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Biomineralization of calcium carbonates by bacterial strains isolated from Kashmir cave Buner.

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Biomineralization of calcium carbonates by bacterial strains isolated from Kashmir cave Buner

A Dissertation submitted to the Department of Microbiology, Quaid-i-Azam University, Islamabad, in partial fulfillment of the requirement for the degree of

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By

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In the name of Allah Talla who is merciful and compassionate.

Or have you thought that the companions of the cave and the inscription were, among our signs, a wonder? (surat Al-Kahf) (the cave) (18:8).

My humble effort is

Dedicated to

The Creator! The Great! The Soul!!

My Lord… Almighty Allah

Dedicated to The Paragon of Virtues…

The excellence! The guidance! The solace!!

My Prophet… Hazrat Muhammad (Peace Be Upon Him)

Dedicated to

My loving mour ao plaar, Martyred students of APS and Malala Yousafzai

DECLARATION

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

Saeed Ullah Jan.

CERTIFICATE

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

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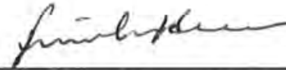

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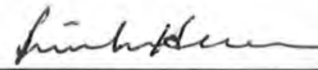

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ABBREVIATIONS

CFU	Colony forming unit
°C	Centigrade
%	Percentage
G	Gram
NA	Nutrient agar
Ml	Micro litter
L	Liter
YR	Yellow red
RY	Red yellow
TSI	Triple sugar iron
DNA	Deoxyribonucleic acid
CTAB	Cetyltrimethyl ammonium bromide
TE	Tris EDTA
FTIR	Fourier transform infrared
XRD	X- ray diffraction
SEM	Scanning electron microscopy
UBB	Urea broth base
TBE	Tris borate EDTA
NCBI	National Center for Biotechnology Information
UV	Ultra violet

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SAEED ULLAH JAN

Abstract

Caves are underground natural compartments and hosts diverse microbial communities. To explore bacterial strains performing vital role in biomineralization of calcium carbonates in cave environments. We screened 25 soil bacteria already isolated by Kashmir cave located in Buner, KPK in order check their potential for $CaCO_3$ precipitation. Serial dilution was performed and total colonies count were 4.6 x 10^4 CFU per gram of cave soil. Out of 25, four different positive bacterial strains (GSN-11, TFSN-14, TFSN-14 and GSN-22) were selected and having potential for calcium carbonate precipitation on B4 medium. Microscopically two strains were found to be Gram positive and the rest of two were Gram negative. All these four strains were characterized microscopically, biochemically and genetically through 16S r RNA sequence analysis. The strains were identified that GSN-11 is *Bacillus toyonensis*,TFSN-14 *Paracoccus carotinifaciens*,TFSN-15 *Brevundimonas naejangsanensis* and an unidentified strain named as GSN-22.different parameters, such as temperature, incubation time and pH were optimized to check the growth of calcifying bacteria and as well as precipitation of calcium carbonates. The highest number of calcifying bacteria was found at both 25°C but also good growth at 35°Cand best precipitates were produced at 25 °C with optimum pH 5. Compound microscopic slides of crystals were prepared produced by different strains exhibited different morphologic structures. Atomic absorption analysis of the soil sample had highest concentration of calcium 1615.35 mg/kg where as sodium, magnesium, potassium, iron, manganese, zinc, nickel, chromium and lead were also present. X-ray diffraction analysis (XRD) was performed which confirmed the polymorphs of calcium carbonate. Examination of crystals by scanning electron microscopy (SEM) technique was carried out at different magnification levels to view the structure of calcium carbonate precipitates. Fourier transform infrared spectroscopy (FTIR) analysis of calcium carbonate precipitated by bacterial strains was performed and analyzed in according to the control both had similar peaks in same region which in turn confirmed that the crystals were of calcium carbonate.

INTRODUCTION

Introduction

A cave is an immense space inside Earth where organisms can penetrate thoroughly up to twilight zone (Gillieson *et al.*, 1996). Cave study is known as Speleology, while in some part of the world like USA and Canada the cave study is also known as spelunking, caving and potholing. And the expert of this study is called speleologist. Caves are found everywhere in world, some well-known examples of caves in USA is Mammoth cave, Vietnam (Son Doong Cave), Pakistan (Pir Ghaib Garrha), Oman (Majlis al Jinn cave), New Zealand (Waitomo Cave) etc. On broad level caves are categorized in two types, terrestrial and sea caves and varies in length. Terrestrial caves range from few miles up to 390 miles in length (Gulden *et al.*, 2012), while sea caves varies in length from 5 to 340 m (Sjoberg, Rabbe *et al.*, 1988). In 2013 the world's longest sea cave has been explored situated in New Zealand named as Matainaka Cave which is ~1500 m long (Barth *et al.*, 2013).

To explore different cave microenvironments, cave researchers should go inside and check all parameters of cave (Kambesis *et al.*, 2007). On the other hand, the anthropological exploration of caves has the possibility to affect microbial diversity and also by introducing different sources of nutrients (Northup *et al.*, 1997; Lavoie and Northup, 2005; Ikner *et al.*, 2007).

Cave study started in 17th century, but later, before the mid of nineteenth century, the research value of cave environment was focused in its contribution to many other studies of science, and research of cave was thought as a part of the other branches of science including geology, archaeology, chemistry, biology and geography. Before the cave research by Édouard-Alfred Martel, very little research was conducted in speleology and Édouard-Alfred Martel is known as father of cave microbiology. So in this regard Martel in 1895 introduced an organization with name of "Société de Spéléologie". Inner environment of cave is entirely different from outside environment. A cave can be divided into three zones; entrance zone starting from opening of cave up to few meters receiving light, followed by twilight zone which is middle of cave and the last zone is dark zone where light cannot penetrate. Caves are deficient in organic nutrients, have high humidity, constant low temperature and significant amount of minerals are regarded as extreme environment for living organisms and caves also have biofilms of highly adaptive microorganisms

(Schabereiter, Gurtner *et al.*, 2003), and a great number of microbes are extremophiles (Rothschild and Mancinelli, 2001; Thomas and Dieckmann *et al* ., 2002). Running water in caves, human being and cave dwelling animals can be responsible for organic matters that help in existence of heterotrophic microorganisms in caves (Groth and Saiz-Jimenez, 1999; Groth *et al.*, 1999, 2001).

Speleogenesis is the process of cave formation and depends on different climatic and hydrological parameters of the region in which a cave is present (Engel, 2010; Palmer and Palmer *et al.*, 2009) and it is also affected by open-air environmental process like melting of snow or rainfall. Cave dissolution is fairly because of weak acid like carbonic acid (Palmer and Palmer *et al.*, 2009).

In contrast to open air environment, caves have a very constant internal environment (Poulson and White, 1969; Culver *et al.*, 1982). Furthermore, continuous darkness, and temperature inside the

Cave is nearly equal to the mean annual exterior temperature and the movement of the air is towards saturation (Gilbert et al., 1994). When cave researchers are exploring a cave, they may confront an environment totally changed as compared to open air environment, a place of complete darkness enclosed by rocks and soil. Mysterious formations, aquatic and waterfalls, narrow crawling routes, subterranean, gigantic rooms with large excretory materials of crickets, bats and cave rats expect the cave explorer. Caves are explored everywhere in the world and in diverse locations, they may be found in cold alpine environments to warm tropical rain forests, and formation of caves take place through a variety of natural processes.

Inside caves different environments are colonized by different microorganisms like bacteria, fungi, algae and protozoans. Bacteria are found in cave water, soil surfaces and sub surfaces, on walls and even also present in guano. Beside these, cave microbes explored to live with troglobiotic cave animals as parasites and epibionts (Golemansky and Bonnet *et al.*, 1994). Human beings going inside caves or direct interaction between bats or bats guano or cave explorers may have greater chance of pathogen transmission with environment (Li *et al.*, 2010). Research has investigated that tourists and cave researchers who deals with bats, guano and cave explorers are always exposed to numerous cave pathogens (Jurado *et al.*, 2010).

Microbial diversity of cave

Abundantly inside cave, chemolithoautotrophic bacteria are found and are dependent on the energy produced by different elements of soil. In cave, there are two types of bacteria, first group of bacteria is epigenic bacteria which are found inside caves due to anthropogenic activities of human beings as well as other different animals, and other group of bacteria is that which are permanently living in caves.

Inside cave, bacteria can be found in many microenvironments (Megušar and Sket *et al.*, 1977).Gram positive bacteria are most abundantly found in caves including actinobacteria are Bacillus, Arthrobacter, Brevibacterium, Rhodococcus Streptomyces, etc. (Groth *et al.*, 1999). Whereas, gram-negative bacteria have also been reported in caves, like, Xanthomonas, Serratia, Pseudomonas and Acinetobacter in all cave environments.

Few species of the pathogenic genus *Gordonia* like; *G. bronchialis*, *G. otitidis* reported from Grotta dei Cervi cave Italy, are regarded as opportunistic pathogens and are causing diseases of bacteremia and bronchopulmonary (Iida *et al.*, 2005; Blanc *et al.*, 2007). Inside caves, numerous other disease causing bacterial strains have been isolated, such as; *Alcaligenes*, *Escherichia coli*, *Pseudomonas*, *Staphylococcus aureus* and *Sphingomonas* (Bastian *et al.*, 2009).

The presence of *Bacillus* species, *S. aureus* and *E. coli* in caves indicate a clue of human interaction with caves (Ikner *et al.*, 2007). Other than bacteria, fungi are also found in entrance areas of caves but not in deep areas because fungi need great amount of organic matter (Dickson and Kirk *et al.*, 1976). They are seen covering many spaces and manure of cave dwelling animals that rarely go outside cave. In addition to this, fungi may live as mutualist with other microorganisms, while exploring the fungus interaction with cave dwelling animals, zygomycota and some hyphomycetes on dead body of cricket Troglophilus neglectus are also found (Zalar *et al.*, 1997; Gunde Cimerman *et al.*, 1998). Some fungi like; *Aspergillus* sp. and *Penicillium* sp. were investigated in weathered limestone (Megušar and Sket *et al.*, 1977).

Pathogenic fungi, *Histoplasma capsulatum* are also reported in cave soil colonized by bats causing pulmonary histoplasmosis disease in cave researchers (Nieves-Rivera *et*

al., 2009). Microalgae and cyanobacteria have been isolated from cave water (Kuehn *et al.*, 1992; Sanchez *et al.*, 2002) and other photosynthetic microorganisms can be found in entrance zone but are not explored in dark zones because there light cannot penetrate.

Majority of minerals deposit in caves consist of calcium carbonate. Calcite is found in great amount while gypsum and aragonite can also be found in every cave, calcite is the major component of speleothems and after calcite, Aragonite is another cave mineral commonly found in caves (Hill and Forti *et al.*, 1997). Whereas Gypsum can also be found as a common cave mineral and it is always white in colorless. The rest of nearly 172 minerals are found in trace amount. Other valuable easily extractable mineral assets from caves are marble, gypsum, dolomite, salt and limestones all are found in great number in entire world.

Shoji and Folk for the first time said that microorganisms may have an important role in carbonate precipitation (Shoji *et al.*, 1964). Bio mineralization of elemental calcium carbonate is a common biological process carried out by cave microbes under suitable optimum parameters like pH, temperature, incubation time and nutritive medium (Boquet *et al.*, 1973). Whereas some soil dwelling bacteria and few species of fungus may also produce crystals of calcium carbonate by mean of different bio-chemical reactions like ammonification, denitrification, photosynthesis, anaerobic oxidation of sulphide (Castanier *et al.*, 2000; Riding *et al.*, 2000). Calcium carbonate precipitation is a biological as well as a geochemical method of producing calcium carbonate precipitation with help of microorganisms specially those residing inside cave soil (Mortensen *et al.*, 2011). This biological process carry out mineral precipitation, which holds the different soil particles packed in one structure and may also enhance the stiffness ability of the soil. Cave microbes can enzymatically carry out the biological reactions to crystalize calcium carbonate in soil (Fujita *et al.*, 2000). Soil residing bacteria can boost up calcium carbonate precipitation by promoting the alkaline conditions in cave soil (Kohnhauser *et al.*, 2007), this is achieved by many bio-geochemical processes like nitrate, sulfate, iron reduction, and break down of urea (DeJong *et al.*, 2010; Van Paassen *et al.*, 2010).

Applications

Cave bacteria ensure dissolution and precipitation reactions that comprise carbonates, clays, silicates, manganese, iron, sulfur, and formation of speleothems (Northup and Lavoie *et al.*, 2001). Cave environment comprises 1–2% methane (CH_4), while considerable ratio of methane is present in bubbles which help microorganism's colonies to flow along with water, hence methane producing microbes transform CH_4 into high molecular weight organic compounds and keeps a balanced complex community of microbes in cave environment (Hutchens, Elena *et al.*, 2004). Bats' guano in cave is a rich source of phosphate and was used as natural fertilizer in the 19th and early 20th centuries, to enhance soil fertility (Abel and Kyrle, 1931; Badino *et al.*, 2004; Onac *et al.*, 2007). Nowaday's guano has been substituted by chemical fertilizers. In some very harsh and nutrient deficient caves, the only energy source for microorganisms is guano of bats, crickets, or other different birds (Poulson, 1972; Gnaspini-Netto, 1989; Herrera, 1995; Ferreira, 1998; Ferreira and Martins, 1998; Ferreira *et al.*, 2000). While in some poor countries in Southeast Asia, people catch bats for food purposes (Price, 2007*b*). In recent times, the speleothems from "Atacama cave", provide salt to whole state (De Waele *et al.*, 2009). Caves are also important related to medicinal study, European surgeons were using moon milk from caves for dressing wounds. Because moon milk functions as a dehydrating agent and it also helps in blood stopping from wounds. Cave experts have also found that sulfur bacteria residing inside caves are also producing nutritive vitamins. Caves have a constant temperature throughout the year therefore human beings used caves to store different food items like wine, apples and potatoes. Caves were also used to grow mushrooms. Moreover, the blue penicillin mold and cheese improvement take place only in a wet and cool environment like that of cave (Gee *et al.*, 1994). Some cave microbes are also producing different useful byproducts such as enzymes, antibiotics that work under extreme conditions. (Van den Burg *et al.*, 2003). *Streptomyces tendae* explored in Italy from Grotta dei cervi cave is producing a new antibiotic cervimycin that helps to fight multi-drug resistant pathogens (Herold *et al.*, 2005). Some species of genus *Gordonia* are playing an important role in biotechnology because they carry out biodegradation and bioremediation processes, and some species like *G. nitida* desulphurate fuels (Lee *et al.*, 2005). Cave microbes are involved in degradation of

the earliest, primitive paintings within Altamira cave of Spain (Schabereiter-Gurtner *et al.*, 2002).

AIMS AND OBJECTIVES

Aims and Objectives

- Aim of the study is "Biomineralization of calcium carbonates by bacterial strains isolated from Kashmir cave".
- To study the role of cave bacteria in calcium carbonate formation.
- To check the effect of temperature, pH, incubation period on calcium carbonate formation by these isolates.
- To characterize all the isolates biochemically and through molecular identification.
- To perform Elemental analysis of cave soil by atomic absorption spectroscopy.
- To analyze the crystals formed by the isolates with FTIR, XRD and SEM techniques.

LITERATURE REVIEW

Literature review

Caves are hollow spaces beneath the ground that are accessible to all forms of life, but exceptions are there some caves are not easy to access. Internal parameters inside cave are completely variable from open air environment. Caves are biospheres of complete darkness enclosed by clay and rocks, watery and waterfalls having narrow crawling routes, massive rooms with enormous breakdowns of bats, crickets, and cave rats. Caves are found in each part of the world such as, United States, Italy, Australia, France, Pakistan etc. But only a small portion of them have been investigated. Microbial study of cave is known as Bio Speleology, and the expert of this study is said to be biospeleologist. Speleology reshapes other cross-disciplinary fields that combine the knowledge of biology, chemistry and geology.

Cave yields an important niche for many unique microorganisms. Cave biology is very diverse and is completely distinct from surface habitats. Generally the deeper the cave becomes the more complex is the ecology.

Microorganisms are found living in all microenvironments of caves so in this case cave microorganisms range from bacterial groups such as Actinobacteria, Proteobacteria, and Firmicutes to yeasts and some fungus species in entrance zone(Barton and Jurado.,2007).Caves are deprived of natural light, oligotrophic conditions, having low temperature and high humidity, by the way changes in these microclimatic parameters like temperature, humidity and cave microbial diversity and abundance are linked with human access. Changes in these conditions can lead to damage of cave features (Russell and MacLean., 2008).

Cave microorganism's lives in nutrient limited environments and are metabolically adaptable obtaining energy from different cave compounds, gases and by oxidizing metals upon rocks. By mean of these pathways cave microorganisms perform vital role in cave biogeochemical cycle and in the formation of cave features (Barton and Jurado, 2007). This delicate microbial diversity and parameters can be disturbed by regular anthropogenic activities with hostile effects on the cave environment (Bastian *et al.*, 2010). Lot of scientific research of speleology has been concentrated on cave walls harboring abundant information on microbial diversity and function. (Gurtner *et al.*, 2000; Portillo *et al.*, 2009*b*; Stomeo *et al.*, 2009; Martinez and Asencio, 2010;

Pašić *et al.*, 2010). That is why majority of microbial activities are supposed to take place on caves rock surfaces and maximum caves are lacking of significant soil or sediment layer.

History of caves used by human

Cave study started in 17th century but up to mid of nineteenth century bio speleology was thought to be a part of other larger subjects like geography, geology and archaeology. The most basic work was written in 1654 by a Parisian Jacques Gaffarel so his creation was extinct with passage of time. Cave microbiology was ignored prior to the research of Edouard Alferd Martel and later on in 1895 he established the first organization of cave science named as Societe of Speleologie. Up to late 19th century Austria and Vienna was considered as center of scientific study and karst study was a vital topic (Herak and Stringfield *et al.*, 1972). Martel took France to the front in karst study with Austria where it has remained since.

Humans are used to caves since stone ages and use them for different purposes. Caves were being used as shelter for religious people and bats. They were also used as temples as well as burial sites, and other used caves as source for minerals and economic prosperity. Low temperature and world of darkness inside cave considered to be optimum environment for fermenting items. Pulmonary patients were using caves for treatment of respiratory diseases. Bats guano was used as fertilizer, recently, which is replaced by chemical fertilizers. In early time people had the advantage to store different food items in humid and cold environment of cave for long period of time.

Types and Speleogenesis of caves

Speleogenesis is a slow process it takes a very long time. Caves vary in shape, size and length. They are found in each part of world. On broader level Caves are categorized in two types, terrestrial and sea caves. Terrestrial caves ranges from few miles up to 390 miles in length (Bob Gulden *et al.*, 2012), while sea caves varies in length from 5 to 340 m (Sjoberg and Rabbe *et al.*, 1988). Caves are formed by various geologic ways. These geologic ways may include a mixture of pure chemical pathways, erosion from water, atmospheric influences, tectonic forces, microbial

process and pressure. Majority of caves are formed in limestone by dissolution process called limestone caves.

Dissolutional caves are the most abundant caves and these are found in soluble rocks like lime stone, but they can also be produced in other different rocks such as dolomite, chalk, marble, salt, and gypsum. The rock is dissolved by mean of weak acids (H_2CO_3) that is naturally found in soil, with passage of time a crack is produced in rock which is then broadens and at the end cave is generated (John Burcham *et al.*,2009).

Dissolutional caves are also formed by H_2S (hydrogen sulfide) gas rising from underneath, where deposits of oil releases off sulfurous vapors. This gas combines with soil water and producing H_2SO_4 (sulfuric acid). The acid then softens the limestone from beneath, giving a crack which converts in cave (Davis, 1980).

$$CO_2 + H_2O \longrightarrow H_2CO_3$$

$$H_2S + H_2O \longrightarrow H_2SO_4$$

Ice cave is a type of cave, which is found in glaciers by the melting of ice, and when the melted water flows down within and under glaciers, which grind the ice as result, ice cave is generated.

Lava cave is a type of cave also termed as lava tube, which is formed whenever the lava burst from volcano producing a tube, which then cools down and broadens giving a cave like cavity.

Sea Cave Sea caves are originated along seacoasts everywhere in the world. In order to form a sea cave, the host rock must first contain a weak zone. Which are formed by wave action in zones of weakness in sea cliffs. These are formed by combined action of waves, tides and rain drops which delicate the rock (Barth N *et al.*, 2013).

Sandstone caves these are small caves, which are generated at cliffs, cut up by water. The flow of water deteriorates the sand particles which are immovable firmly attached

with each other and then the current of water takes away these particles from each other and caves are formed.

Cave habitat

Caves provide a diverse ecological niche to different organisms. In caves diverse microscopic organism like bacteria, fungi, algae and protozoa colonizes different environments. Microbes are frequently found in water, rocks, on residues and in bats guano. An additional vital group of microorganisms are those which establish some type of interaction like epibionts and parasites with troglobiotic animals (Golemansky and Bonnet, 1994). These all organisms are classified as terrestrial and aquatic cave organisms.

Aquatic habitat: Organisms that live in a cave located in aquatic area are Georgia Blind Salamander, Cave Crayfish, and Cave Amphipod etc. These caves have psychrophilic temperature and high pressure. Animals living in these caves are adapted to these extreme parameters.

Terrestrial habitat: The organisms that are living in terrestrial environments are referred to as terrestrial animals. These are organisms that live on dry land areas such as cave spiders, raccoons, salamanders and bats etc.

Caves are oligotrophic in nutrient point of view if compared with open-air environment. So nutrients are imported from external environment. These nutrients are carried in caves by means of walking animals, water currents, gravity and by circulating air. In addition to these when animals dies inside caves they also serve as source of food for microorganisms. Majority of microbes inside caves are chemolithotrophes. They depend upon the inorganic elements present in soil sediments and they adapt themselves to oligotrophic conditions.

A cave habitat can also be categorized upon natural sun light penetration. A cave can be divided into three zones, entrance zone starts from opening of cave up to few meters receiving light, here the microbial diversity closely resembles to the outer environment is followed by twilight zone which is the middle of cave. In this zone mostly walking animals like bats, cave snake and cave spiders are found. And the last zone is dark zone where light cannot penetrate.

Cave organisms are further divided on the basis of life cycle in caves. Some animals stay in cave for short duration, while others may live for longer time and the rest of animals pass their whole life in caves. Organisms which live on dry land are termed as trogloxenes and organisms which lives in sea caves are referred as stygoxenes.

Trogloxenes and stygoxenes: Trogloxenes are also termed as cave guests, because they visit caves for very short period of time and are living in caves opening or entrance zone. They come out in search of food and for reproduction. Examples of trogloxenes are rats, raccoons, cave crickets, Bears, and extinct animals like cave bears, cave lions, cave leopards, and cave hyenas. Stygoxenes include salamanders, mouth catfishes, swamp eels. And about 170 species of Stygoxenes fish are investigated in all continents (Rantin *et al.*, 2013).

Troglophiles and stygophiles: These are tiny cave dwelling animals that have adapted their life in dark environments. These organisms use caves as shelters, hibernation, safety and reproduction. They pass their complete life cycle in side caves and also come out for very short time such as beetles, millipedes, segmented worms, daddy longlegs (Phil Chapman, 1982). Stygophiles populate both surface and underground marine environments, but are not essentially limited to one environment. (Thomas *et al.*, 1967). Examples are hill stream loaches, heptapterid catfishes, characids etc.

Troglobites and stygobites: These animals only lives in caves and they complete their whole life cycle in caves and have adapted to cave environment. They are obligate or strictly subterranean (Rubens *et al.*, 1999). True troglobites have physical adaptations to caves environment like reduced or no vision, extended appendages and loss of pigment (no color). Snails, Blind cave fish, Kauai Cave Wolf Spider are troglobites. Blind Texas salamander, blind flatworms are stygobites.

Most bats are nocturnal. And caves provide optimum environment to bats. They fly and forage for their food at night. This means that they need safe places to sleep during the day. Caves provide the kind of protected shelter in which bats can thrive. Hanging from the ceiling of a cave, some of the most successful species of bats live in large cave colonies. Some of these colonies have millions of members, even up to 20 million. Carlsbad cave in New Mexico once had 7-8 million bats.

Microorganisms in caves

Cave microorganisms range from bacterial groups such as Proteobacteria (Alphaproteobacteria, Betaproteobacteria, Gammproteobacteria), Actinobacteria, and Firmicutes to yeasts and saprophytic fungus such as *Penicillium, Fusarium* and *Trichurus* (Barton and Jurado, 2007; Zhou *et al.*, 2007; Bastian *et al.*, 2009; Portillo *et al.*, 2009a; Jurado *et al.*, 2010; Adetutu *et al.*, 2011; Vaughan *et al.*, 2011). These microbial communities are affected by the geological nature of the caves, prevailing environmental conditions, soil or sediment factors and cave factors (Ikner *et al.*, 2007; Shapiro and Pringle, 2010; Adetutu *et al.*, 2011).

Gram positive bacteria are most abundantly found in caves including actinobacteria are *Bacillus, Arthrobacter, Brevibacterium, Rhodococcus Streptomyces*, etc (Groth *et al.*, 1999). Whereas gram-negative bacteria are also explored in cave like *Xanthomonas, Serratia, Pseudomonas* and *Acinetobacter* in all cave environments.

Inside caves numerous other disease causing bacterial strains are investigated, *Alcaligenes, Escherichia coli, Pseudomonas,Staphylococcus aureus* and *Sphingomonas* (Bastian *et al.*, 2009). Many cyanobacter are capable of producing lipopolysaccharides that irritate the skin, a species of Lyngbya is responsible for one of the skin irritations commonly known as "swimmer's itch."

Some Nitrifying bacteria are also investigated in cave environments such as *Nitrosomonas* or *Nitrobacter* which has a role in Nitrocalcite formation (Faust 1949, 1967, 1968, Hill 1981a, b. Hill, Eller, Fliermans, and Hauer, 1983. Fliermans and Schmidt *et al.*, 1977). and also oxidizes ammonia to nitrous and nitric acid that can dissolve limestone. Other bacteria that have been found in caves include the Gram-negative aerobic *Clostridium* and *Azobacter* that are able to obtain nitrogen from the air and convert it into nitrogen compounds. Relatively few organisms can 'fix' nitrogen gas in this way and all of them are bacteria.

Leptospirosis for cavers the most important heterotroph is the *spirochaete* bacterium *Leptospira interrogans* that causes Weil's disease. Megušar and Sket, 1977 identified *penicillium* sp. and *Aspergillus* sp. and in weathered limestone Mulec et al, (2002) isolated fungus belonging to Cladosporium herbarum group.

Cave System Energetics

In the dark zone of a cave bacteria cannot use the sun energy to convert carbon dioxide to organic compounds, and so they use a variety of other metabolic pathways. These bacteria that make their own food by using chemicals and minerals as their source of energy and so are called chemoautotrophic bacteria. They obtain energy by the oxidation or reduction of simple inorganic substances (e.g. iron, sulphur, nitrogen). They only need simple inorganic elements to survive e.g. iron or sulphur, moisture, carbon dioxide and a few trace elements to survive (J. R. Spear *et al.*, 2001). They do not need sunlight like green plants. In British mines and caves *Ferrobacillus ferrooxidans* uses carbon dioxide as its carbon source and oxidizes iron to obtain energy. Sulphur reducing and oxidizing bacteria are usually found near shale rock containing pyrite. Because caves are geologically stable structures, it is possible to identify, measure, and model how nutrients and other energy sources enter them. We believe that there are three major routes for energy to enter these subterranean systems and support microbial growth. (i) as atmospheric gases such as nitrogen, CO_2, and organic molecules, including methyl halides and aromatic hydrocarbons; (ii) as soil derived aromatic and polyaromatic compounds percolating into the system with surface water. and (iii) as reduced metal ions, such as Mn(II) and Fe(II) within the rock itself. Other microbial species that we find in caves can mobilize inorganic phosphate, oxidize methane and hydrogen, and derive energy by hydrolyzing proteins, lipids, and other macromolecules that are released by other members within the microbial community, allowing recycling of those macromolecules (Barton, H. A., *et al.*, 2006).

Importance of microorganisms in calcium carbonate precipitation

Microorganisms have been agents for geochemical change for over 85% of the earth's history, and linkages between the geochemical and biological evolution of the earth are profound. It is widely accepted that microorganisms were largely responsible for production of oxygen in the earth's atmosphere and that through their metabolism they can dramatically alter elemental distributions. Interactions between the biosphere and geosphere are complex because organisms are able to transform the chemistry of their environment. Calcium carbonate precipitation is a general phenomenon in the

bacterial world under appropriate conditions (Boquet E *et al.,* 1973). Indeed, some bacteria and fungi can induce precipitation of calcium carbonate extracellularly through a number of processes that include photosynthesis, ammonification, denitrification, sulphate reduction and anaerobic sulphide oxidation (Castanier and Riding *et al.,* 2000). And Additionally, the activity of sulphate reducing bacteria has been shown to mediate precipitation of dolomite (Vasconcelos *et al.,* 1995 and Warthmann *et al.,*2000). The primary role of bacteria in the precipitation process has been ascribed to their ability to create an alkaline environment through various physiological activities. (Castanier *et al.,*1999 and Douglas *et al.,*1998). In addition to field observations, calcium carbonate has been formed in the laboratory in association with different bacterial cultures, such as marine bacteria, soil bacteria, *Pseudomonas flurescens, Myxococcus xanthus,* and various other autotrophic and heterotrophic bacteria (Castanier *et al.,*1999).

Caves host diverse microbial populations and are sites of active mineral precipitation. Chemical processes mediated by microbial activity are fundamentally related to the distribution of microbes throughout the cave system. Mineral precipitation is, however, commonly considered to be abiogenic despite the fact that microbes are present in caves. Analysis of cave substrates from a geological perspective shows that microbes can mediate constructive and destructive processes. Microorganisms have been shown to be important active and passive promoters of redox reactions influencing geological processes. Potentially these processes can significantly influence the formation and preservation of any cave deposit. Although there is extensive documentation of microbial precipitation of calcium carbonate in the noncave carbonates literature (Ehrlich *et al.,* 1996). Studies of microorganisms in caves have been predominantly descriptive, with only a few experimental studies reported. The past decade has produced extensive research into microbial interactions with minerals in cave environments. Fungi, algae and bacteria are implicated in the precipitation of carbonate dripstone in caves (Danielli *et al.,* 1983 and Cox *et al.,* 1989). A large variety of heterotrophic microbial communities in stalactites are well documented in cave ecosystems (Laiz, L *et al.,* 2000). Monitoring modern sites of active precipitation can provide valuable insights into the factors that control precipitation and evolution of various cave deposits (Borsata *et al.,* 2000). Various microbiological techniques have also been used to show that microbes are present in

most cave environments and modify the composition of fluids and or influence precipitation of various minerals, including calcite (Northup *et al.*, 1997).

Speleothems and Minerals

Cave Minerals of the World (Hill and Forti, 1997) reviews the many types of speleothems and minerals found in caves. Spelothems are secondary mineral deposits formed by a physicochemical reaction from a primary mineral in a cave (Moore, 1952). Hill and Forti recognized 38 speleothem types, with numerous subtypes and varieties, A particular speleothem can be composed of many different minerals or unconsolidated materials.

Moonmilk

Moonmilk, also known as "Mondmilch" and by a wide variety of other names (Bernasconi 1981and Reinbacher 1994) describes a range of microcrystalline mineral aggregates with a physical appearance ranging from a very soft paste to cottage cheese that dry to the consistency of talcum powder.

How the precipitates are formed in caves?

Microorganisms have been shown to be important and have influence in geological formations (Ehrlich, 1996). Bacteria and fungi can precipitate calcium carbonate extracelularly through a variety of processes that include photosynthesis, ammonification, denitrification, sulfate reduction, and anaerobic sulfide oxidation (Simkiss and Wilbur 1989). These processes increase the concentration of H_2CO_3, which helps in speleogenesis. An initial step in the precipitation of carbonate involves the attraction of Ca^+ and Mg^+ positive ions to negatively charged cell surfaces molecules. The bacterial cell acts as a nucleation site, a critical stage in mineral precipitation, Later on microbes produce extracellular polymeric substances (EPS), which trap sediments and hence precipitates the minerals.

Application of cave microorganisms

Cave microbes produce different by-products that are helpful in biotechnology and industry point of view, such as pectinase isolated by *Sclerotinia borealis,* which is used in Cheese ripening and wine industry (Takasawa T et al., 1997). Enzyme such as β-Lactamase isolated from *Psychrobacter immobilis,* a cave soil bacterium acts as antibiotic agent (Feller G *et al.*,1997). Ice nucleation proteins are extracted from glacier cave microbes having applications in food industry and synthetic snow (Sheridan PP *et al.*,2000). Caves are considered as extreme environment due to the absence of light and nutrient deficiency (Leveillé and Datta, 2010). Cave environments are believed one of the most favorable sources of useful compounds. Numerous studies have focused on secondary metabolites produced by microbes that live in such habitats as potential sources of useful mixtures. extremozymes (Singh *et al.*, 2011) bio surfactants (Banat *et al.*, 2010) exopolysaccharides (Nicolaus *et al.*, 2010) antitumor agents (Chang *et al.*, 2011) radiation protecting drugs (Singh and Gabani., 2011) antibiotics, immunosuppressants, and statins (Harvey, 2000). Microbial biofilms in caves are recently relatively uninvestigated microbiomes, which may prove to hold novel biodiversity. The parvom of cave mat dwelling microbes can be a promising natural source of novel antimicrobial agents that could offer truly new platforms for therapeutic antibiotics. Natural product discovery is not an easy effort, however, and well-thought approaches are needed in order to increase the chances for success.

Caves in all over the world are rich in resources, a total of 175 different minerals occur in limestone caves, majority of them have been investigated in caves (Moore and Sullivan, 1997). Caves provide a unique subsurface environment for endangered animals. Caves preserve fragile archaeological and paleontological materials for long times. Throughout history people have used caves for many purposes from guano mining to tourism. The potential of cave environments as natural laboratories could be their most vital future use.

Most mineral deposits in caves are made of calcium carbonate ($CaCO_3$). And common cave mineral is calcite. Aragonite and gypsum are also found. The other 172 cave minerals are much less common than the primary three calcite, aragonite, and gypsum an interesting narrative is that more than 40 of the known cave minerals

contain phosphorus. The phosphorus in these minerals is derived from bat excretory material called guano. Bat guano was the most highly used fertilizer in the 19th and early 20th centuries. More recently, guano has been replaced by cheaper and more easily obtained chemical fertilizers. Other valuable minerals extracted from the caves are quarried rock, Limestone, dolomite, marble, gypsum, travertine, and salt are all mined in large quantities throughout the world (Huff *et al.,* 1940).

Due to constant temperature of cave internal environment, humans have used caves to store food items for example potatoes and apples. Caves have also served as subsurface farms for mushrooms, rhubarb, and celery. People have also used caves to store cheese during the aging process. Some of the stronger varieties of cheese must be cured as long as two years to produce their characteristic sharp flavors, dry textures. Furthermore, the blue *penicillin mold* in Roquefort cheese develops only in a cool, wet atmosphere such as a cave. Not surprisingly, then, penicillin was originally developed in the caverns below Roquefort, France (Gee, 1994). In the 1700s, French immigrants were the first to use American caves for storing and aging wine. Later, German immigrants found caves especially suited for aging lager beers, which require storage at low temperatures for several months.

Tourism in cave and karst areas is big business. In the United State alone, more than 150 caves are open to the public, and several million visitors pass through each year (Moore and Sullivan, 1997). Many of these caves are skillfully lighted and host well maintained trails.

Caves also provide fascinating opportunities for therapeutic investigation. Some early uses of cave materials as medicines have proven to be scientifically valid. In the 16th and 17th centuries, for example, European physicians used dried moonmilk from caves for dressing wounds. The moonmilk acted as a dehydrating agent and stopped bleeding (Shaw, 1997). It was also thought to have curative qualities. Now we know that moonmilk contains *actinomycetes,* mold-like bacteria that have antibiotic properties. Scientists have also discovered that sulfur bacteria found in caves produce vitamins of the B group, an important area of research that could supply useful nutritional and metabolic products. Only continued research and time will show that what miracle drugs will be developed from cave resources and cave microbe after exploration of unseen wealth.

Screening of calcium carbonate precipitating microbes

Microorganisms have been agents for geochemical change for over 85% of the earth's history, and linkages between the geochemical and biological evolution of the earth are profound. Fungi and bacteria have implicated in the precipitation of carbonate mineralization in caves (Danielli and Edington, 1983).

Different chemolithotrophic microorganisms are investigated to do mineralization of calcium, iron, sulfur, and manganese (Jones and Motyka, 1987).

Bacillus is a gram positive, rod shape bacteria mostly present in soil sediments of caves and are the most common bacteria found among the calcifying isolates. *Bacillus thuringiensis* and *Bacillus pumilis* mediate the precipitation of calcite under well-defined conditions. Microorganisms are active in a wide range of mineralization processes and have been involved in the deposition of minerals throughout the history of the Earth (Ehrlich 1990 and Chafetz, 1994). Bacteria from soils, freshwater, and saline habitats have frequently been reported to be able to precipitate calcium carbonate both in natural and in laboratory conditions (Boquet,1973; Rivadeneyra, 1993; Ferrer, 1988).

Some other bacterial species like *Rhodovulum steppense* and *Rhodovulum sp. S 17* isolated from Soda Lake having ability to precipitate $CaCO_3$ under controlled laboratory conditions. (Kompantseva *et al.*, 2010).

Arthrobacter, Bacillus firmus, Bacillus megaterium, Bacillus sphaericus and Brevibacillus brevis all these bacterial strains have the potential to carry out calcium precipitation at optimum parameters (Paola Cacchio *et al.*,2010).

Some other bacteria were also investigated in the Stiffe cave of Italy having role in calcifying $CaCO_3$ such as *Kingella sp. and Xanthomonas sp* (Claudia Ercole *et al.*, 2010).

MATERIALS AND METHODS

Materials and Methods

The below mentioned research work entitled as Bio mineralization of calcium carbonates by bacterial strains isolated from Kashmir cave Buner, Pakistan was carried out in Microbiology Research Laboratory (MRL),Department of Microbiology ,Quai-i-Azam University,Islamabad.

Fig 3.1: visit to Kashmir cave Buner

Enumeration of bacterial strains

Serial dilution technique was carried out, in which one gram of cave soil sample was suspended in 10 ml sterile normal saline and after proper dilutions, transfer of 100 µl of each dilution were spread on nutrient agar plates, then the plates were incubated at 37 °C for 24-48 hours. Different colonies were then marked with pointer and enumerated.

Viable counts (CFU/g) were calculated by using the formula;

CFU/g = Number of colonies/ volume of sample used × dilution factor.

Identification of bacterial strains

The positive isolates were identified by biochemical characteristics and 16S r RNA sequencing. Gram staining was also performed for the identification of organisms.

Gram staining

Smears of all four isolates were prepared on glass slides. The smears were air dried followed by heat fixation, crystal violet dye was applied to the smear for one minute. After one minute of contact time the primary dye was washed with tape water and Gram iodine was applied for one minute and was again washed with tape water. Ethyl alcohol was applied as decolorizer for few seconds and was washed with water.in the last Safranin as counter strain was used for one minute hence washed with water. The air dried slides were observed by microscope under 100X magnification applying immersion oil.

Biochemical characteristics

Isolates were biochemically characterized. Following biochemical tests were performed.

- Oxidase test
- Catalase test
- Citrate test
- Urease test
- TSI test
- Casein test
- Amylase test
- Gelatinase test

Oxidase test

Oxidase was mixed with distilled water and two to three drops of oxidase reagent was placed on a filter paper. Then with help of sterile loop culture was streaked on filter paper having oxidase reagent. After few seconds the positive isolates indicated blue color at the site of streak.

Catalase test

One drop of catalase reagent (H_2O_2) was place on clean glass slide. Then with help of loop wire a single colony of culture was transferred to the drop of H_2O_2. After 30 seconds the positive organisms produced bubbles.

Citrate test

Simmon's citrate agar slants were prepared and incubated for 24 to 36 hours at 37 °C after sterile inoculation. The citrate test detects the ability of an organism to use citrate as source of carbon and energy. The positive isolated indicated blue color.

Urease test

Urea broth base was autoclaved and 1gram of urea in 100 ml distilled water was mixed with UBB through syringe filter and dispense aseptically in sterile tubes. Cool the tubed medium followed by inoculation. And incubated at 37 °C for 72 hours. Pink color in test tubes indicated positive results.

Triple sugar iron

Triple sugar iron medium containing glucose, lactose, yeast extract and beef extract was prepared and autoclaved. Using aseptic technique in hood, poured medium in test tubes in order to make slants and butts. Stab the needle containing the pure culture into the medium, up to the butt of the test tube, and then streaked the needle back and forth along the surface of the slant and Incubated at 37 °C for 18 to 24 hours. If glucose fermentation has occurred. There will be red slants and yellow butts with or without gas production (breaks in the agar butt).while in case of Lactose or sucrose fermentation, both the slants and butts were red.

Casein test

Nutrient agar and casein was autoclaved and poured in plates, after that a small lawn was made upon the plated and incubated at 37 °C for 48 hours later on glacial acetic acid was applied and checked the results. The positive isolates gave clear zones around the lawn indicating digestion on casein protein.

Amylase test

Soluble amylase was autoclaved and poured into plates and took a single colony of culture with the help of loop and made a lawn on plate, incubated the plates at 37 °C for 48 hours followed by placing crystal iodine in the lid if plate. After few minutes the positive isolates indicated zones around the lawn. The test aimed to determine if the organism is capable of breaking down starch into maltose through the activity of the extracellular amylase enzyme.

Gelatinase test

2.5 gm, of gelatin was autoclaved and butts were made in test tubes, kept them in the refrigerator until just prior to inoculation for 5 minutes for solidification. Picked up a colony with straight loop and stabbed the butts to a depth of 2 inches along with a control. Incubated both the test tubes and control tube at 37 °C for 3 days. At the end of 3 days period placed both tubes (cultured tubes and control) in a refrigerator for about 1 hour to determine whether digestion of gelatin (liquefaction) has occurred, medium in liquid form indicated positive results.

Molecular identification of positive isolates

DNA extraction

The DNA extraction of bacteria was carried out by centrifugation of culture at maximum speed for 5 minutes. And the cells were pelleted out. The supernatant were discarded, cells were again suspended in 560 μL of TE buffer followed by addition of 30 μL of 10 % SDS and 3 uL of proteinase K and incubated at 37 °C for 1 hour.

Then 100 μL of phenol was added to these tubes hence vortexed for 60 sec, to lyse the cells. By removing the aqueous phase from organic phase, the samples were centrifuged at 13,000 rpm for 5 minutes. Then 1.5 ml tubes were taken and upper aqueous phase was placed in it. About 40 μL of TE buffer was added in tubes and also mixed 100 μL of chloroform centrifuged it for 5 min at 13,000 rpm. The aqueous DNA phase was transferred to a clean eppendroff tubes after each centrifugal phase separation. Phenol extracted DNA was then precipitated by the addition of 450 μL of isopropanol, mixed

thoroughly. DNA was pelleted by centrifugation at 14,000 rpm for 10 min at room temperature and supernatant was discarded. To precipitate DNA again, pellets were resuspended in 100 μL of TE buffer and 660 μL of 70 % ethanol was added mixed thoroughly and kept in refrigerator for overnight. After incubation, DNA was pelleted by spinning at 14,000 rpm for 10 min at 4 °C. Pellets were resuspended with 100 μL of TE buffer and kept at -20°C.

Preparation of the Agarose Gel

To check the presence of DNA, agarose 1.5 g was dissolved in 1 x TBE buffer and was heated in microwave oven for 60 seconds in order to dissolve the agarose completely. The agarose solution was allowed to cool and about 2 μL of ethidium bromide was added to the solution followed by well shaking for even mixing. For sample loading comb were placed in tray. And solution was poured into gel tray, allowed to solidify for 10 to 20 minutes. About 2 μL Loading dye was mixed with DNA samples followed by loading of samples into the wells. The agarose gel was run for 30 minutes at 110 V and 400mA and observed under UV.

Phylogenetic Analysis

After the DNA extraction by above mentioned protocol, amplification of 16S r RNA gene was amplified by PCR and Sequencing of samples was performed by Macrogen Commercial Seoul Korea, 16r RNA gene sequences of the positive isolates and most similar sequences from Gen Bank were identified through BLAST from NCBI. The alignments were analyzed thoroughly and corrected manually. The ambiguous aligned regions were removed from analysis. Finally phylogenetic trees were constructed using neighbor joining method with bootstrap values.

Importance of B4 medium for $CaCO_3$ mineralization

In many organomineralization experiments related to bacteria, the precipitation of $CaCO_3$ crystals were performed by using B4 medium, which consists of 0.4% yeast extract, 1% dextrose, 0.25% calcium acetate and agar.

B4 is the most common medium used in general organomineralization studies and has been used to characterize mineral precipitation potential. B4 medium has been used to culture microorganisms involved in active $CaCO_3$ precipitation from different ecosystems such as caves, soils, and water. B4 was also used to determine the effects of temperature on precipitation, observing that microbial $CaCO_3$ precipitation increased with time and temperature of incubation.

More than 50% of environmental bacterial isolates are being tested to date are able to precipitate $CaCO_3$ on B4, these include members of the *Bacillus, Arthrobacter, Kingella and Xanthomonas.*

Precipitation of $CaCO_3$ crystals

B4 medium was prepared and pH was adjusted at 7, autoclaved and poured into plates followed by standard streaking of isolates (GSN-11, TFSN-14, TFSN-15, and GSN-22) upon the plates in such a way that one line was drawn in mid of plate, properly wrapped by mean of Para film and cling film and was incubated at 25°C. After few days when results were checked all strains had precipitated the $CaCO_3$, photo graphs and compound microscopic slides were made.

Optimization of various parameters for the precipitation of $CaCO_3$

Selection of medium

B4 is the most common medium, which consists of 0.4% yeast extract, 1% dextrose, 0.25% calcium acetate and agar is used in organomineralization studies and has been used to characterize mineral precipitation potential. B4 medium has been used to culture microorganisms involved in active $CaCO_3$ precipitation from different ecosystems such as caves, soils, and water.

Effect of incubation temperature

Optimization of temperature (15, 25, 35, 40°C) was checked for mineralization of calcium carbonate crystals at B4 medium and was found that maximum $CaCO_3$ crystals were precipitated at 25 °C.

Effect of pH

Effect of pH (5, 7, 8, and 9) on the production of $CaCO_3$ was observed by streaking of positive isolates upon specific B4 medium. After few days it was known that best results were given at 5 pH.

Digestion of cave soil

In order to know various elements in cave top floor soil sediments, atomic absorption spectroscopy was carried out along with soil digestion mentioned below.

About 1g of cave soil was grinded to powdered level and mixed with aqua regia 3:1(HCL and HNO_3).this mixture was heated at 150°C and were kept in fume hood for overnight. Next day 5 ml of $HCLO_4$ was again added and kept on heating at high temperature until the overall volume of mixture remained 1 ml. during this process brown fumes were observed, then rest of mixture was passed through Whatman filter paper (NO.42).the volume of filtrate was raised to 50 ml by adding deionized or distilled water, at the end it was applied upon absorption spectrophotometer to check the elements.

X-ray diffraction (XRD) of the crystals

It is a process which is carried out to identify the molecular structure of different products like crystals, metals, minerals and biological macro molecules. Calcium carbonate crystals were properly washed with distilled water and were dried in oven, later on X-ray diffraction analysis was performed with a Philips 1830/40 X-ray diffractometer on crystal sample using 40 kV and 30 mA with a scanning speed of $0.005°2\theta\ s^{-1}$. The time constant was set at 2 s to check the composition of precipitated calcium carbonates.

Scanning electron microscopy (SEM)

Scanning electron microscopy technique was performed to view the images of crystals precipitated by bacterial strains by scanning the crystals with beam of electrons,

Morphology of crystals from bacterial isolates were studied by scanning electron microscopy (SEM, Philips XL30CP).

Fourier transform infrared spectroscopy (FTIR)

FTIR is a spectroscopy technique, which used to get infrared spectrum of absorption, emission, photoconductivity of a solid, liquid or gas (Griffiths et al., 2007). It is one of the well-known experimental methods for the study of secondary structures of different products.in this process we took the $CaCO_3$ crystals from the plate with wire loop and run FTIR. The obtained spectrum was then analyzed for the different functional groups present in soil samples. The samples were scanned from 4000-400cm^{-1} at resolution of 6.0cm^{-1}.

RESULTS

Results

Study was conducted to evaluate the biomineralization of calcium carbonates by bacterial strains isolated from Kashmir cave, Buner.

4.1. Screening of bacterial isolates for precipitation activity

Previously isolated 25 bacterial strains were investigated for their potential of calcium carbonate mineralization. Only four isolates were screened out having this activity e.g. GSN-11, TFSN-14, TFSN-15 and GSN-22.

4.2. Identification of isolates

For identification purpose Gram staining was performed.

Table 1: Gram staining of isolates.

Isolates	Gram's reaction
GSN-11	Gram negative single rods
TFSN-14	Gram negative short chains
TFSN-15	Gram positive long chains
GSN-22	Gram positive

4.3. Viable cell count in the soil sample

Number of viable cells per gram, were calculated through serial dilution of the soil sample and it was found as 4.6×10^4 CFU/g.

4.4. Biochemical tests

After microscopic examination, the isolates were identified through different biochemical tests, all the four isolates were catalase positive. Isolates GSN-11 and TFSN-14 were citrate positive and TFSN-15 as well as GSN-22 were citrate negative .isolates GSN-11,TFSN-14 and GSN-22 gave positive results in case of Oxidase test while TFSN-15 showed negative indication.GSN-11 and GSN-22 were urease positive and TFSN-14 and TFSN-15 were urease negative.so for enzyme assay TFSN-14 and GSN-22 were casein positive gave clear zones while GSN-11 and TFSN15

were negative, and for Gelatin all isolates were Gelatinase positive,in case of amylase all isolates were negative gave no clear zones.

Table 2: Biochemical tests for characterization of bacterial isolates

Isolates	Oxidase	Catalase	Citrate	Urease	TSI	Casein	Amylase	Gelatin	Identified isolates
GSN-11	+	+	+	+	R/Y	_	_	+	*Bacillus toyonensis*
TFSN-14	+	+	+	–	Y/R	+	_	+	*Paracoccus limosus*
TFSN-15	_	+	_	_	Y/R	_	_	+	*Brevundimonas diminuta*
GSN-22	+	+	_	+	R/Y	+	_	+	GSN-22

Key: + = positive, _ =negative, R/Y=red slant yellow butt, Y/R=yellow slant and red butt.

4.5. Molecular identification

DNA extraction and 16S rRNA based on phylogenetic analysis

DNA of the isolates was extracted by CTAB method and was run on agarose gel electrophoresis. For phylogenetic analysis, the 16S rRNA sequences of all four isolates were sent to NCBI which confirmed the isolates TFSN-14 as *Paracoccus*

Limosus and TFSN-15, GSN-11 as *Brevundimonas diminuta* and *Bacillus toyonensis* respectively.

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.65426500 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method. And are in the units of the number of base substitutions per site. The analysis involved 18 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 594 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

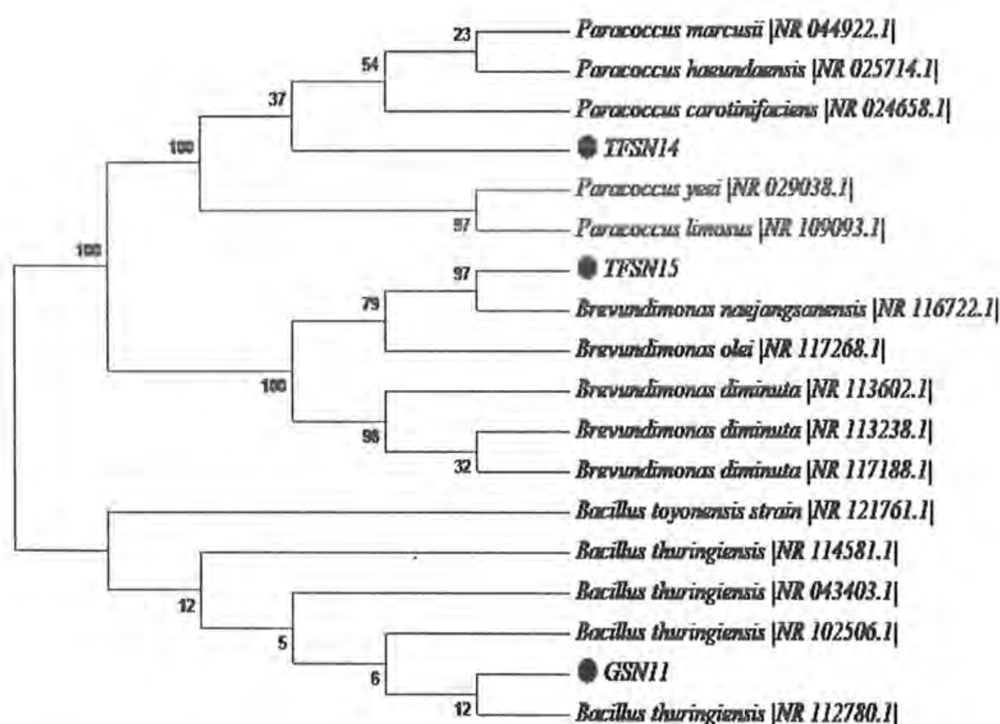


Fig.4.1.Phylogenetic tree of the isolates based on 16S r RNA sequence.

4.6. Calcium carbonate precipitation

The fresh culture of the positive isolates were streaked upon the middle of plates having B4 medium and incubated. After 8 to 10 days of incubation time, when plates were observed there were clear zones on both sides of streaked line which indicated that nearby calcium has been attracted by bacteria for the purpose of precipitation. Plates were again incubated. Later on the bacteria had induced precipitations which are as bellow:

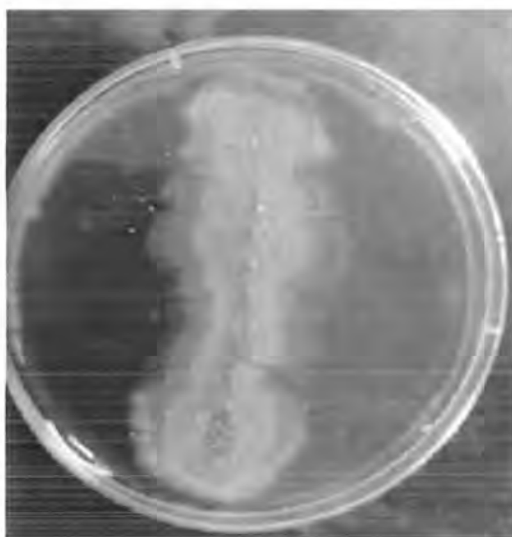


Fig. 4.2: Crystals produced by *Paracoccus limosus.*

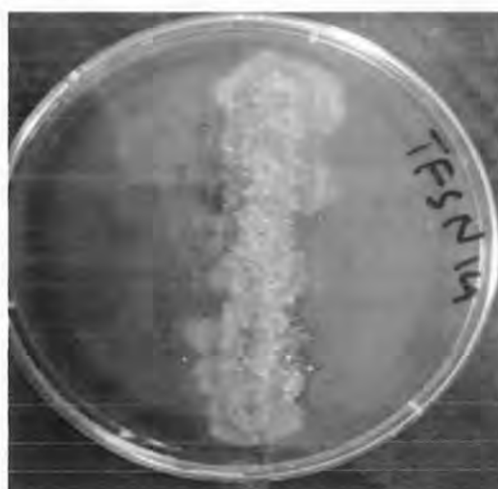


Fig.4.3: Precipitates induced by *Bacillus toyonensis.*

4.7. Effect of various parameters on growth of calcifying bacteria

Effect of temperature at growth

The growth of all four isolates was checked on selective B4 medium consisting of (calcium acetate 0.25 g, dextrose 0.5 g, yeast extract 0.4 g and agar 1.5 g per 100 ml) on different temperature ranging from 5°C to 35°C.

Table 3: Effect of temperature on bacterial growth.

Temperature (°C)	*Bacillus toyonensis*	*Paracoccus limosus*	*Brevundimonas diminuta*	GSN-22
5	_ _	_ _	_ _	_ _
15	_	_	_	_
25	+ +	+ +	+ +	+ +
35	+ + +	+ + +	+ + +	+ + +

Key: _ =neglectable growth, _ _= no growth, + += good growth, + + + =very good growth.

The isolates showed very good growth at higher temperature 25°C and 35°C and maximum growth was observed at 35°C.but at lower temperature 5°C and 15°C the growth was neglected.

Effect of different pH at growth

Plates were prepared with different pH ranging from 3,5,7,9 and 11, after 12 days of incubation at 35°C, plates with 3 and 5 pH had no growth while 7, 9 and 11 plates had very good growth, and maximum growth was seen on 11 pH followed by 9 pH, and specially on 9 and 11 plates there were huge clear zones (Fig. 4.4).

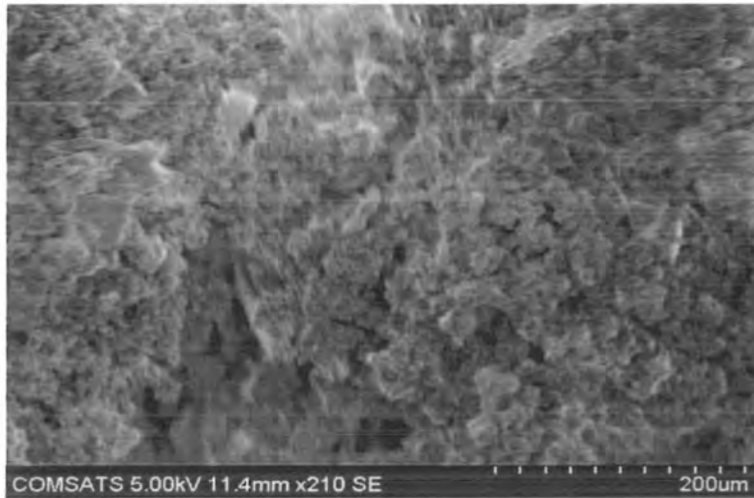


Fig. 4.9: Calcium carbonate precipitates at 210 X

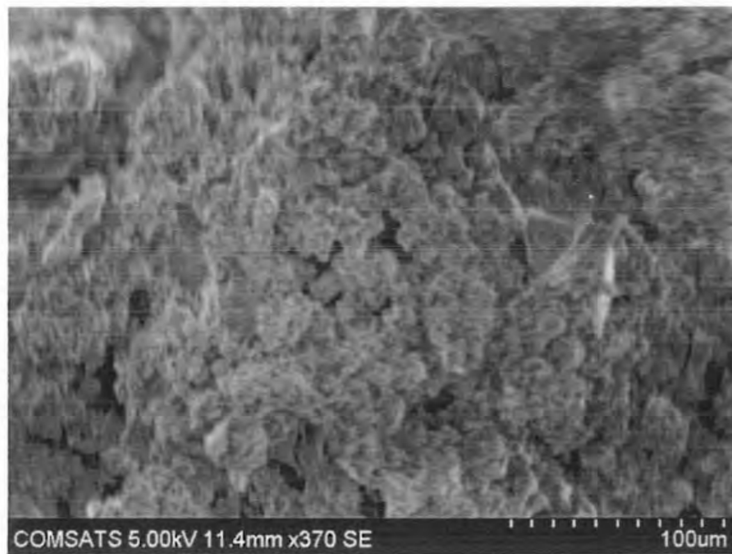


Fig. 4.10: Calcium carbonate precipitates at 370 X

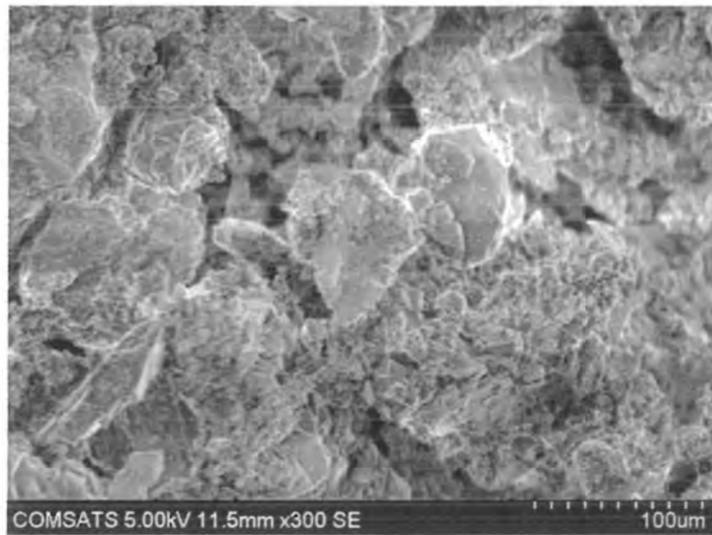


Fig. 4.11: SEM images of crystals precipitates formed by *Brevundimonas diminuta.*

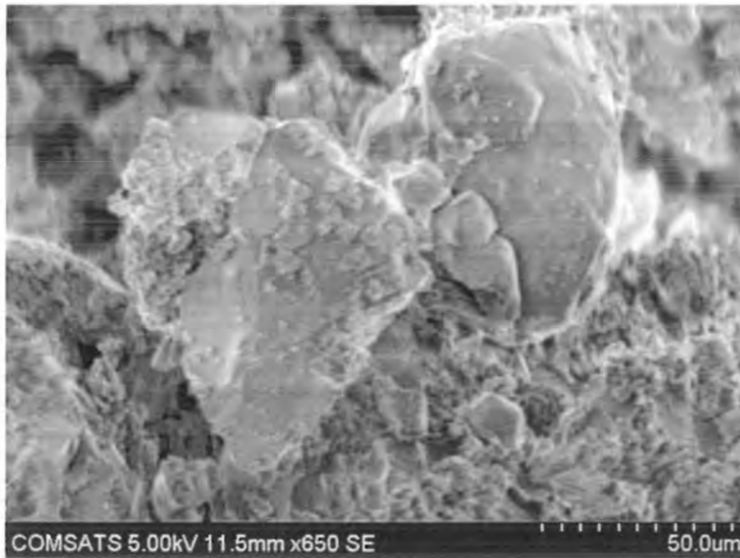


Fig. 4.12: Calcium carbonate precipitates formed by *Brevundimonas diminuta* at 650 X.

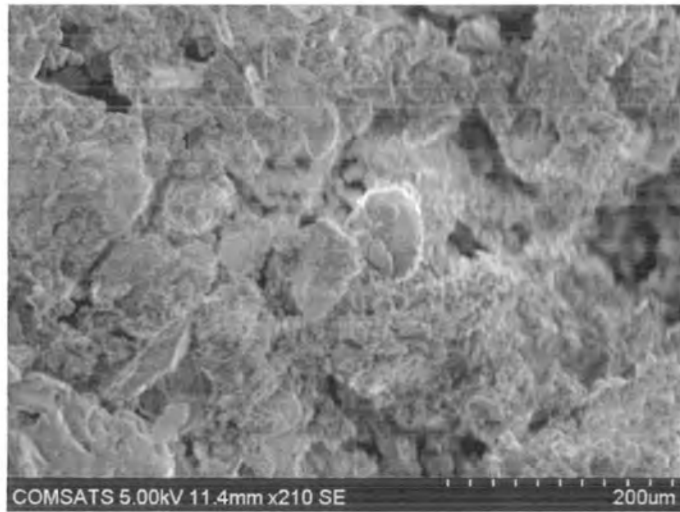


Fig. 4.13: Calcium carbonate precipitates formed by *Brevundimonas diminuta* at 210 X.

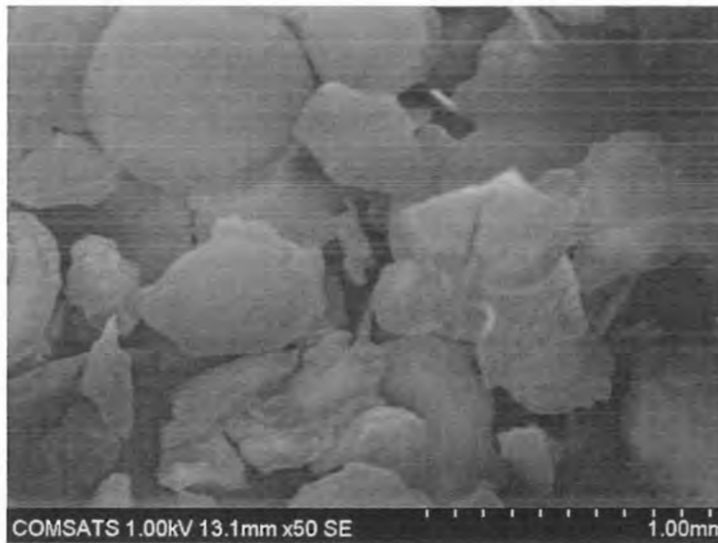


Fig. 4.14: Calcium carbonates crystals produced by GSN-22.

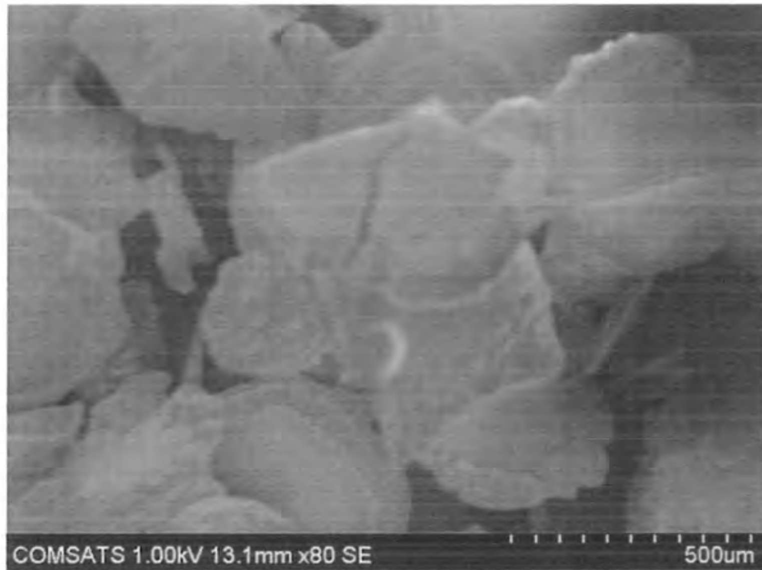


Fig. 4.15. Calcium carbonate precipitates formed by GSN-22 at 80 X.

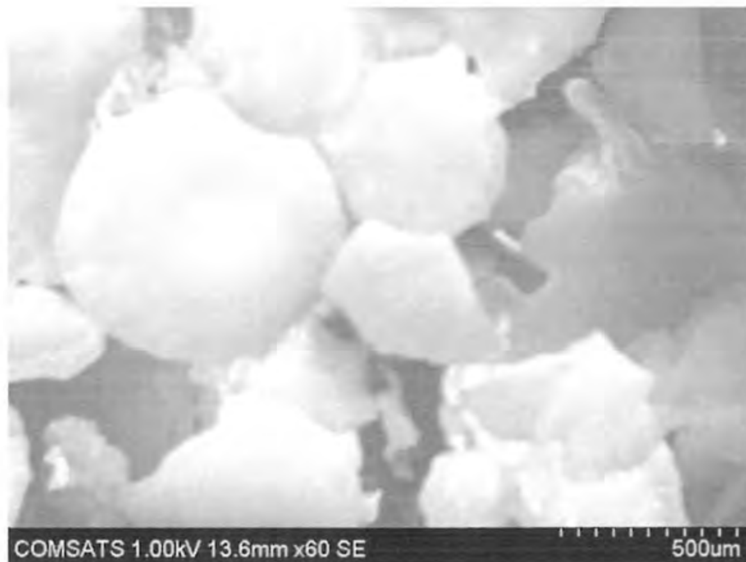


Fig. 4.16: precipitates Calcium carbonate precipitation formed by GSN-22 at 60 X.

4.11. Fourier transform infrared spectroscopy (FTIR) analysis of calcium carbonate precipitates

FTIR analysis of Calcium carbonate was performed on FTIR (Tensor 27 of Bruker company equipped with ZnSe ATR).

Calcium carbonate of sigma was taken as control and was analyzed in according to the control. The control showed characteristic peaks of CaCO3 at 871cm^{-1},712cm^{-1} ,1794cm^{-1}, 1405cm^{-1}, all the samples analyzed showed peaks in same regions which indicated the presence of calcium carbonate in the samples precipitated by cave bacteria. FTIR peaks overlay of both standard and sample are showed with colored labeled peaks.

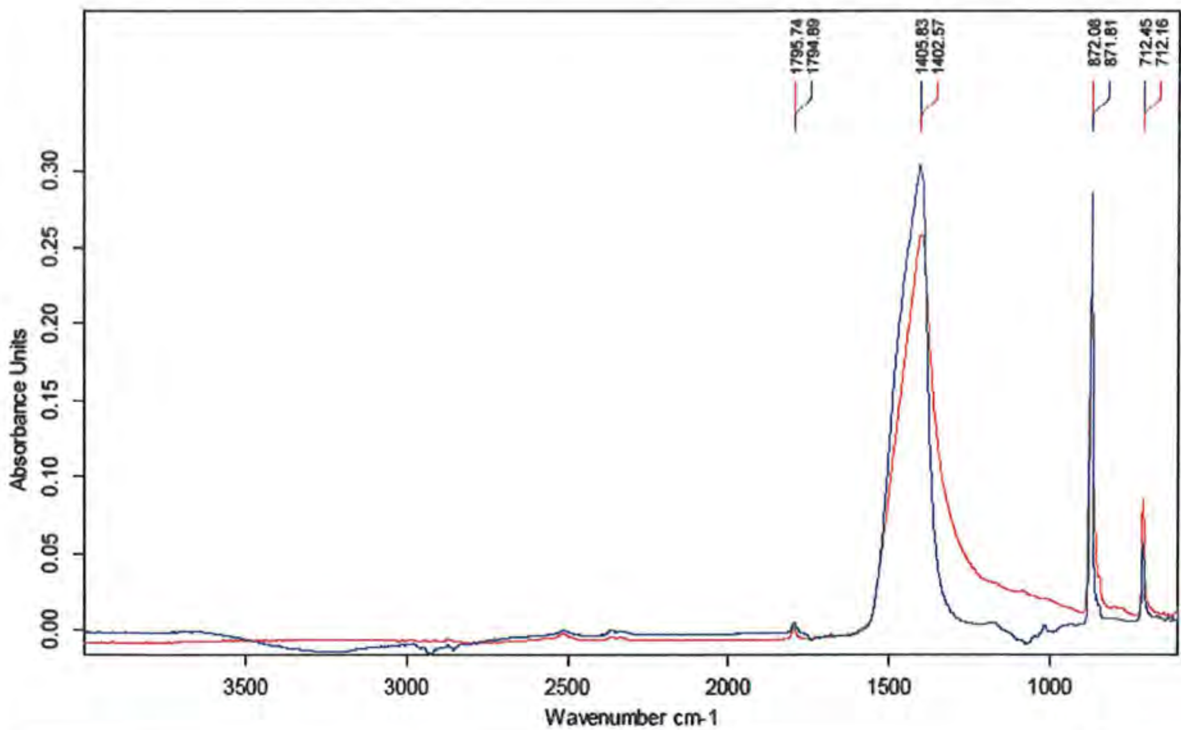
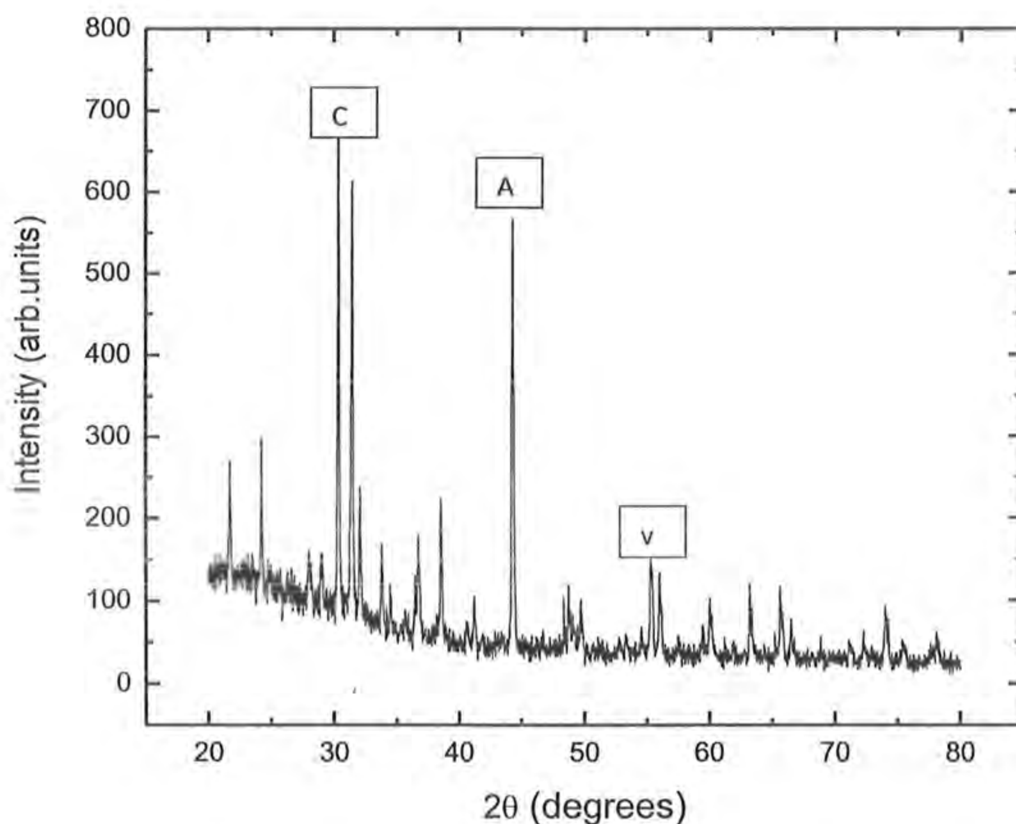


Fig.4.17: FTIR analysis of $CaCO_3$ with control.

4.10. X-ray diffraction analysis of calcium carbonate (XRD)

XRD was performed for the quantitative determination of the mixture of calcium carbonate polymorphs. Fig4.16, showed the XRD patterns of the three polymorphic calcium carbonate crystals as calcite, aragonite and vaterite. All synthesized calcium carbonate samples were crushed into fine powder form for characterization.



key: C= calcite, A= aragonite and V= vaterite.

Fig.4.18: XRD analysis of the polymorphs of Calcium carbonate.

DISCUSSION

Discussion

The current study was carried out to determine the biomineralization of calcium carbonates by bacterial strains isolated from Kashmir cave Buner. The Kashmir caves are series of natural lime stone caves located at boundary line of Buner and Mardan districts of KPK. The length of the cave is 98m having height of 30m. Its width is 25m. The research was conducted in Microbiology Research Laboratory (MRL) Microbiology Department, Quaid-i-Azam University Islamabad.

In this study already isolated bacterial strains and we characterized these strains in term of microscopic, cultural and biochemical characteristics. Over all round about 24 bacterial strains were investigated for calcium carbonates mineralization but only 4 strains (*Bacillus toyonensis* GSN-11, *Paracoccus carotinifaciens* TFSN-14, *Brevundimonas naejangsanensis* TFSN-15 and GSN-22 an unidentified strain) had calcifying ability. The Gram's staining of *Bacillus toyonensis* was negative single rods ,*Paracoccus Limosus* was also negative short chains. Whereas *Brevundimonas diminuta* was Gram's positive long chains and GSN-22 was also Gram's positive small chains. The literature shows that Gammaproteobacteria (45 %) group is the most dominant group of bacteria investigated in caves followed by *Bacteriodstes, Firmicutes*, and Actinobacteria. Actinobacteria have been considered to be mainly confined to cave soils. (Porter 1971). It has been reported that the actinobacteria (Gram positive groups) isolated from the Altamira cave revealed a great taxonomic diversity with the predominant isolates belonging to the genus Streptomyces. Members of the genera *Nocardia, Rhodococcus, Nocardioides, Amycolatopsis, Saccharothrix, Brevibacterium, Microbacterium* and coccoid actinobacteria were also found. (Groth et al. 1999). While genera of Gram negative bacteria are also found in in cave sediments such as *Pseudomonas, Serratia, and Enterobacter.*

The total viable cell count was performed by pour plate technique and the results showed that the total count of the sample was 4.6×10^4 CFU/g of the cave soil sample. The number of bacterial strains from cave soil sample is lesser then the number of bacteria present in samples of open space environment, the reason is that caves have oligotrophic nutrient conditions, complete darkness, deprivation of energetic sun light, different anti-growth byproducts produced by bacteria or other organisms, psychrophilic temperature, etc.

Because cave bacteria lives in nutrient deficient environment when they are sampled and grown upon different growth medium which is more nutritive then cave environment so bacteria dies and cannot survive on high nutrient medium the reason may be that these strains had adapted themselves to low nutrient parameters for long period of time.

In many organomineralization experiments related to bacteria, the precipitation of $CaCO_3$ crystals were performed by only using B4 medium, which consists of 0.4% yeast extract, 1% dextrose, 0.25% calcium acetate and agar.

B4 is the most common medium used in general organomineralization studies and has been used to characterize mineral precipitation potential. B4 medium has been used to culture microorganisms involved in active $CaCO_3$ precipitation from different ecosystems such as caves, soils, and water. B4 was also used to determine the effects of temperature on precipitation, observing that microbial $CaCO_3$ precipitation increased with time and temperature of incubation. More than 50% of environmental bacterial isolates are being tested to date are able to precipitate $CaCO_3$ on B4, these include members of the *Bacillus, Arthrobacter, Kingella* and *Xanthomonas*.so this B4 medium was used in MRL to induce calcium carbonates.

As caves are nutrient deficient environments. No nutrients are transported from outside except sometimes by water currents, air movement or by tourists, energy source of cave microbes is obtained from the animal activities. It is also supported by plant actions that take place at the cave sediment or entrance zone of cave. Sometimes when it is raining water draining inside cave may also provide organic wastes helpful for the growth of different types of organisms. Sometimes the solid waste management concerned authorities dump waste nearby entrance zones of these very sensitive and precious resources in research point of view can also leach to the cave enhancing the food supply for cave microbes. These all parameters change the nutrient ecosystem of the environment, at the end a large and diverse number of microbial communities are grown upon these food sources. In some depth microenvironments of caves when the external food sources are not available then those microbes depend on inorganic elements present in cave soil sediments so mostly bacteria residing in caves are Chemolithotrophes.

Calcium carbonate mineralization/precipitation is a universal process carried out by different bacterial strains along with some fungus under optimum parameters. Here the study was focused upon bacterial mineralization only. Certainly, some bacteria and fungi can do precipitation of calcium carbonate extracellularly by streaking fresh bacterial culture at the middle of B4 medium (calcium acetate, yeast extract, dextrose and Technical agar) plate followed by incubation. After proper incubation period the bacteria will attract the calcium toward its self producing clear Zones. Later on the calcium is precipitated by mean of different exopolymeric substances (EPS) at the top of media.

Microorganisms use chemical pathways to generate energy, such as human beings use the chemical gradient between nutrients and oxygen to produce energy for existence. In using these chemical pathways different cave microbes usually modify the environmental parameters in which they are living, which in turn change minerals along with their structural morphologies. Such microbially-mediated bio mineralization regularly produces banded layers of minerals on cave rocks, sediments etc.

Cave microbes have very pivotal role in biotechnology and bioremediation, many bacteria along with other microbes possess the capability to produce many significant byproducts which can work under psychrophilic temperature and therefore has remarkable contribution for industry like antibiotics and tumor suppression constituents. Some novel strains of microbes which breakdown complex hazardous aromatic materials, like benzothiazole and benezene sulfonic acid. In addition to this many parasitic fungal species such as *Troglophilus neglectus* are producing enzymes like chitinase , lipase and protease (Glavan, 1997).

The growth of isolated all four strains was checked at four different temperature ranging from 5°C to 35°C. The growth was examined, all the isolates showed maximum growth at 35°C, whereas least growth was observed at lower temperature 5°C along with 15°C. But interestingly according to Laiz all tested bacteria from cave soil samples were able to grow comparatively well in a range of temperatures from 13° to 45°C (Laiz, L., et al., 2003). The number of bacterial strains increased from 25 to 35°C. There was a decrease in the number of strains at very law and higher temperature.

Optimization of Calcium carbonate mineralization was checked at different temperature ranged from 5°C to 35°C. But the optimum temperature for calcium precipitation was 25°C. Crystal precipitation at 25°C started after a 12 to 15 days of inoculation. Petri plates were examined periodically up to 25 days for the presence of crystals. At lower temperatures (5 to15°C), crystal precipitation was very slow taking incubation period of round about 40 days. And no precipitates were produced beyond 35°C. The size and quantity of crystals increased with time. Up to 10 days of inoculation the calcium precipitates were regularly checked and noticed that the precipitates were white in color and had powdered structure later on with passage of time they appeared in clear crystal form. In the present study, the metabolic activity of bacterial strains and temperature are key factors in carbonate deposition. The optimum temperatures have a positive effect on bacterial precipitation of calcite, increasing the ability of the strain to form crystals.

In the same way pH also played a very important role in mineralization process. Calcium carbonate was checked at different pH ranging from 3 to 11 pH and found that optimum results were observed at 5 pH. It showed that the cave environment is slightly acidic in nature because of carbonic acid and sulfuric acids which are present in cave sediments which also helps in speleogenesis.

Caves have presented an exclusive task to scientific study because, excluding for entrance zones, majority of caves are hidden from vision. As a result, caves and different cave environments are being ignored by all walks of life. Because Caves are deprived of natural sun light, complete darkness, unpredictable internal conditions and contain a number of physical and psychological hurdles. And sometimes it is the cave researcher who takes the risk to face all these obstacles having desire to find and see frightening places previously unknown. After all there is very limited research conducted in this new field, it is time to explore the unseen wealth in caves.

The elemental analysis of cave soil samples was carried out by proton induced X-ray Emission technique in Biochemistry Department. The result showed that the soil sample had highest concentration of calcium 1615.35 mg/Kg and magnesium 502.85mg/Kg .the other elements which were found in trace amounts were NA, K, Fe, Zn, Mn, Ni, Cu and Cr. Microbe interact with all these metals in cave environments, changing their chemical state, while these all elements also able to affect microbial

growth, metabolic activity and survival. From these all metals microbes form bio minerals like calcium carbonate, silicates and iron oxides. This wholes pathway is known as bio mineralization of metals.

Simple compound microscopy was carried out in which crystals of calcium carbonates were placed upon a sterile slide and observed under microscope at 40X and 100X,

X-ray diffraction or XRD analysis is a distinctive technique which helps in determination of crystallinity of different products, different types of polymorphic form, comparative study between powder and crystal elements and to quantify the percentage crystallinity of samples. But we focused on calcium carbonate different polymorphic forms. So the different peaks confirmed the presence of all three polymorphic forms such as calcite, aragonite and vaterite in the precipitated $CaCO_3$.

FTIR analysis of calcium carbonate was performed on FTIR model Tensor 27 of Bruker company equipped with ZnSe.

Calcium carbonate of made of sigma was taken as control and was analyzed in according to the control. The control showed characteristic peaks of calcium carbonates at $1794cm^{-1}$,$1405cm^{-1}$,$871cm^{-1}$, $712cm^{-1}$, all the samples analyzed showed peaks in same regions which indicated the presence of calcium carbonate in the samples precipitated by cave bacteria.

Scanning electron microscopy technique was carried out to view the morphological structures of all three types of crystals precipitated by cave bacterial strains. The samples were in powdered and pure crystalline form and which all exhibited different morphologic structures.

CONCLUSIONS

Conclusions

- Four positive strains were isolated from Kashmir cave, Buner, were characterized biochemically and molecularly as *Bacillus toyonensis, Paracoccus carotinifaciens, Brevundimonas naejangsanensis and* unidentified Gram positive bacterial specie.
- All the four isolates were able to grow on B4 medium showed Calcium carbonate mineralization ability.
- Optimum pH for calcium carbonate formation was 5, Temperature was 25°C and incubation period was 20 days.
- Elemental analysis of cave soil was performed by atomic absorption spectroscopy which showed that calcium was present in greater amount where as Sodium, Magnesium, Potassium, Iron, Manganese, zinc, nickel, chromium and lead were also present.
- Crystals formed by bacteria were analyzed by FTIR, XRD and SEM techniques which confirmed that these crystals were of calcium carbonate.

FUTURE PROSPECTS

Future Prospects

- This habitat has potential for further exploration in term of microbiology.
- To explore novel bacterial strains in cave soil.
- To investigate more valuable compounds production such as enzymes, vitamins and amino acids.
- These isolates can further be studied for production of antibiotics.
- To study the role of these isolates in precipitation of different minerals such as sulphur and iron.

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APPENDICES

Appendices

Table.1: Effect of temperature on calcifying bacterial growth

temperature	*Bacillus toyonensis*	*Paracoccus limosus*	*Brevundimonas diminuta*	GSN-22
5°C	— —	— —	— —	— —
15°C	—	—	—	—
25°C	++	++	++	++
35°C	+	+	+	+

Table.2: Effect of pH on growth of calcifying bacterial

pH	*Bacillus toyonensis*	*Paracoccus limosus*	*Brevundimonas diminuta*	GSN-22
3	—	—	—	—
5	—	—	—	—
7	+	+	+	+
9	++	++	++	++

Table.3: Effect of incubation time on growth of positive bacteria

Time in days	*Bacillus toyonensis*	*Paracoccus limosus*	*Brevundimonas diminuta*	GSN-22
5	--	--	--	--
10	–	–	–	–
15	+	+	+	+
20	++	++	++	++
25	+++	+++	+++	+++

Table.4: Effect of temperature on calcium carbonate production

temperature	*Bacillus toyonensis*	*Paracoccus limosus*	*Brevundimonas diminuta*	GSN-22
5	--	--	--	--
10	--	--	--	--
15	±	±	±	±
25	+++	+++	+++	+++
35	+	+	+	+