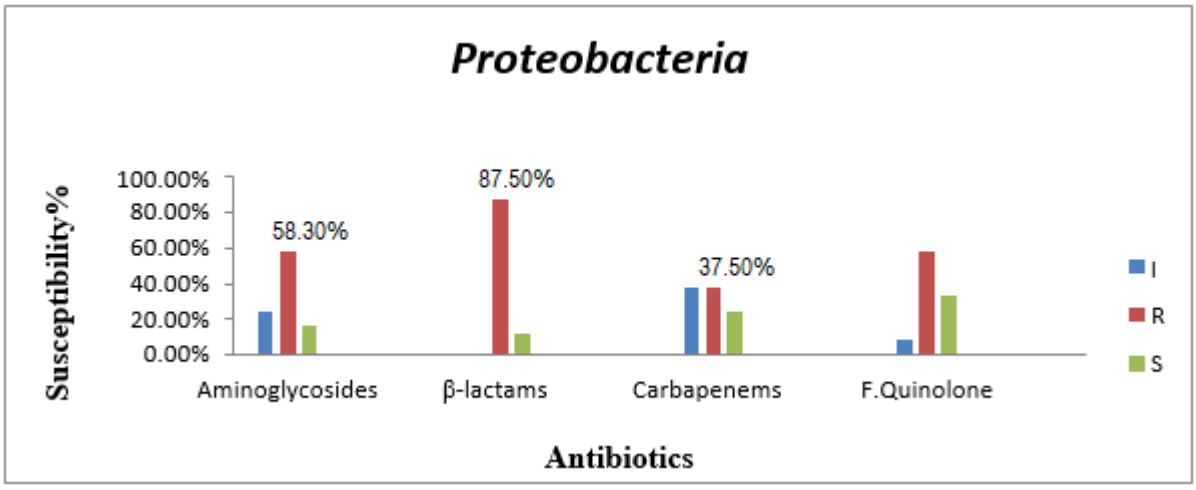


Figure 4.4 Antibiotic susceptibility study for *proteobacteria*

Only one of the isolated strains belonged to gram negative *Bacteroides*. Antibiotic susceptibility testing revealed the strain to resist 66.6% of the antibiotic belonging to class aminoglycosides, 50% to β-lactams and 33.3% resistance was showed to fluroquinolones. The results are illustrated in table 4.6 and figure 4.5.

Table 4.6 Antibiotic susceptibility profile for *Bacteroides*

STRAIN	AMK	TOB	CN	IPM	ETP	LEV	CIP	OFX	CAZ	CAR
GML3	R	S	R	R	I	R	I	S	R	R

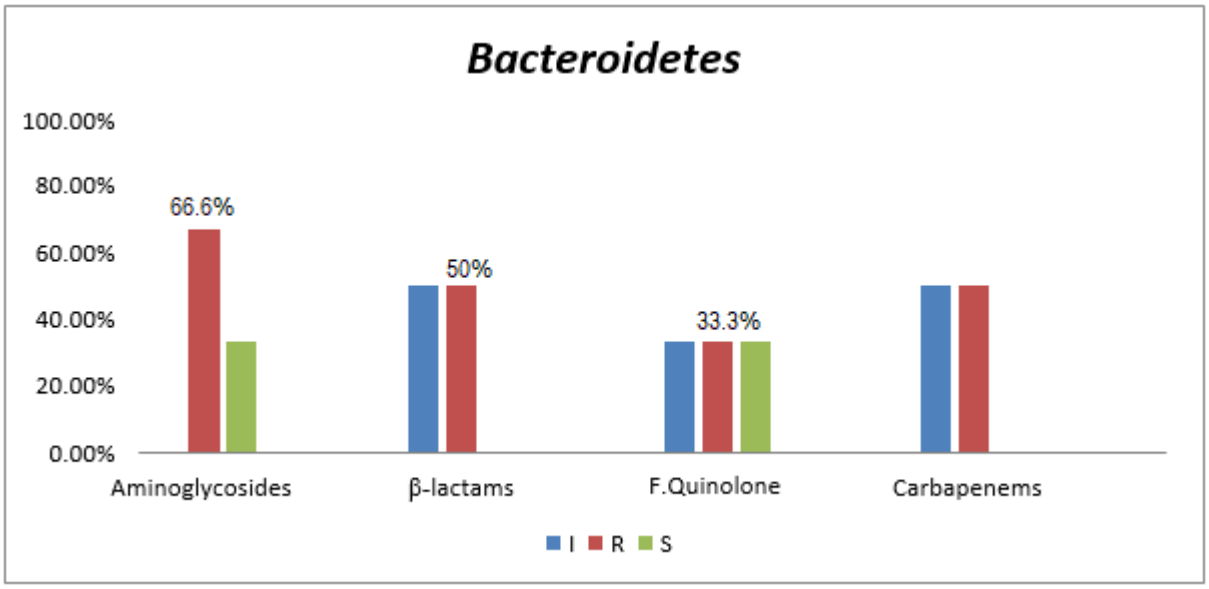


Figure 4.5 Antibiotic susceptibility study for *Bacteroides*

4.3.3 PCR for antibiotic resistant gene amplification

DNA of 14 isolated strains was extracted and subjected to PCR to detect presence of selected antibiotic resistant genes.

4.3.4 Molecular detection of *bla-CTX M* gene

The presence of bla-CTX M gene was confirmed in 78.5% of the isolated bacterial strains (fig 4.6). The gene is responsible for mediating resistance against β-lactams. The gel image of the PCR product is shown in figure 4.7.

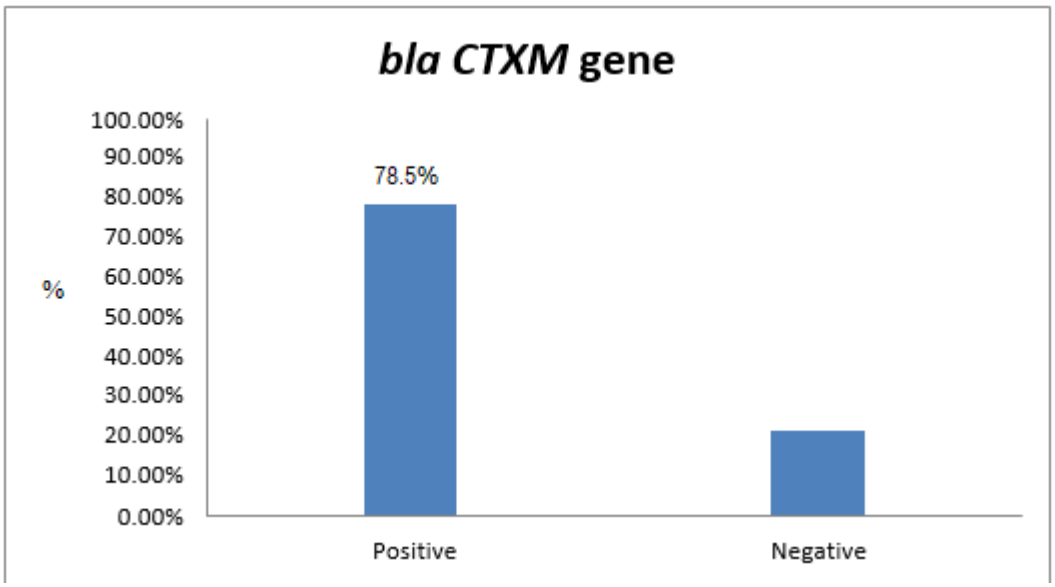


Figure 4.6 prevalence of *bla CTXM* gene in tested organisms

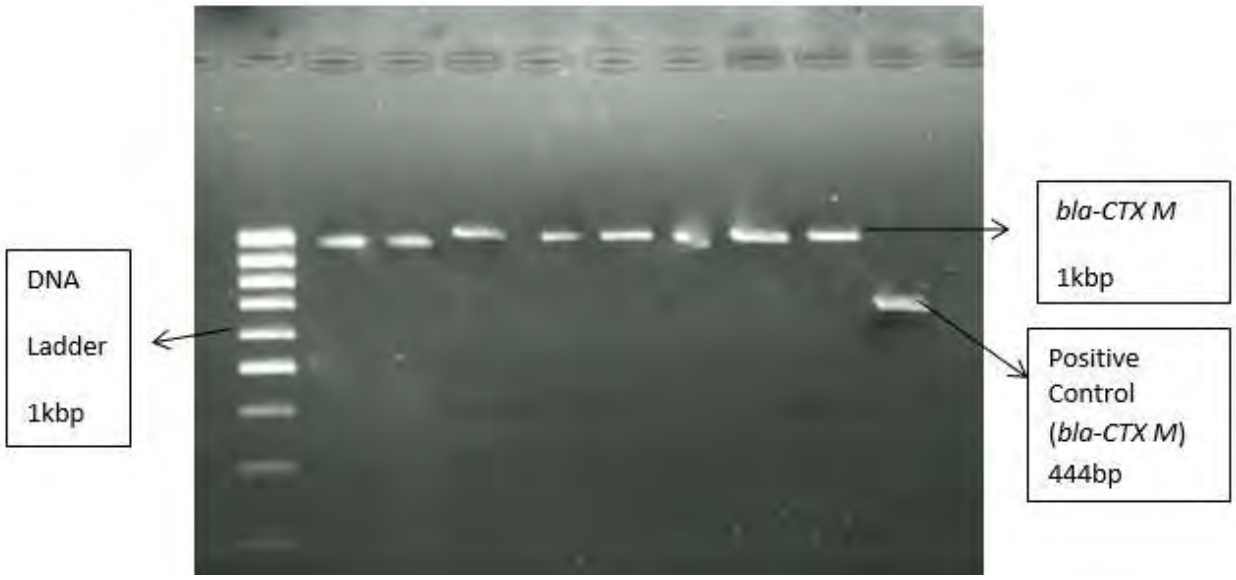


Figure 4.7 *Bla-CTXM* $_{gene}$ detected on 1.5% agarose gel electrophoresis.

Positive and negative results for *bla-CTXM* gene against tested organisms are shown in table 4.7.

Table 4.7 Representation of results for *bla-CTXM* gene against tested organisms

Gene	Strain IDs										
Bla- CTX M	GBL1	GTL2	GTL3	GTL4	GTL5	GTL6	GTL 8	GTL 9	GML2	GML4	GML5
	++	++	++	++	++	++	++	++	++	++	++
	GTL7				GML1				GML3		
	– –				– –				– –		

4.3.5 Molecular detection of *bla-NDM* gene

Like *bla-CTXM, bla-NDM* gene is also responsible for imparting resistance against β-lactam class of antibiotics. In our research, 64.2% of isolated bacteria possessed the

gene and hence were capable of resisting β-lactam antibiotics. The data is graphically represented in figure 4.8 and gel image of the gene is shown in figure 4.9. Results for individual strain are presented in table 4.8.

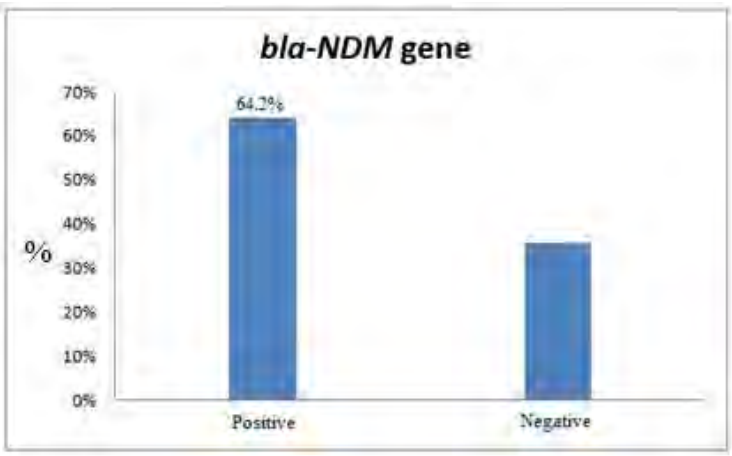


Figure 4.8 prevalence of *bla-NDM* gene in tested organisms

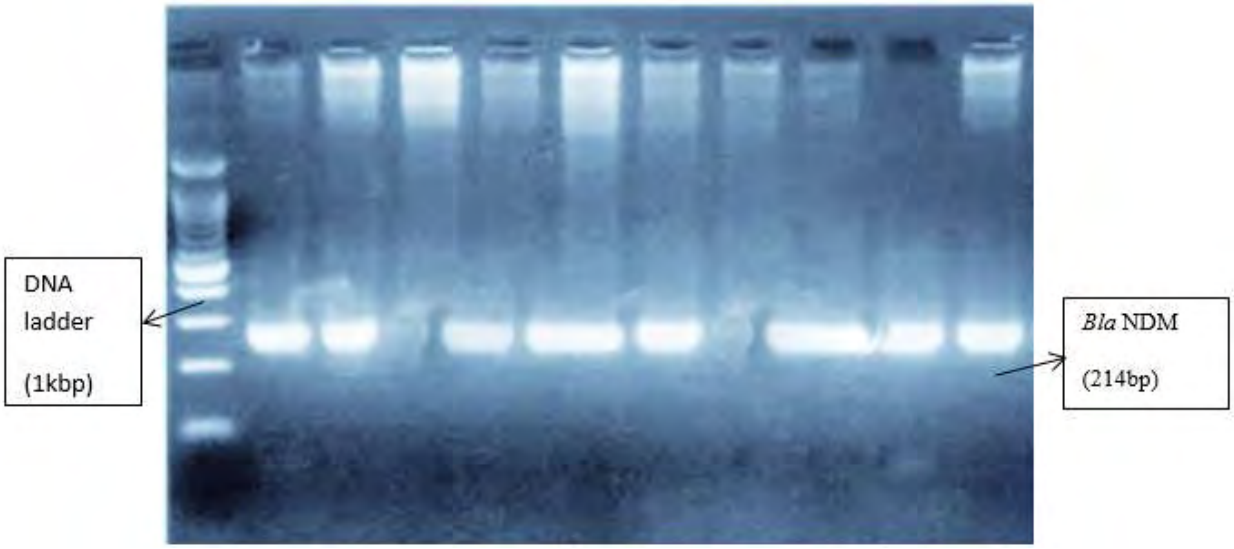


Figure 4.9 *bla-NDM* $_{gene}$ detected on 1.5% agarose gel electrophoresis.

Table 4.8 Representation of results for *bla-NDM* gene against tested organisms

Gene	**Strain IDs**								
bla - NDM	GBL1	GTL3	GTL4	GTL6	GTL8	GTL9	GML2	GML4	GML5
	++	++	++	++	++	++	++	++	++
	GML1		GML3		GTL2		GTL5		GTL7
	— —		— —		— —		— —		— —

4.3.6 Molecular detection of *GyrA* gene

GyrA gene is responsible for imparting antibiotic resistance against Fluroquinolones class of antibiotics. In our study *GyrA* gene was found in 42.8% of the total isolates. The gene was nonspecifically present in all tested bacterial genera. Figure 4.10 shows the graphical representation of the results. The Gel image of the detected gene is shown in 4.11 and table 4.9 shows individual response of isolated strain against gene testing.

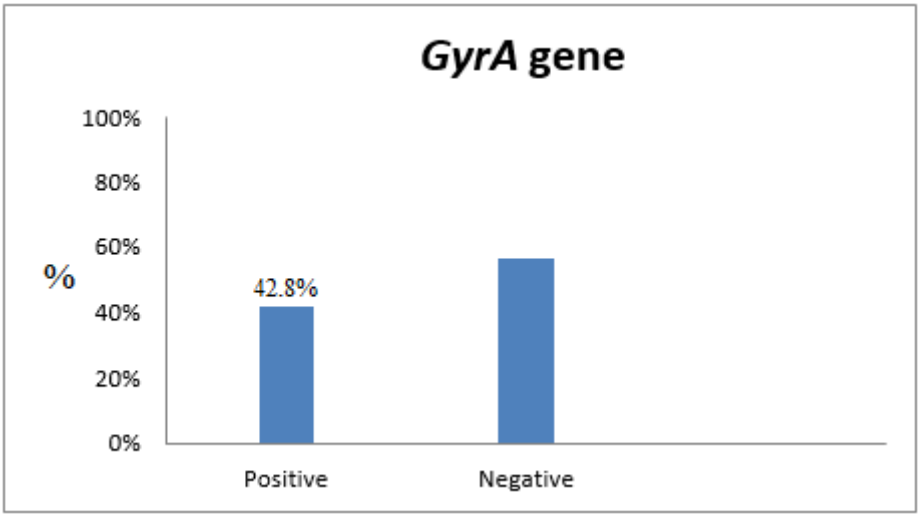


Figure 4.10 prevalence of *bla GyrA* gene in tested organisms

Figure 4.11 *GyrA* $_{gene}$ detected on 1.5% agarose gel electrophoresis.

Table 4.9 Representation of results for *GyrA* gene against tested organisms

Gene	**Strain IDs**							
*Gyr*A	GML3	GML4	GTL3	GTL6	GTL8	GTL9		
	++	++	++	++	++	++		
	GBL1	GML1	GML2	GML5	GTL2	GTL4	GTL5	GTL7
	– –	– –	– –	– –	– –	– –	– –	– –

4.3.7 Molecular detection of *VanA* gene

VanA gene is responsible for occurrence of glycopeptides class of antibiotics. In the present study, 35.70% percent of the strains tested positive for having *VanA* gene. It was the lowest percentage number found against all tested classes and types of antibiotics. Figure 4.12 shows graphical representation of the results. Figure 4.13 shows gel image of the isolated gene and table 4.10 shows results for individual tested strain.

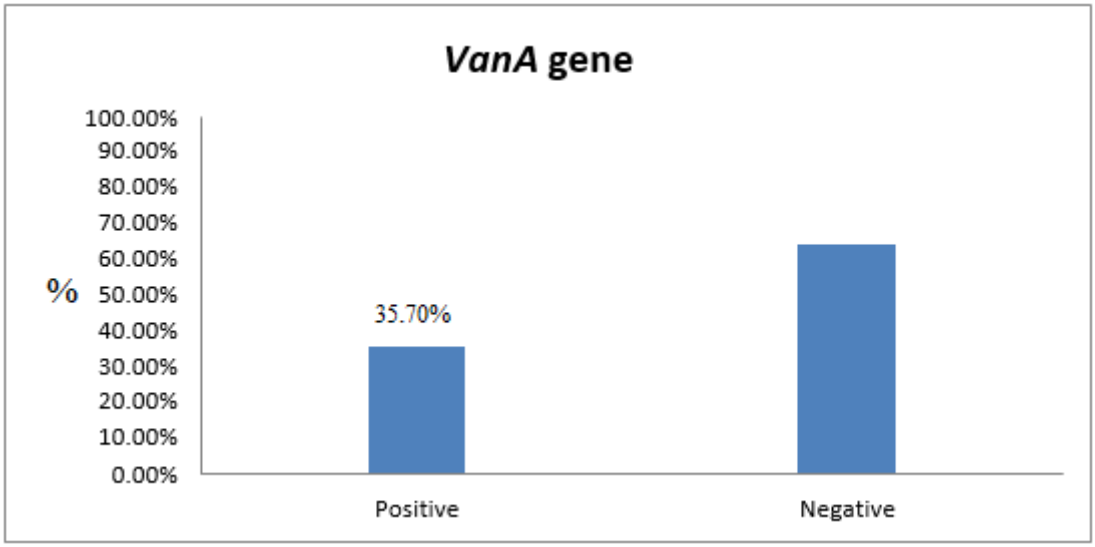


Figure 4.12 prevalence of *VanA* gene in tested organisms

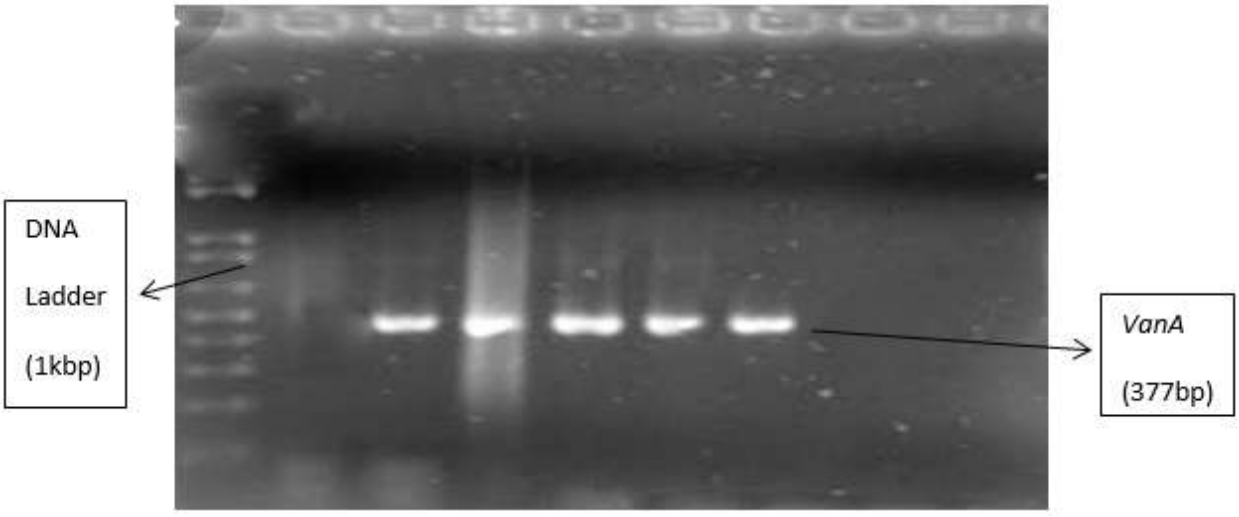


Figure 4.13 *VanA* $_{gene}$ detected on 1.5% agarose gel electrophoresis

Table 4.10 Representation of results for *VanA* gene against tested organisms

Gene	**Strain ID**								
*van*A	GBL1	GML3	GML4	GTL4	GTL6				
	++	++	++	++	++				
	GML1	GML2	GML5	GTL2	GTL3	GTL5	GTL7	GTL8	GTL9
	– –	– –	– –	– –	– –	– –	– –	– –	– –

4.4 Discussion

Antimicrobial resistance (AMR) is gripping today's world at a rapid pace. Scientists are now investigating if AMR has penetrated the pristine environments, like caves (Pašić et al., 2019). It is now being established that cave microorganisms not only harbor antimicrobial resistant genes (ARGs), but also disseminate them, contributing towards the global problem of AMR (Greig et al., 2015). This study was documented to evaluate presence of antimicrobial resistant bacteria in Kashmir cave of Pakistan.

Caves are isolated environments, usually linked with exterior through drip water, winds and bats (Mulec et al., 2020). Bats are free roaming wild mammals belonging to the order *Chiroptera* that is a diverse group with a diverse feeding habits (Cláudio et al., 2018). Presence of AMR-*E.coli* has been reported in bat guano samples of Slovenian caves (Skok et al., 2020). Antimicrobial resistant *Pseudomonas aeruginosa* has been reported from caves of Roraima Tepui, Guayana Highlands (Suárez et al., 2020). Studies on Australian fruit bats (Pteropus poliocephalus) has reported presences of novel strains of *Klebsiella africana* and *Klebsiella pneumoniae* (McDougall et al., 2021). Espinoza et al., has reported presence of multiple AMR bacterial strains in bat

guano samples of Peruvian Boobies (Sula variegata), northern Peru (Espinoza et al., 2021).

The *Firmicutes* found in our study showed resistance against carbapenems, which is an unlikely result. *Firmicutes* usually are susceptible towards carbapenems, as reported in an study carried out in Scarisoara cave ice cores (Paun et al., 2021). An intermediate level of resistance was shown by *firmicutes* towards aminoglycosides. The high G+C content organisms like *firmicutes* employ hydrolytic ring opening mechanism for aminoglycoside deactivation. The mechanism is shown in an study conducted on cave microbes of Carlsbad Caverns National Park, New Mexico (Bhullar et al., 2012). Presence of AMR firmicutes is also reported in a study of pristine cave in Western New Guinea, where organisms of this phylum were showing resistance owards a variety of commonly used antimicrobial compounds (Turrini et al., 2020). The other phylum that we tested in our research was *proteobacteria,* that showed high level (87.50%) of resistance against β-lactam antibiotics. A metagenomic study conducted in Krubera-Voronja Cave, Arabica massif, has reported presence of polyketide synthases (PKSs) and nonribosomal peptide synthetases (NRPSs) genes in *proteobacteria* enabling them to resist antimicrobial compounds (Kuisiene et al., 2021). Proteobacteria around the world are known to resist β-lactam and extended spectrum β-lactam drugs (Adesoji & Ogunjobi, 2016). It has been documented that ARGs are shred between different bacterial species, specially members of phyla *Proteobacteria, Firmicutes, Bacteroidetes* (Hu et al., 2016). In our study, *bacteroides* showed particularly high resistance (66.6%) against aminoglycosides. Members of phylum *Bacteroidetes* are anaerobic microorganisms capable of infecting human tissues. They are highly resistant to antibiotics, specially aminoglycoside class of antibiotics (Osińska et al., 2020). They are specially equipped with class 1 integrons, that are responsible for development and transmission of ARGs (Chamosa et al., 2017).

The last part of our research comprised of DNA profiling isolated organisms to establish occurrence percentage of selected ARGs namely, *bla-CTXM*, *bla-NDM*, *Gyr A* and *VanA* gene. The prevalence frequency for *bla-CTXM* gene was 78.5%, *bla-NDM* 64.2%, *Gyr A* 42.8% and *VanA* gene was 35.70%. Occurrence of resistant genes in bat guano have been reported by Gharout-Sait et al., while studying carbapenemase producing *Klebsiella pneumoniae* (Gharout-Sait et al., 2019). Presence of bla- TEM,

bla-CTXM ,bla-KPC and bla- NDM resistant genes have been documented in studies (Sousa et al., 2022).

4.5 Conclusion

The current study provides an insight into prevalence of multidrug resistant bacteria in Kashmir cave, Pakistan. The top is very scarcely explored in the scientific world and our study could be an important addition to the world of knowledge. The bat guano isolates showed resistant to multiple commonly used antibiotics, which is alarming considering caves as pristine natural ecosystems. Exitance of ARGs further raise concerns as these bacteria may become reservoirs of ARGs and transport it in ecosystems via horizontal gene transfer. This research study can be considered as stepping stone and more valuable data should be built on the topic.

Chapter 5

Biodegradation of Low-Density Polyethylene by Bacterial Strain isolated from bat guano of Kashmir cave, Pakistan.

Abstract

Plastic waste is one of the biggest challenges in the field of solid waste management and Polyethylene is the major contributor to this waste. There has always been a constant inflow of research on biodegradation of polyethylene by microorganisms isolated from soil of dump sites or wastewater. This study focused on evaluation of biodegradation potential of *Alcaligenes faecalis* bacterial strain, isolated from cave bat guano samples. The guano sample was collected from deep end of the cave and screening for LDPE bacteria was carried out from three layers of the bat guano. GTL 3 strain was selected for the experimentation based on maximum growth shown during the screening process. Biodegradation was carried out in MSM medium amended separately with glucose, Tween 80 and $CaCO_3$. Assessment of biodegradation process was done on the basis of growth of the bacterial strain (OD 620nm), weight loss percentage of LDPE pieces, mechanical characterization including young's modulus, tensile strength, tear strength and elongation at break at every 10^{th} of the experiment till 90^{th} day. Lipase production of GTL 3 strain was also evaluated at every 10^{th} day for 90 days using NPP as substrate. Chemical changes and changes in functional groups were checked using Fourier Transform Infra-Red spectroscopy at the end of the experiment. Physical changes were observed using Scanning Electron Microscopy at the end of the experiment. The results of the study revealed an initial decrease in OD that stabilized with increasing time of incubation. Maximum growth (OD 1.9) was observed in culture with added $CaCO_3$ in it. Weight loss % increased continuously throughout the experiment with maximum loss of 6.9% observed by the end of the experiment in $CaCO_3$ medium. Maximum decrease in Young's modulus was from 385.423Mpa to 316.964Mpa, tensile strength from 15.6Mpa to 2.62Mpa, tear strength from 126.486 N/mm to 65.581 N/mm and elongation at break from 17.98mm to 1.98mm in $CaCO_3$ amended medium by end of experiment. Lipase production increased in the initial days of the experiment, with a maximum production of 18.4 U/mL at day 30 and decreased continuously from hereafter. FTIR showed peak formation at 2345cm^{-1}, 3493cm^{-1},

726cm^{-1},1145cm^{-1}, 1875cm^{-1}, 2250cm^{-1}, and 3560cm^{-1} suggests oxidative breakdown taking with the polyethylene polymer. SEM images revealed clear signs of physical damage, erosion, abrasion of LDPE pieces. Bacterial adherence and biofilm formation by GTL 3 on LDPE surface was also observed in SEM images. It can be concluded that GTL 3 isolated from cave bat guano is capable of biodegrading light density polyethylene pieces. Extreme, pristine environments like caves should be explored for isolation of microorganisms with unique properties of potential biotechnological application.

5.1 Introduction

Polyethylene (PE) has long been accepted as a non degradable environmental pollutant. Materials made from polyethylene are extensively used in domestic and industrial applications and has a worldwide production of nearly 150 million tons/year (Peixoto, Silva, & Krüger, 2017). Properties like durability, high tensile strength, low weight, stability to mechanical shock and low cost make polyethylene a difficult commodity to replace. Polyethylene and polypropylene make up around 92% of all the synthetic plastics produced worldwide, and they are most commonly used for mass production of plastic bags, disposable containers, bottles, packaging materials, etc. (Bailey Jr et al., 1991). It is estimated that consumption of plastic bags alone is between 500 billion to 1 trillion globally, which not only disturbs natural ecosystems, but also poses a major disposal concern in solid waste management (Roy et al., 2008). Removal of plastics has been a field of study for many years now and various physical, chemical, and biological methods have been proposed for it. Out of these, biological methods are preferred due to their economic feasibility and environmental friendliness (Andrady et al., 2022). Due to its stable molecular structure, PE resists biodegradation and that is why management of plastic waste is an ever increasing problem with no current techniques offering complete solution (Albert et al., 2013). Options to deal with PE could include a change of manufacturing process to develop modified formulation of PE especially considering their mechanism for biodegradation once the polymer goes into landfill (Jamil et al., 2017). Since the early 1970s, many researchers have reported the PE biodegradation potential of various bacteria and Fungi, including *Bacillus*, *Pseudomonas*, *Staphylococcus*, *Streptococcus*, *Streptomyces*, *Brevibacterium*, *Nocardia*, *Moraxella*, *Penicillium*, and *Aspergillus* from soil, marine, and sludge under natural conditions. (Schlosser et al., 2015). However, high structural stability and strong hydrophobicity makes biodegradation of PE a challenging job, especially in shorter time durations.

Cave are considered as harsh biome having constant low temperature, high humidity and low nutrient availability (Biswas, 2010b). Microorganisms inhabiting caves showcase a variety of biochemical processes mediated by stressed environmental conditions. Biodegradation of PE is carried out by organisms known as chemoheterotrophs, the organisms that use organic compounds as their source of carbon as well as source of energy. Caves are one of the biotopes where such organisms are

abundantly present (Leo et al., 2012). Microorganisms living in cave and karst environments are exposed to oligotrophic, nutrient starved conditions and have evolved to survive in any available carbon source. Extensive studies have reported these heterotrophic organisms to be responsible for biodegradation/deterioration processes occurring inside cave environments (W. Ma et al., 2023). Study conducted by Wiseschart et al., has reported 26 xenobiotic biodegradation pathways in cave dwelling microbes using shotgun metagenomic sequencing (Pootanakit et al., 2019). Under harsh growth conditions such as in caves, many bacteria produce biosurfactants, thereby accelerating biological oxidation and pollutant biodegradation. Bacteria living in such conditions are capable of producing superoxide dismutase, efflux transporters, and metal-binding proteins. Production of these enzymes and proteins allow bacteria to degrade herbicides, pesticides, heavy metals, petrochemicals, and some aromatic compounds (Farda et al., 2022).

Caves in Pakistan are one of the least explored biotope specially regarding bacterial diversity and potential biotechnological applications. This study is therefore, aimed on isolation of bacterial strains from anthropogenically isolated Kashmir cave of province Khyber Pakhtunkhwa, Pakistan and their potential to degrade low density polyethylene.

5.2 Material and Methods

5.2.1 Sampling

Bat Guano samples were collected from Kashmir Cave, province Khyber Pakhtunkhwa, Pakistan. Kashmir cave is part of a series of limestone caves, most probably of marine origin (Zada et al., 2016). Piles of Guano samples were collected from dark end of the cave (188m from entrance) to minimize impact of external environment, labelled and packed within a zipper bag and stored inside an icebox. The samples were then stored at 4˚C for 5 days, until further processed for processed.

5.2.2 Screening of Low Density Polyethylene (LDPE) degrading bacterial strains

5g of sample was taken from 3 different layers of bat guano, namely top, middle and bottom layers mixed with 50ml autoclaved normal saline to form a solution. After carrying out serial dilutions, 100µl in nutrient broth medium (Sigma Aldrich) and incubated at 25˚C for 48h for bacterial growth. Subsequent sub cultures were prepared

to obtain pure colonies of bacteria. Commercially available polyethylene was used to screen LDPE degrading bacterial strains. The LDPE was cut into sheets of 1x1mm dimensions, washed with autoclaved distilled water once and twice with 70% ethanol for surface sterilization. Mineral Salt medium (MSM) with composition (g/L) [KH_2PO_4, 2.0; K_2HPO_4, 7.0; $MgSO_4.7H_2O$, 0.1; $ZnSO_4.7H_2O$, 0.001; $FeSO_4.7H_2O$, 0.01; $MnSO_4.6H_2O$, 0.002; NH_4NO_3, 1.0; $CuSO_4.7H_2O$, 0.0001; pH 7.2] was prepared as screening medium. 100µl of bacterial suspension from already prepared pure bacterial cultures were used as inoculum in 10ml of MSM and 10 pieces of LDPE and was incubated for 7 days at 25˚C in shaker incubator at 150rpm. GTL 3 strain was selected for further experimentations based on maximum growth (OD at 600nm) in culture medium.

5.2.3 Molecular Identification of the selected strain

The selected bacterial strain for the study was identified using 16S rRNA sequencing. For this purpose, the genomic DNA was extracted through Gene JET Genomic DNA Purification Kit (Thermo Scientific K0721) according to manufacturer's protocol. The DNA obtained was commercially sequenced from Macrogen Korea. The obtained sequences were analyzed and trimmed accordingly to enhance sequence quality. Identification was done using BLAST search in GenBank NCBI. Identical sequences were obtained from NCBI, and phylogenetic tree was constructed through Neighbor Joining method in MEGAX software.

5.2.4 Effect of culture composition on PE degradation

A total of 4 biodegradation experiments were set (including control) in 150ml Erlemeyer flasks containing 100ml MSM, 10µl bacterial culture and 10 pieces of sterilized LDPE pieces to study effects of various metabolites on the process of biodegradation. The MSM medium was augmented with Tween 80 (0.5%), Glucose (1% w/v) and 0.03% Calcium Carbonate ($CaCO_3$) to match natural Calcium concentration present inside limestone caves.

5.2.5 Biodegradation Analysis

The biodegradation experiments were set up for 90 days and various parameters were assessed at regular intervals to determine the overall progress of the process of biodegradation.

5.2.5.1 Growth of Bacterial strain

Viability of bacterial strain in terms of growth was measured using spectrophotometer. Samples were collected from batch experiments on every 10^{th} day for optical density (OD) at 620nm wavelength.

5.2.5.2 Weight loss of PE films

To assess biodegradation, weight loss of LDPE film was calculated at every 30^{th} day of incubation. For this purpose, LDPE films from each batch experiment were withdrawn and precise dry weight was calculated using lab scale analytical weigh balance. To remove any adhesives, the films were suspended with 5% sodium dodecyl sulfate solution for 1h. The films were then washed twice with 70% ethanol and left at 60˚C overnight in oven for drying. The weight loss was calculated using formula:

$$Weight\ loss\ \% = \frac{Initial\ weight\ of\ LDPE\ film - Final\ weight\ of\ LDPE\ film}{Final\ weight\ of\ LDPE\ film}\ X\ 100$$

5.2.5.3 Mechanical Characterization

Youngs Modulus, tensile strength, tear strength and elongation at break were used to detect any change in mechanical properties of LDPE films during the course of treatments. The LDPE films were extracted after every 10 days for analysis. For this purpose, the Universal testing Machine (3382, Instron, UK) was used following ASTMD 882-91 and 1922 standards.

5.2.5.4 Production of Lipase enzyme by GTL 3 strain

Lipase production was determined by using lipase enzyme activity assay in the cell free supernatant using spectrophotometer. Aliquots of the sample from the batch experiments were withdrawn after every 10 days and centrifuged at 5000rpm for 5min. Pellets were discarded and enzyme activity in the supernatant was checked using *p*-nitrophenyl palmitate (NPP) (Sigma-Aldrich) at 25˚C. Reaction mixture was prepared

using 100µl of NPP solution and 2.1ml of buffer A (50mM of Tris-HCL, pH 8.0) by heating at 40˚C for 10min in water bath. 100µl of cell free supernatant containing lipase enzyme was added in the mixture and incubated at 40˚C for 20min. The reaction was terminated using 100µl $ZnSO_4$ on ice bath. The reaction mixture was centrifuged at 8000rpm for 5min, and the supernatant was used to estimate liberated *p*-nitrophenol using spectrophotometer (absorbance at 410nm). Enzyme activity was expressed as international units, where one international units corresponded to one micromole of *p*-nitrophenol released per minute (Zhao et al., 2021).

5.2.5.5 Fourier Transform Infrared Spectroscopy of LDPE films.

Biochemical changes incurred because of LDPE-microbe interaction were checked using Fourier Transform Infrared Spectroscopy (FTIR). The analysis was performed after 90 days of incubation of LDPE films in bacterial culture. The untreated LDPE pieces were used for comparison. The analysis was performed to detect any change in functional groups between 400 and 4000 cm^{-1} wave number on Jasco FT/ IR – 620 equipment.

5.2.5.6 Scanning Electron Microscopy and EDS analysis

Surface morphology to detect physical degradation of LDPE pieces was carried out by Scanning Electron Microscopy (SEM) (JSM 5910 Joel, Japan). After the end of 90 days of incubation, LDPE pieces were removed from the culture medium and rinsed thoroughly with autoclaved distilled water. Before the scan, the pieces were mounted on copper stubs with gold coating. Gold coating was carried out under vacuum conditions.

5.3 Results

5.3.1 Molecular Identification of the selected strain

16S rRNA sequencing and phylogenetic analysis revealed the selected GTL 3 strain to be *Alcaligenes faecalis*. The phylogenetic tree constructed on MEGS X software is shown as figure 5.1.

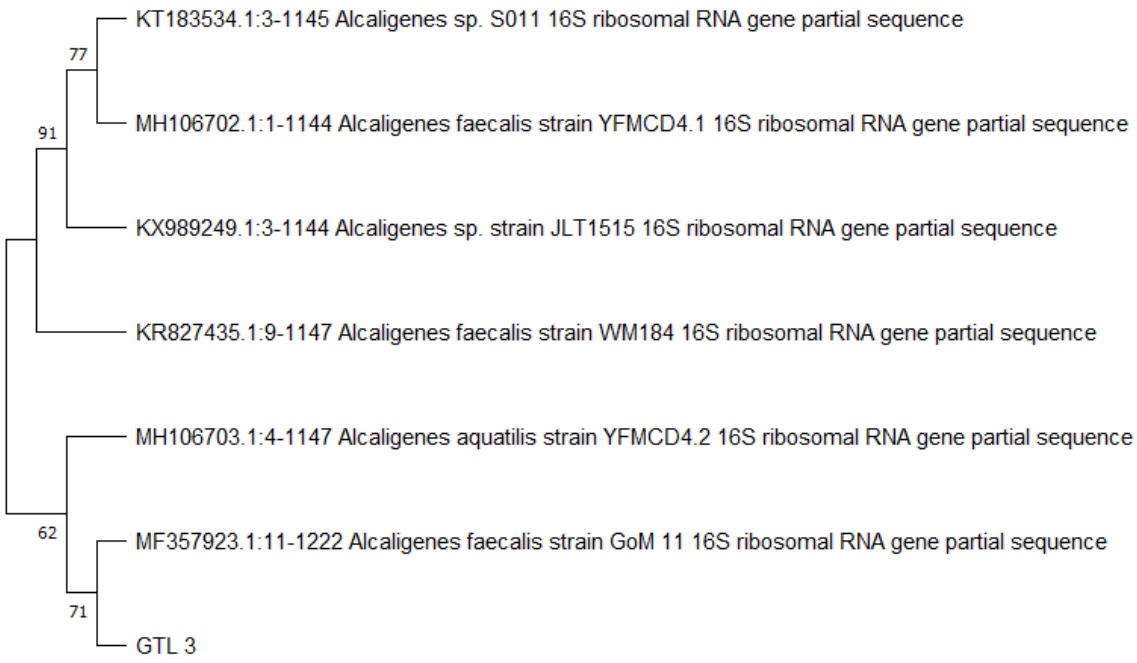


Figure 5.1 Phylogenetic tree of the isolated GTL 3 strain

5.3.2 Biodegradation Analysis

5.3.2.1 Growth of Bacterial strain

Growth of the bacterial strain was assessed at every 10^{th} day during the 90 days of experimental setup. Growth of GTL 3 strain decreased in the initial 10 days in cultures containing Tween 80 and $CaCO_3$ but got stabilized as the days progressed. A slight increasing trend was observed at the later end of the experiment. Maximum growth was sustained in the medium augmented with $CaCO_3$. On the other hand, a surge in growth was observed when the culture was supplemented with glucose, but after the first 20 days, the growth ceased and eventually reduced. The growth patterns are illustrated in figure 5.2.

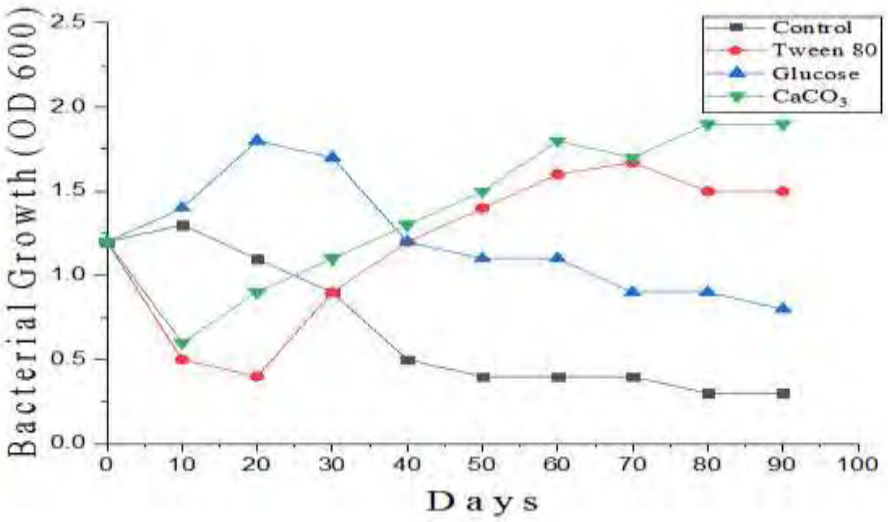


Figure 5.2 Growth of GTL 3strain in different culture conditions

5.3.2.2 Weight loss of PE films

The weight loss data is shown in figure 5.3. consider able weight loss of LDPE pieces was observed in all of the mediums. Maximum decrease in weight occurred in medium supplemented with $CaCO_3$, most likely because of the enhanced growth that occurred in this medium. Compared with 0.9% wight loss % in control experiment, a % loss of 2.3%, 1.2% and 2.8% was observed in cultures supplemented with Tween 80, glucose and $CaCO_3$ respectively, after 30 days of incubation. Maximum weight loss percentage of 6.9% was observed in $CaCO_3$ supplement medium by the of 90 days experiment.

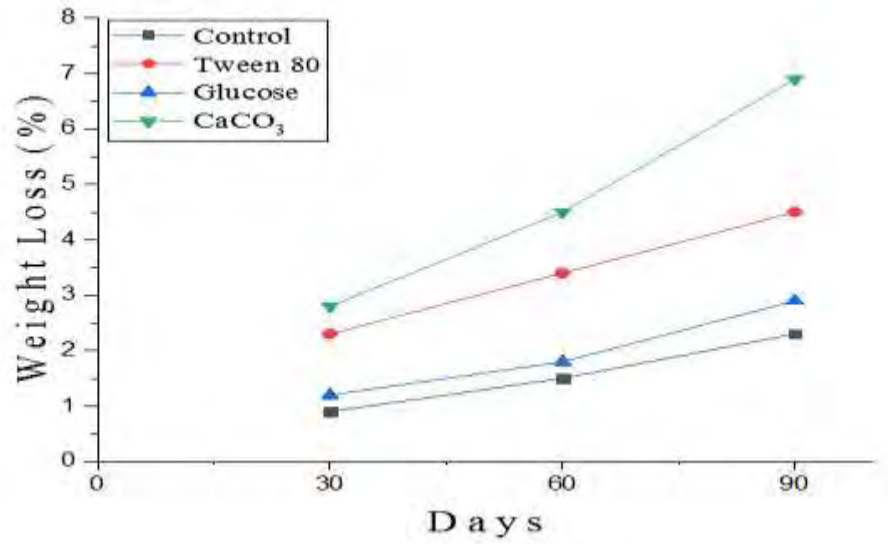


Figure 5.3 Weight loss percentage of LDPE pieces during 90 days of experiment

5.3.2.3 Mechanical Characterization

At the start of the experimentation, LDPE pieces had Young's Modulus of 385.423Mpa, Tensile Strength of 15.6Mpa, Tear Strength value of 126.486N/mm and Elongation at break was recorded as 17.98mm. The values for all the mechanical tests decreased as the degradation experiment progressed showing weakening of the plastic pieces. Maximum loss in mechanical strength was observed in culture medium supplemented with $CaCO_3$, again because of high bacterial growth curve of the medium in relation with others. Young's Modulus was decreased to 316.964Mpa, Tensile strength to 2.62Mpa, Tear Strength to 65.581N/mm and Elongation at break to reduce to a bare 1.98mm. The results are shown in figure 5.4.

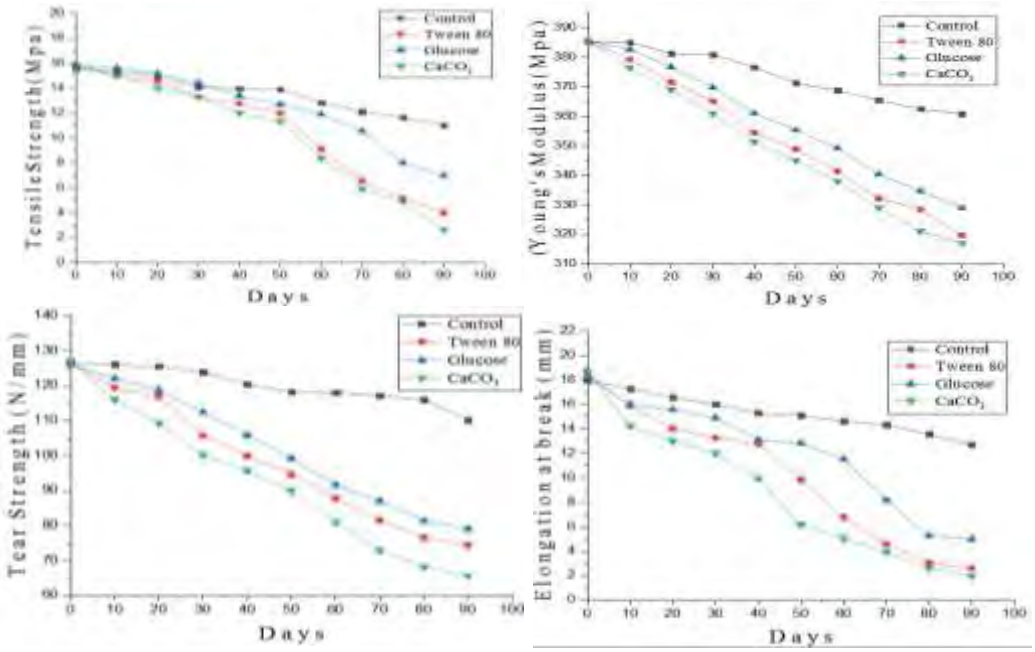


Figure 5.4 Young's Modulus, Tensile Strength, Tear Strength and Elongation at break of LDPE pieces after at end of 90 days of experiment

5.3.2.4 Production of Lipase enzyme by GTL 3 strain

Lipase production by GTL 3 strain incubated in various culture compositions was assessed on every 10th day for a period of 90 days. The results showed that production of lipase started at around 10th day of incubation and peaked at 20th day of the experiment. Lipase production significantly decreased after 30 days of incubation of the bacterial strain with LDPE pieces. Maximum lipase production of 18.9 U/mL was observed on 20th day of incubation in culture medium incubated with Tween 80. The results are shown in figure 5.5.

Figure 5.5 Lipase production by GTL 3 strain incubate with LDPE pieces

5.3.2.5 Fourier Transform Infrared Spectroscopy of LDPE films.

FTIR analysis was carried out to determine formation of new functional groups in LDPE pieces after getting treated with GTL 3 strain. Comparison was made with untreated polyethylene pieces. The results show formation of new peaks at critical areas suggesting breakdown of treated LDPE pieces (figure 5.6). New peaks at 2345cm^{-1} and 3493cm^{-1} when incubated with glucose represent formation of OH stretching vibrational and O–H stretching vibration due to alcohol/hydroxyperoxide respectively. New peaks at 726cm^{-1} and 1145cm^{-1} in LDPE pieces incubated with Tween 80 indicate formation of Rocking deformation/C–H bending vibration (CH_2) and Ring vibrations overlapped with stretching vibrations of (C–OH) groups respectively. Similarly, peaks formed at 1875cm^{-1}, 2250cm^{-1} and 3560cm^{-1} when incubated with $CaCO_3$ show formation of Aromatic group, Nitrile group and Alcohol group respectively (Table 5.1).

Table 5.1 formation of new peaks in LDPE pieces incubate with GTL 3 strain (Kunlere, Fagade, & Nwadike, 2019; Zhang, Ding, & Hu, 2022)

New Peak	Functional Group
Incubated with Glucose	
2345	OH stretching vibrational
3493	O–H stretching vibration due to alcohol/hydroxyperoxide
Incubated with Tween 80	
726	Rocking deformation/C–H bending vibration (CH_2)
1145	Ring vibrations overlapped with stretching vibrations of (C–OH)
Incubate with $CaCO_3$	
1875	Aromatic group
2250	Nitrile group
3560	Alcohol group

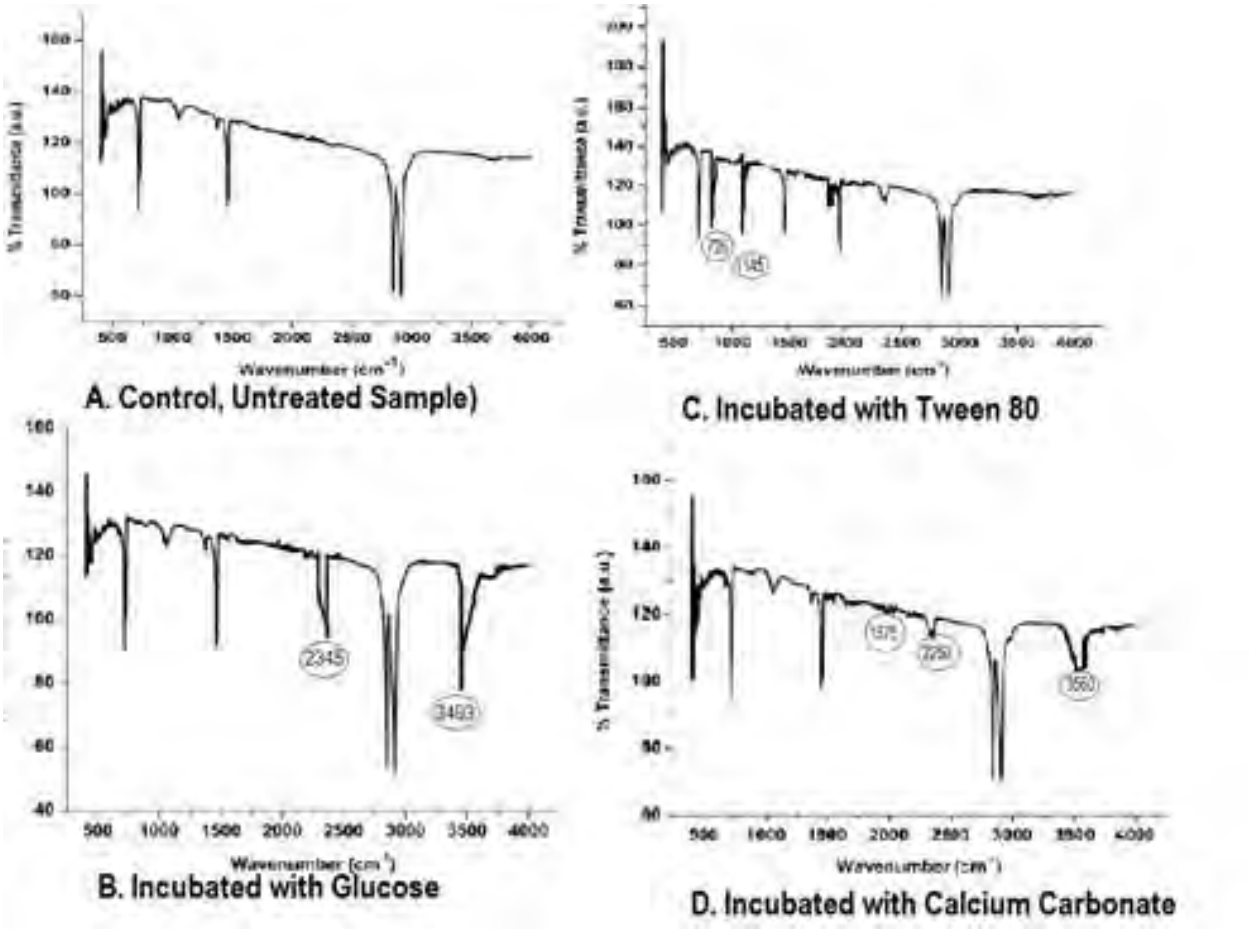


Figure 5.6 FTIR spectrum of LDPE pieces incubated with GTL 3 after 90 days of experiment.

5.3.2.6 Scanning Electron Microscopy of LDPE pieces

SEM showed extensive physical damage incurred on the surface of LDPE pieces after 90 days of incubation with GTL 3. Clear signs of erosion and abrasion are seen in the images. When compared with untreated sample, SEM images of treated sample shows adherence of bacterial strain on the surface of the polyethylene film, suggesting formation of biofilms that enhances the process of biodegradation. SEM images of the treated polyethylene pieces in comparison with control are shown in figure 5.7.

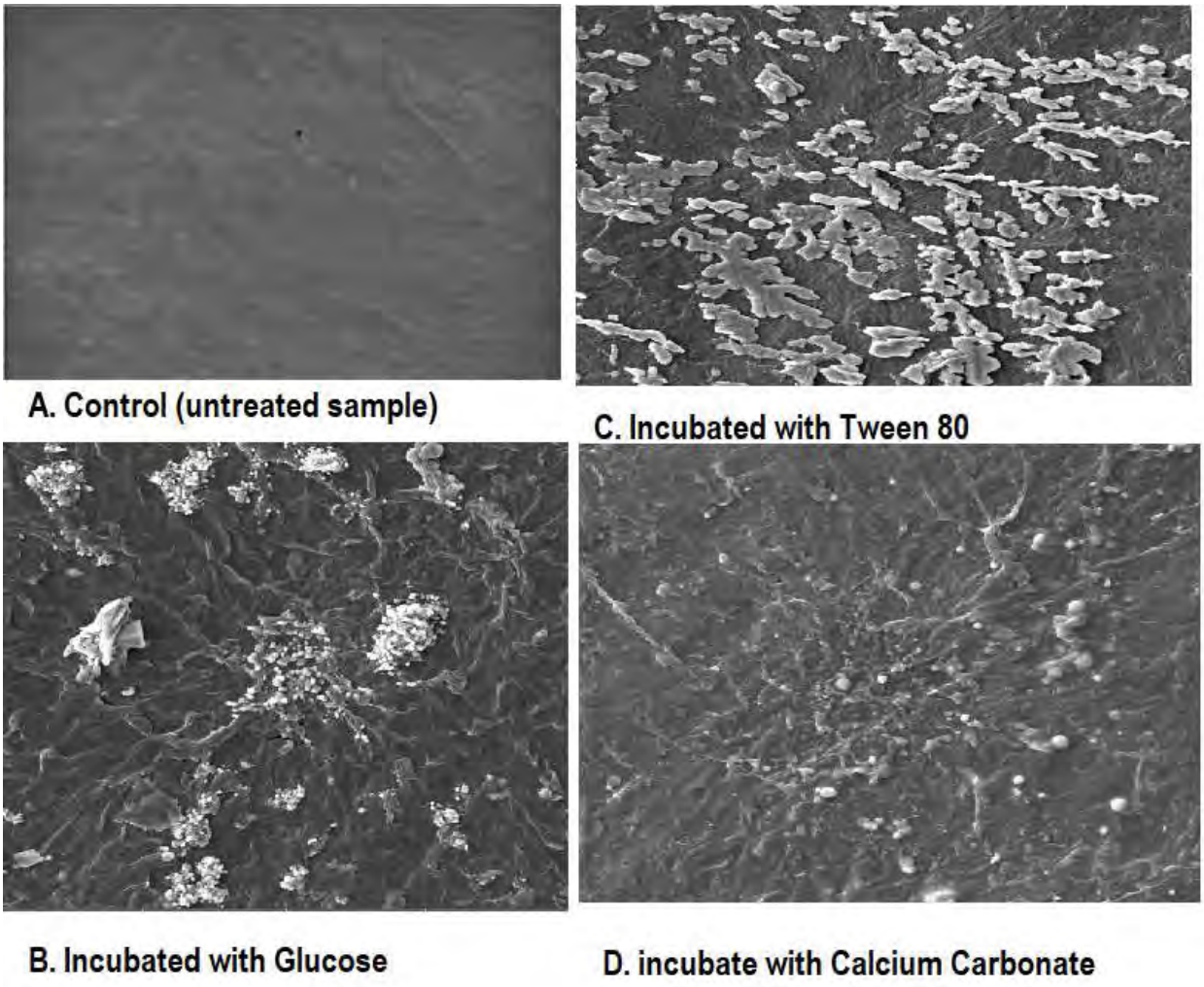


Figure 5.7 Scanning Electron Micrographs of LDPE pieces after 90 days incubation with GTL 3 strain

5.4 Discussion

After all these years of scientific advancement, researchers are yet to provide a definitive solution to plastic waste. Introduction of new formulations, chemical and physical degradation and biological methods of plastic degradation have all been tried, yet the problem exists where it used to from the beginning. Scientists are now looking for better solutions in environments that were not explored before. These environments are extreme in conditions and harbor life with unique capabilities. Ecosystems like caves, marine ecosystems, glaciers, hot water springs etc. are examples of these environments. In the context described above, the current study was carried out to evaluate potential of GTL 3 strain isolated from bat guano to deteriorates light density polyethylene.

GTL 3 strain in this study has been identified as *Alcaligenes faecalis*. The strain has shown heightened capability of biodegrading polyethylene in an study on used polyethylene bags (Ray et al., 2021). These bacteria are known to have diverse biodegradation pathways and have been used to treat a variety of organic pollutants. For instance, Alcaligenes faecalis strain AZ26 has been used to successfully degrade reactive textile dyes (Hossen et al., 2019). *Alcaligenes* sp. strain DN25 has been employed for removal of cyanide (Li et al., 2019). Neisi et al., used *Alcaligenes faecalis* strain BPAN 5 for biodegradation of Bisphenol A from saline industrial wastewater. *Alcaligenes faecalis* has also been used in combination with *Micrococcus luteus* for biodegradation of Cyanotoxin Cylindrospermopsin (Manage et al., 2022). *Alcaligenes faecalis* has also been used in combination with iron oxide nanoparticles to degrade crude oil contaminants (Oyewole et al., 2019).

In our study, the growth of the bacterial cell decreased in initial days of incubation, suggesting activation of the lag phase of bacterial growth. The growth steadily increased after 10 days of incubation till the 90th day. The results are in accordance with our previous study where an attempt was made to degrade LDPE with bacterial consortium isolated from soil samples of Kashmir cave (Jamil et al., 2017). A constant growth pattern has been documented in a study where marine bacterial species are tested for LDPE biodegradation (Jha et al., 2021). Samanta et al., has also reported an initial lag phase followed by high growth rate of *Bacillus tropicus* strain incubated for 40 days with LDPE (Halder et al., 2020). In our study, a steady loss in weight of LDPE pieces occurred along the time of incubation. The weight loss is direct indication that the plastic pieces are consumed by bacterial strain as a source of food. Similar findings have been reported where 2.5% of the dry weight loss was observed while treating polyethylene with thermophilic *Brevibacillus borstelensis* (Hadad, Geresh, & Sivan, 2005). Sudhakar et al., reported 10% and 5% loss in weight of polyethylene when incubated with *Bacillus sphericus* (Alt) and *Bacillus cereus* (BF20), respectively for one year (Venkatesan et al., 2008). Similarly, 1.0, 1.5 and 1.75% polyethylene weight has been reported in 30 days of incubation with marine bacterial species, *Kocuria palustris* M16, *Bacillus pumilus* M27 and *B. subtilis* H1584 (Harshvardhan & Jha, 2013).

Maximum weight loss was observed when the PE pieces were incubated with calcium carbonate supplementation. Possible reason is the active growth of bacterial strain with added calcium content. Since the bacterial strain belonged to a limestone cave, calcium carbonate was added in the medium to provide near natural conditions to the isolate. The weight loss is complimented with maximum growth in medium supplemented with calcium, that resulted in maximum weight loss as well. Furthermore, calcium carbonate is known to facilitate biofilm formation. Adherence and attachment of bacterial colonies on the smooth surface of LDPE is the first step towards biodegradation of the polymer. Added calcium carbonate facilitated enhanced biofilm formation (as evident in SEM images) that resulted in increased weight loss.

The tensile strength of polyethylene makes them a game changer in this world. High tensile with low weight is one of most important features that makes LDPE irreplaceable from our daily life. In this research, loss of tensile strength and decrease in mechanical properties have been seen with increasing time of incubation of LDPE pieces. This decrease in mechanical properties confirm loss of homogeneity of LDPE pieces due to the process of microbial biodegradation (Samanta et al., 2020). Study on biodegradation of LDPE given a mixed treatment of pro-oxidant, UV irradiation and subsequent biodegradation by *Aspergillus oryzae* resulted in 62 and 51% decrease in break at elongation % and tensile strength (Lakshmi Narasu et al., 2011). In a study carried out on *Klebsiella pneumoniae***,** the bacterium was capable of degrading HDPE plastic pieces by strongly adhering to the pieces resulting in significant weight loss and 60% decrease in tensile strength of the plastic.

Lipase belongs to the group of enzymes that carry lipolytic activity and catalyze hydrolysis of fatty acids and glycerol. In the present study, GTL 3 strain isolated from bat guano was capable of producing lipase enzyme up day 30 of the degradation experiment, the production decrease as the incubation reached 90^{th} day. Many studies have reported production of lipase enzyme by bacteria during degradation of polyethylene (Kavitha & Bhuvaneswari, 2021). Lipase disrupts the electrical balance with PE and creates carbonyl groups with PE structure that results in CH_2 chain becoming hydrophilic, hence the process of biodegradation starts (Gajendiran et al., 2017). Many studies have reported using *pseudomonas* sp. For biodegradation of

polyethylene because of its ability to produce lipase enzyme, along with serine hydrolase and esterase (Melki et al., 2020).

FTIR of the LDPE pieces in our study showed formation of new peaks suggesting chemical changes in LDPE. Biodegradation of polyethylene progresses by β-oxidation pathway and oxidative changes suggested by FTIR indicate towards biodegradation of the LDPE films. In our study, peaks formed at 2345, 3493, 1145, 2250 and 3560cm^{-1} shows formation of oxidative functional groups. Similar findings have been reported by Shah et al., while working on degradation of polyurethane using novel bacterium consortium (Ahmed et al., 2008). Peaks in these ranges attributes towards hydrolysis and oxidation of bonds in LDPE films, as demonstrated by Victorathisayam and Sujatha in their work biodegradation of high, low and linear density polyethylene (Victorathisayam & Sujatha, 2012). A shift of peaks to higher wave number, as has happened in our study, indicates that -OH (of alcohol), N=C (of isothiocyanate), and C=C (of alkene) were broken and the carboxylated compounds were formed (Hamed et al., 2021). All the evidence of reduced peak intensities and formation of new peaks points to the fact that our GTL 3 strain is utilizing LDPE as source of carbon, breaking the polymers into smaller pieces leading to generation of CO_2, H_2O and CH_4 as by products (Mohsen et al., 2010).

In the current study, SEM was carried out to observe physical damage incurred on LDPE films. The results showed significant physical damage to the plastic films. Structural changes on the surface of PE films such as grooves, cracks, damaged layer, pits and roughening of the surface is reported after 30, 60, and 90 days of incubation by Pripdeevech et al., while working with fungal species of Thailand (Pripdeevech et al., 2021). Significant physical abrasion observed through SEM has been reported in a study of plastic bottles biodegradation by marine bacteria (Bhowal et al., 2020). In this study, bacterial colonization on the surface of PE films is visible in SEM images, pointing towards positive microbe-plastic interaction. Addition of Tween 80, glucose and $CaCO_3$ showed facilitation and formation of biofilms in SEM images. SEM of Tween 80 and glucose showed extensive colonization of bacterial strains on rather smooth background of PE whereas calcium carbonate shows not only bacterial colonization but enhanced degradation of PE in the background as well. and Several studies have concluded that biodegradation of PE is associated with biofilm formation

(Sivan, 2011). Bacterial attachment with polyurethane films observed through SEM is reported by Gunawan et al., (Gunawan et al., 2020). SEM images also confirmed biofilm formation resulting in biodegradation of polyethylene in a successional community of PE mulching bacterial communities (P. Wang et al., 2022).

5.5 Conclusion

It can be concluded from the current study that GTL 3 strain isolated from bat guano samples of Kashmir cave has potential to biodegrade LDPE films. Bacterial growth, mechanical properties of treated LDPE and changes in chemical and physical structure of plastic indicated positive plastic-microbe interaction that resulted in degradation of PE. It can also be concluded from this research that extremely pristine environments like caves can harbor exciting microorganisms with unique, enhanced capabilities for potential biotechnological applications. Research should focus more on such environments to explore treasures of nature that has remained hidden from us since long.

6. Overall Conclusions

1. Bat guano of Kashmir cave is rich in carbon, nitrogen and phosphorus content along with considerable amounts of other minerals and heavy metals. Carbon content decreases with depth of guano whereas heavy metals accumulation was notable at the bottom layers

2. Out all the bacterial strains isolated from guano samples, 64.28% belonged to phylum Firmicutes, 35.7% to phylum Proteobacteria and 7.1% to Bacteroides

3. *Bacillus Subtilis* GTL4 isolated from top layer of guano samples was found to produce bacitracin like antimicrobial compound that was effective against gram positive as well gram negative ATCC test strains.

4. GCMS profile of the crude extract from *Bacillus subtilis* GTL4 showed production of wide variety of antitumor, antiinflammatory and antineoplastic compounds.

5. Most of isolates from guano samples were capable to resist commonly used classes of antibiotics. Genetic assessment of resistant genes revealed maximum resistance against Glycopeptides drugs (64%) whereas least resistance was observed against Fluroquinolones (45.23%)

6. *Alcaligenes faecalis* GTL3 strain isolated from guano top layer showed significant potential to carry out biodeterioration of LDPE. Medium augmentation with calcium carbonate resulted in stable bacterial growth and enhanced degradation effects.

7. Future Prospects

1. Metagenomic studies are to be conducted on cave bat guano isolates to determine the unculturable microbial diversity.
2. Future research should focus on metabolomics to unwind hidden metabolic genes that could provide more insight about various degradation pathways.
3. Studies on biogeochemical cycles should be conducted to better establish microbial niche of guano bacteria.
4. Cave rocks (speleothems) can be explored for isolation of microbes with potential biotechnological applications.
5. Potential for degradation of other organic pollutants (petrochemicals, rubber etc.) should also be examined.

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