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MICROBIAL ECOLOGY OF BAT GUANO AND IT'S IMPACT ON ASSOCIATED ECOSYSTEMS

THESIS SUBMITTED FOR THE AWARD OF DOCTOR OF PHILOSOPHY IN ZOOLOGY IN THE FACULTY OF SCIENCE, SANT GADGE BABA AMRAVATI UNIVERSITY

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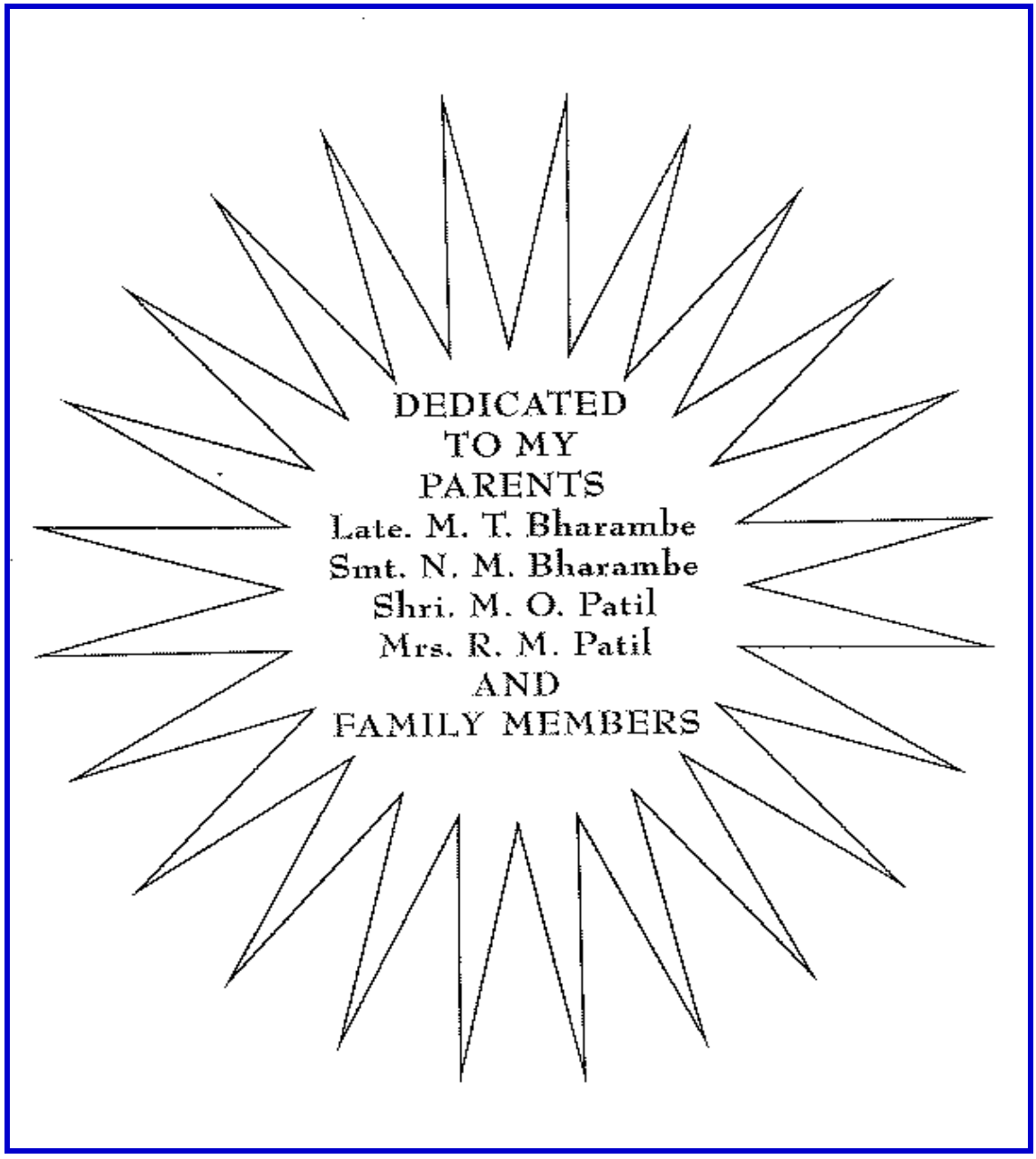
DEDICATED
TO MY
PARENTS
Late. M. T. Bharambe
Smt. N. M. Bharambe
Shri. M. O. Patil
Mrs. R. M. Patil
AND
FAMILY MEMBERS

Dr. G. N. Vankhede
Professor and Head,
Department of Zoology
Sant Gadge Baba Amravati University,
Amravati.

CERTIFICATE

This is to certify that I have been supervising the research work of Mr. C. M. Bharambe, entitled, "**Microbial ecology of bat guano and it's impact on associated ecosystems**" for the Degree of Doctor of Philosophy in the Faculty of Science, Sant Gadge Baba Amravati University, Amravati.

He has completed the research work satisfactorily and the thesis is ready for evaluation.

Amravati
Date : 13.7.2007

(Dr. G. N. Vankhede)
HEAD
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Sant Gadge Baba Amravati University
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DECLARATION

I hereby declare that with the exception of the guidance and suggestions from my supervisor Dr. G. N. Vankhede, the thesis entitled, "**Microbial ecology of bat guano and it's impact on associated ecosystems**" is my own unaided work, which was carried out in the Laboratory of Department of Zoology, Sant Gadge Baba Amravati University, Amravati.

This work either in part or whole has not been submitted to any other university or institute for the award of any diploma or degree.

Amravati

Date : 13.07.2007

(C. M. Bharambe)

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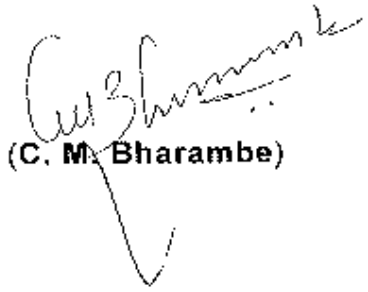
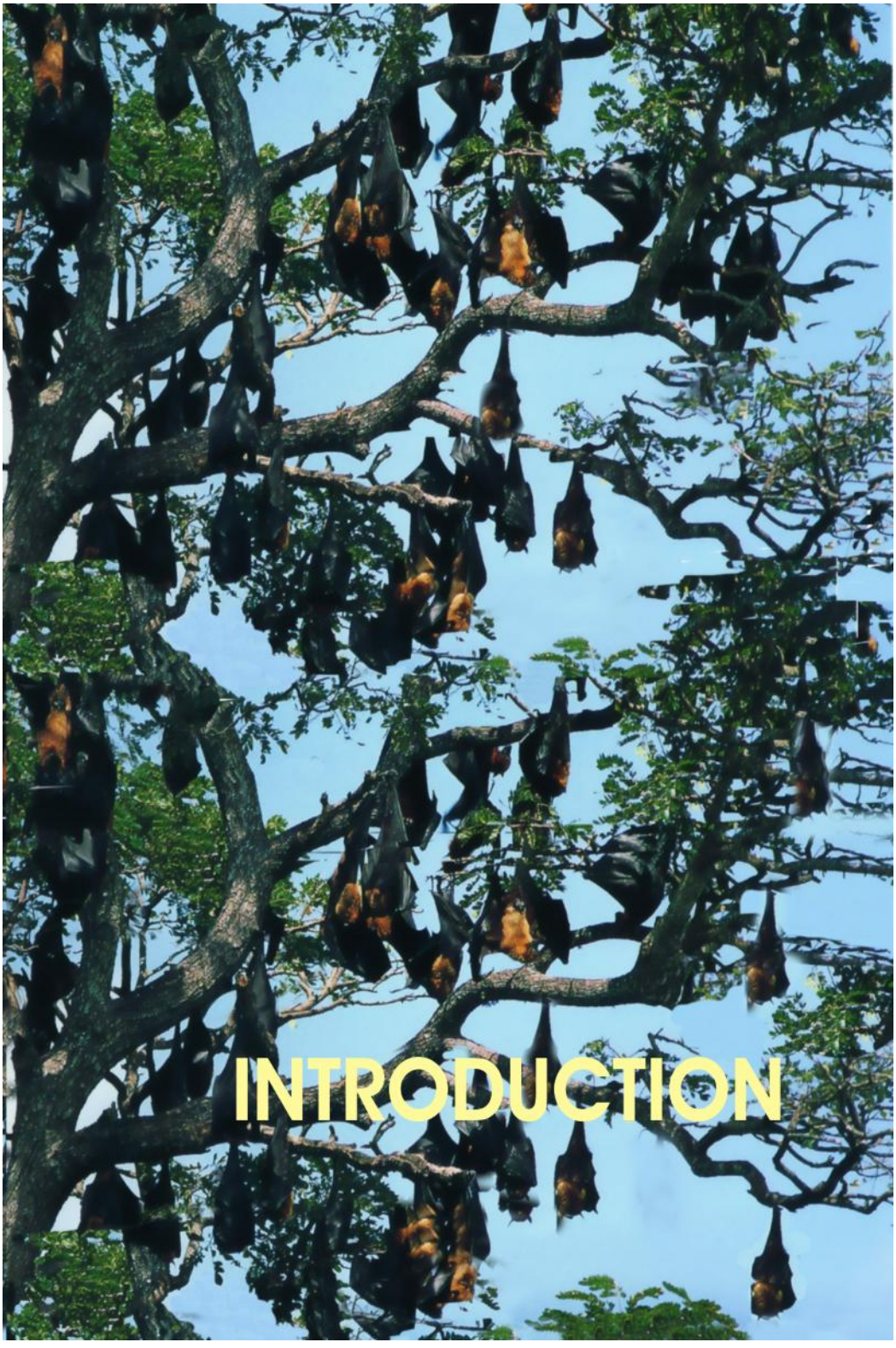


(C. M. Bharambe)

CONTENT


INTRODUCTION

INTRODUCTION

A marvelous symbiosis exits between the microorganisms and bat guano. Bacteria in the mammalian intestinal tract aid in the breakdown of food during digestion. These organisms synthesize enzymes capable of degrading a vast array of substances. Innumerable microbes are regularly excreted along with waste products and together with soil organisms, they constitute the microbial population of a bat guano deposit.

A large roosts population of bat deposit thousands of kilograms of dropping annually. A chemical analysis of the guano revealed a substance rich in carbon, nitrogen, phosphorus, potassium, vital minerals and trace metals. Some of these fall into small pools and rivers near the cave and old temples. When it enters the water, a nutritious broth is formed, supporting the growth of numerous microorganisms. These in turn become food for tiny microorganism, and together they supply nutrients for fish and crustaceans. Until recently, very little research had been conducted on the microbial role of bat guano on ecology of such an environment some microorganisms produce compounds of industrial importance.

In central Texas, Bracken Cave is a summer home to some 20 million Mexican free-tailed bats (Tadarida brasiliensis). The cave environment helps to yield a population of microorganisms on the deposited guano. Some of the organisms yielded by the cave environment appear to be completely new species and possibly new genera. Several of these organisms produced unusual enzymes (Steele, 1989).

1.1, Bat guano is a microbial ecosystem:

Microorganisms present in bat guano are capable of transforming ammonia and ammonium compounds to more oxidized forms of nitrogen. One of the most serious urban problems facing us today is the removal of ammonium compounds from industrial and municipal waste. If ammonia is discharged into lakes and rivers, a process called eutrophication occurs, and microorganisms in the water transform the ammonia to other nitrogen compounds while consume vast amount of oxygen. Bacteria in bat guano are known to be very efficient in the nitrification process.

Potential product from guano microorganisms is an enzyme called chitinase, a protein capable of converting the chitin exoskeleton of insects and crustaceans to simpler sugars. Excluding cellulose, chitin is one of the earth's most abundant natural soures of complex carbohydrates (polysaccharides). Waste from crustaceans, such as shrimp and crabs, forms a major source of inexpensive raw material for the enzymatic conversion of chitin to a more easily fermentable sugar. This, in turn, may be used for alcohol and fuel production. Another potential use for chitinase producing microorganisms is the biological control of insect and nematode pests.

Microbially produced products are being utilized by human beings e.g. beer, wine, bread, cheese and yogurt. Microorganisms are also used to produce many important enzymes, amino acids, vitamins, antibiotics, pesticides, herbicides, etc. These products benifit such diverse areas as agriculture, medicine, pharmaceuticals, textiles, paper production, photographics and food production.

The deposition of guano and subsequent colonization by microbes is crucial to a cave ecosystem. The resultant food chain play a crucial role in a cave ecosystem. Bat guano often provides the major influx of nutrients in an environment devoid of photosynthesis plant life. An ounce of bat guano contains billions of bacteria, and a single guano deposit may contain thousands of bacterial species. At present we are not knowing these species.

Bat guano is used in agriculture in many regions of the world for hundreds of years. In the 1600s in Peru, the Incus valued guano so highly that the punishment for harming the animals that produced it, was death. During the late 19th century, it had become important enough to American farmers that the government offered free land to those who discovered guano deposits, provided the harvest was made available to U.S. citizens.

According to Keleher, (1996) bat guano can be safely used as a fertilizer, both indoors and outdoors, and benefit vegetables, herbs, flowers, all ornamentals, and fruit and nut trees. Its primary ingredients are roughly 10% nitrogen, 3% phosphorus, and 1% potassium. The high nitrogen content is responsible for plants, healthy green colour and their rapid growth after application. Phosphorous promotes root growth and flowering, and potassium encourages strong stems. Besides these three major nutrients, guano is known to contain all of the minor and trace elements necessary for a plants overall health. Unlike an artificial gardening products, guano contains no fillers. And, while most chemical fertilizers leach out of the soil soon after being applied, the guano remains much longer, enhancing the soil and slowly continuing to feed the plants.

Guano being rich in bioremediation microbes, cleans up toxic substances, and purify garden soil in transition from chemical to organic practices (Barry et al., 1997). He reported that these microbes combat fungus when sprayed directly on plant leaves. Finally, Barry reported that bat guano contains powerful decomposing microbes, which help to control soil borne diseases and harmful nematodes and which served as ideal compost activators, significantly speeding up the decomposition process.

The guano cycle being with plant matter that is eaten by insects, the insects in turn are eaten and digested by bats. After the bats deposit their waste on a cave floor, it is processed once again by millions of beetles and billions of decomposing microbes. Whats left is perfectly preserved and protected inside the cave a natural fertilizer warehouse.

India is famous for its majestic wildlife and studies of its natural history abound, but the bats of India are hardly ever mentioned, and very little is known about them. India has an incredible diversity of bats with at least 109 species. This includes one of the largest in the world, the Indian flying fox (Pteropus giganteus); one of the most colourful, the orange and black painted bat (Kerivoula picta); and one of the rarest, Salim Ali's fruit bat (Latides salimalii). The habits of Indian bats are as diverse as the habitats they live in, from high in the Himalayas, to the deserts of the North-West, to the tropical forests of the East and South, there are bats that feed on fruits, nectar, insects, frogs, and even other bats. Indias remaining bats have their origins in Western Asia and are primarily arid-climate species such as the tomb bats (Taphozous sp.). India has 12 species of flying foxes. Only the Indian flying fox, the frugivorus fruit bat (Rousettus leschenaulti); and the short nosed fruit

bat (Cynopterus sphinx) are common thoughout the country. The remaining species are rare and are found only in the mountains of Southern India or on the Andaman and Nicobar Islands near Thailand. Two of them are specialized on nectar and are both long tongued fruit bats (Eonycteris spelaea and Macroglossus sobrinus). The other flying foxes are also known to visit flowers for nectar and pollen, but they feed primarily on fruits. However, the detail studies on their guano is lacking and fairly reported in any scientific literature.

Sridhar et al. (2006) studied physiochemical characteristics, microflora and manure quality of guano of an endemic insectivorous cave bat, Hipposideros speoris. The impact of bat guano on plant growth was also assessed by them. Results indicated that organic matter, total carbon, total nitrogen and phosphate were high in faecal pellets. Calcium, magnesium, bacteria, actinomycetes and fungi were higher in humus like guano than fresh faecal pellets. Physicochemical features and microbial load between faecal pellets and humus like guano significantly differed ($p<0.05$). Their results clearly indicated that incorporation of small amount of bat guano into the soil enhances crop production.

Although agricultural chemicals drastically increase food production, it leads to impaired soil health, depletion of organic matter and microbes and persistance of heavy metals and pesticides.

During the present decade farming has been promoted to restore soil health and fertility status. Application of farmyard manure (FYM), compost, green manure and bioinoculants are the most important management practices in organic farming (Thampan, 1993). Improvement of

soil fertility through the application of vermicompost is becoming more popular. In contrast to chemical fertilizers, application of FYM meets one half of nitrogen, one sixth of crops during the first season. On equivalent nitrogen basis, FYM or compost is 40-60% efficient as chemical nitrogenous fertilizers to increase crop production. Even though long term (>5 years) application of organic manure overcome such disparity, efficient application of organic matter for self sustenances in agriculture has not reached the farming community. Several organic manures, of animal origin are available now a days for use (e.g. night soil, bovine dung and urine, sheep manure, poultry manure, bat guano, silkworm wastes and vermicompost). Availability of such manures for crop production is restricted due to many constraints such as geographical region, awareness of many value, extent of manure production and managements. There is ample scope to assess and utilize nonconventional organic manures in agriculture. Recently, the use of pill millipedes (Arthrosphaera magna) in composting organic matter has been proposed (Ashwini and Sridhar, 2002, 2003). Likewise, bat guano has been assessed to understand bat diets and food habits (DesMarais et al., 1980; Fenton et al., 1998; Korine et al., 1999), nitrogen and mineral budgets (Studier et al., 1991), seed dispersal (Korine et al., 1999), habitat preference, flight activity (Zielinski and Gellman, 1999), ecology and conservation strategies (Korine et al., 1999; Bhat and Shreenivasan, 1990).

No much work has been carried out on the bat guano in India and the microbial studies are totally lacking and hence it was thought to study the comparative biochemical, biological studies of bat guano from different habitats (cave, old temples and forest) and to assess the feasibility of the bat

guano as supplimentary fertilizer, pesticidal, larvicidal and growth inducer to increase crop production.

1.2, Lonar crater ecosystem:

India has two craters namely Lonar and Ambar. Lonar crater lake is the largest one and is bowl shaped depression formed 50,000 years ago by impact of a huge meteorite that descended on earth from space and curved out a bowl roughly 7 km. in circumference and 1.8 km. in diameter. The size and the age makes it the largest and 3rd oldest meteorite crater in the world. Initially it was throught to be a volcano crater. But it is declare as an impact crater from 1823 when J.E. Alexander pointed out it to be the crater. This is the only crater in the world created by hypervelocity meteorite impact in basaltic rock. The scientific research have revealed that the stone mass which struck the earth was approximately 60 m. in diameter weighing about a million tones. The force of impact is estimated to have generated energy equivalent to six megatons of explosion.

Government of India has declared this crater as a “National monument” and Archeological Survey of India monitor the conservation and development of this Crater Lake (Fig. 1.1).

Lonar crater is situated within Parvani quadrangle in the Buldhana District of Maharashtra, India. (Latitude : N190 58’ Longitude : E 760 31’). It has an almost perfectly circular shape. The Lonar crater lake is formed by the accumulation of water in the deeper parts of basin. Rocks in the crater reveal many characteristic features of the moon rocks.

1.2.1, Lonar Sanctuary:

383.22 ha. area of the Lonar crater lake near Lonar village is notified in June 2000 as a wildlife sanctury (No. WLP/1089/P.K.48/F-1 dated 8th June 2000). It includes 77.39 ha. revenue land of the lake, 39.75 ha. private land under depression and the surrounding 266.18 ha. of reserve forest land. There are many old temples on the peripheral boundry of the crater which have now become roosting places for bats (Fig. 1.2)

Kamalja Devi temple is situated at the southern base of the crater. (19.58.220 N, 076.30.440 E/484 m).

Morache temple (Peafowl's temple) is now famous for existance of thousands of bats and peacocks. (19.58.209 N, 076.30.682 E/485 m).

Waghache temple (Leopards temple) is also famous for bats and people have seen leopard hinding in it many times. (19.58.262 N, 076.30.803 E/492 m).

Ramgaya Temple (19.58.551 N 076.31.020 E/492 m) has become the source of sweet drinking water, as this is the only sweet water stream available in the crater, rest of the crater water is highly saline.

1.3, Ajanta cave ecosystem:

Ajanta is world's greatest historical monument recognised by UNESCO located just 40 kms from Jalgaon city of Maharashtra, India. There are 30 caves in Ajanta of which 9, 10, 19, 26 and 29 are Chaitya-Grihas and the rest are monasteries. These caves were discovered in AD 1819 and were built up in the earlier 2nd century BC-AD. Most of the paintings in Ajanta are right from 2nd century BC-AD and some of them about the fifth century AD and Continued for the next two centuries.

1.3.1, Cave - 7:

The varanth of this cave must at one time, have been elaborate. The ceiling was obiviously painted. The carving is simple.

1.3.2, Cave - 24 :

Unfinished pillars of the front side are worth nothing for the evolution of the pillar style. The earliest pillars are simple, rough on four sides. Later the Mahayana period they were tapered into octagonal form.

1.4, Ellora cave ecosystem:

Ellora is only 29 km. from Aurangabad and 3 km. North of Khuldabad village. Ellora caves belong to the Chalukya and Rashtrakuta age span, nearly five centuries in time and cover 2 km. in length. It lies near an important ancient trade route between Ujjain in Madhya Pradesh. There are 34 caves, cut out of the volcanic lavas of the Deccan Trap. Twelve are Buddhist, created from approximately 600-800 AD, 17 Hindu, created from 600-900 AD and five Jain, created from 800-1100 AD. The Hindu, Jain and Buddhist caves at Ellora are among the finest and most cherished in India.

1.4.1, Cave - 16:

Bat guano was collected from this cave. It is one of the largest and most spectacular rock cut mountain in the world. It is a part of Hindu caves.

1.5, Melghat forest ecosystem:

Semadoh is located in Melghat forest which is a dry deciduous forest. Bats generally roost on the tall trees in the forest.

The problem was investigated by considering the following -

1) Chemical analysis of bat guano with respect to trace metals, N. P. K. etc.

2) Microbial analysis of bat guano.

3) Impact of bat guano on water.

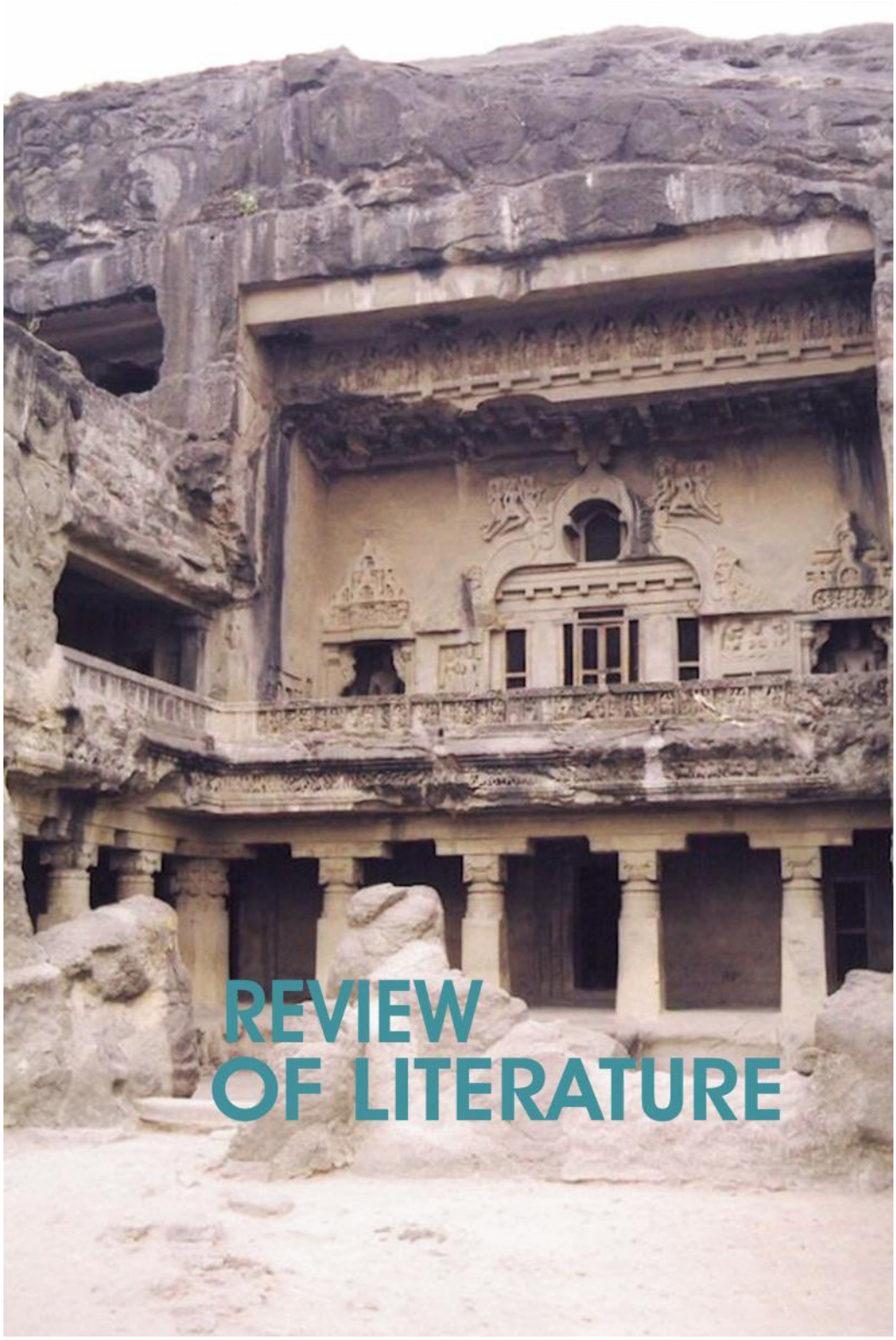
4) Impact of bat guano on soil.

5) Impact of bat guano on the industrial effluent and it’s use in bioremediation of aquatic ecosystem.

6) Impact of bat guano on plant growth.

7) Testing of bat guano for larvicidal activity.

8) Bat guano analysis for undigested remains.


REVIEW
OF LITERATURE

REVIEW OF LITERATURE

Guano of sea birds is known as a manure at international level. It is rich in NPK and contains several microbial species which are being tested for biodegradation processes. Several laboratories are busy in analysing the chemical and microbial contents of guano from different habitats.

Jackson (1973) analysed the bat guano deposited on sand with respect to the fresh weight, dry weight, pH, electrical conductivity, organic matter, total carbon, total nitrogen, C/N ratio, phosphate, potassium, calcium, magnesium.

Bat guano is rich in N, P, K and Ca and hence it is used as a supplimentary fertilizer in many parts of the world. However, what is more important is its microbial fauna. Steele (1989) showed a marvelous symbiosis between microbes and bats.

All mineral species are reported in bat guano by William et al. (1963); Hill and Forti (1986) at normal environmental temperature and pressure. But the most important factor that explained their diversity was the availability of the constituents chemicals. Some compounds came from the main bedrock constituents like nitrate, phosphate and sulphate develops mainly from the decomposition of bat guano.

International Bat conservation (1989 and 2001) reported that a single bat caught up to 600 moths, mosquitoes in a hour. A tablespoon of bat dropping (guano) contain hundrads of species of bacteria and many nutrients of great potential value to caves ecosystem. They also reported that bacteria

seen in the guano may soon be used to produce detergents and even gasohol and antibiotics.

The guanobitic and guanophilic arthropods community was observed in guano and it contained high NPK. NPK content help to improve soil quality and provide nutrients for plant growth (Ross, 2003; Moulds and Trimothy, 2006).

Microbial fauna, nitrogen and carbon stable isotopes (15N/14N and 13C/12C) and minerals (calcium, copper, iron, magnesium, manganese, phosphorous, potassium and zinc) are recorded from of bat guano of the big brown bat, Eptesicus fuscus (Poulson, 1963; Black, 1971; Cummins, 1994; Steinwascher, 1978; Beebee, 1991; Studier, et al. 1991; Beebee and Wong, 1992; Webb et al., 1995; Culver, 1994; Lajtha, and Michener, 1994; Stalinski, 1994; Holyoak and Sachdev, 1998; Fenolio, 2006).

Sulphates were determined in bat guano which were attributed to the depletion of the easily degradable organic material in the medium as reported by (APHA, 1989; Vogel, 1989; Christensen et al. 1996; Harera, 2001; Kamath and Procter, 1998; Chang, et al., 2000; Frank, 2000; Tarley and Arruda, 2004).

Organochlorine and organophosphate insecticide residues in bat guano were detected in all carcass samples (Allinson et al., 2006). There were also significant differences in the liver concentration of some metals like Ag, Cd, Co, Cu, Pb, Se, Zn between sexes within sites and between sites. Metals like arsenic, barium, beryllium, cadmium, cobalt, chromium, copper, lead, selenium, vanadium and zinc were estimated by Graening (2005) in cave stream sediment, having bat guano.

Young bats received their food through lactation from adult femals. Heavy metals mobility occured from the maternal to the young body, in which Cu and Cr were transferred effectively, but Pb and Cd were released only moderately from maternal tissue (Streit and Nagel, 1993).

Studier et al. (1991) analyzed nitrogen, sodium, calcium, magnesium, iron and potassium levels in big brown bat guano throughout summer roosting period.

Conde-Costas (1991) investigated water quality variation resulting from the introduction of large quantities of bat guano at the Cueva El Convento System. Many large colonies of bats, Brachyphylla coverarum, Monophyllus redmani and Mormoops blainvilli roosted throughout the cave and guano was estimated to be 6.4 kg/m/yr.

Fausch et al. (2002) analyzed stable isotopes of carbon (12C) and nitrogen (13N) from bat guano into the invertebrate food web by popular tools.

Decu (1981 and 1986) obderved that variation in pH between fresh and old guano deposits . They also noted that small increases in air temperature close to guano piles up to 140 C due to heat released from guano decomposition.

Fresh guano is commonly basic, with the pH varying according to the volume of urine deposited with faeces. Fresh guano commonly had a pH of 8.5 - 9.0 that rapidly became acidic (5.0 - 5.5) with age and depth, although the centre of guano piles had a stable pH of around 4.0 (Harries, 1970).

Urbani et al. (1967) all mineral species reported form at normal environmental temperature and pressure, but the most important factor that explained their diversity was the availability of the constituent chemicals, some components come from the main bedrock constituents (carbonate , silica), while others from minor or trace bedrock minerals (sulphate from pyrite oxidation), or from the overlaying soil (nitrate), while phosphate and sulphate develop mainly from the decomposition of bat guano.

Martin (1992) analyzed elemental composition of bat guano which reflected the residues present in the undigested portion of ingested prey species, and he remarked that they provided some clues as to the location of contaminants in the environment. Arsenic concentration in guano from Ozark cave, big-eared bats due to its insectivorous habitat. Concentration of selected elements found in grey bats and bat guano from Oklahoma bat caves were as Cd (3.9), Cr (57), Cu (149), Hg (0.54), Pb (23), Se (9.8), Zn (10.70).

Graening and Brown (2003) and Fry (1999) studied stable isotopes are now widely used in trophic of freshwater and cave stream ecosystems because naturally occuring carbon (13C) and nitrogen (15N) isotopes persist and accumulate in food chains, the technique can be used to decipher both diet and trophic position of a given organism (Fry, 1999). They collected multiple samples of bat guano, cave stream sediment; whole bodies of the larvae of E. spelaea; and composite samples of whole amphipods (Gammarus minus) from the cave system, and carried out nutritional analysis of collected samples of bat guano from different guano piles of Stansberry cave as well as caustic sediment and whole amphipods (G. minus).

Mitzutani et al. (1992) reported that bat guano is rich in calories and nutrients. To meet the energetic demands of flight and reproduction, insectivorous bats have evolved extremely short digestive tracts and rapid food transit times.

Seena et al. (2005) analyzed nutrients in bat guano (total nitrogen, phosphorus and potassium).

Poulson (1972) outlined bat guano ecosystems should be preserved as models for the study of communities.

Gnaspini (1989) comparative studies showed that the existence of some faunistic specificity to these types of guano whereas some taxa are indifferent, living on any kind of guano, others have preference or are restricted to one of these types such as hematophagous, frugivorous and insectivorous bat guano.

Aaranson (1970) determined the bacteria, actinomycetes and fungi from faecal pellets of bats.

Tisdale and Nelson (1975) assessed the efficiency of humus like bat gauno in crop production.

Shuxin et al. (1992) evaluated the uptake of NPK by the crops which were based on the dry mass and nutrient leves in plants .

Mathur et al. (1990) investigated the bat humus guano and manures of other animals. He found that nitrogen was higher and total carbon was lower in poultry, cow and sheep manuers resulting in narrow C/N ratio, while phophate was more in cow and sheep manures when compared with bat guano.

Investigations on the possibilities of using nonconventional organic manures like vermicompost, millipede compost, bat guano in agriculture are becoming popular. Several investigators are investing the occurrence of plant promoting microbes in bat guano and their application in agriculture (Kale et al.,1992; Ashwini and Sridhar, 2002; Sridhar et al. 2006).

Fenolio, et al. (2006) reported colonial bat guano as dominant energy resources in many cave ecosystems.

Influencing trophic dynamics, community structure and even physiological specialization (Harris, 1970; poulson, 1972; Gnaspini and Trajano, 2000). Densities of the cave adapted Eurycea spelaea (Bonett and Chippindale 2004) have been suspected to increase in the main rooms of some caves during summer months when grey bats (Myotis grisescens) utilize these caves as maternity roosts (Hendricks & Kezer 1958; Brandon 1971).

The digestive efficiency of myotid bats is only 69 -78%, resulting in the expulsion of unabsorbed nutrients in guano and urine (Webb et al.,1995; Stalinski, 1994).

Microbial biofilms that are formed on the bat guano which increases its nutritional value in much the same way that microbial conditioning of leaves increases their palatability and assimilation by detritivores (Allan, 1995).

Oligotrophy is thought to select for increased diet breadth in subterranean fauna (Culver, 1985; 1994; Holyoak and Sachdev 1998). Other studies determined that ozark cavefish (Amblyopsis rosae) and black bullhead catfish exhibited stable isotopic signatures of carbon (13C/12C) and nitrogen

(15N/14N) of Eurycea spelaea muscle (n=5) and Myotis grisescens guano (n=4) in two similar Ozark cave stream ecosystems.

The Ameiurus melas fed on bat guano in Ozark caves (Poulson, 1963; Black, 1971).

Coprophagy has been reported in larval frogs for the purpose of inoculation of their intestines by beneficial microbes (Steinwascher, 1978; Beebee, 1991).

Tuttle (1986) reported that the endangered Gray Bat (Myotis grisescens) was once one of the most abundant mammals of the southeastern U.S. people in at least five states, especially Alabama, Tennessee and Missouri. These bats played an important role in the checks and balances of nature as the primary controllers of night-flying aquatic insects, including mosquitos. Single colonies consumed literally tons of mosquitoes.

Bat guano was found to contain undigested remains of insect body fragments, hair, pollen and some mineral matter (Maher, 1997).

Tumbling creek cave's gray bats were studied extensively because of their large numbers and the importance of the nutrient input in the form of the guano (William and Aley, 2005).

Ansary et al. (1987) reported many asthamatic patients because of inhalation of bat droppings through cracks in the ceiling into the rooms below where it was inhaled and caused allergic respiratory disorders.

2.1, Bat guano and bioremediation:-

Barry et al. (1997) suggested that bioremeditation technologies have become popular in the early 1980's for site clean up. Land treatment

bioremeditation of soils followed neutralization or chemical and physical treatments. A comprehensive study was conducted to assess the effect of bat guano as a soil cleanser against fungi. Bat guano is a safe and environmental friendly alternative for harmful chemicals. The bacterial population, which is enhanced by the presence of bat guano, could prove to be a better bioremeditator to counteract harmful treatment of chemicals, which kill the beneficial microorganisms. Secondly it is 100% organic and natural. Coyane (1999) reported that the increase in the number of bacteria by the presence of bat guano, brought about biodegradation of toxic compounds in favourable environmental conditions.

Bat guano has been processed to create a powder with particles of less than 3 millimetres across and is odourless. This biological product contained live microbial flora which, when incorporated in the soil, acted on the organic matter to make nutrients available to the plants, including NPK, calcium and magnesium and all trace elements (Omer, 2004). Bat guano has a high humus content, which makes it an excellent soil builder (Lacki et al., 1994).

Pierce (1999) studied the biochemical nature of bat guano and suggested that it can be used as good plant fertilizer (10-3-1 NPK composition), soil builder (improves texture and richness), Lawn treatment (Promotes growth and healthy colour), soil cleaner (bioremeditation microbes help clean up toxic residues), fungicide (combats fungus through foliar feeding), nematocide (decomposing microbes help control nematodes)and compost activator (decomposing microbes expedite composting processes).

Bat guano is very efficient in the nitrification process and to remove nitrogen from industrial waste before it reaches our lakes and rivers (Tuttle, 1986).

The NPK requirments of plantation crops amending Farm Yard Manure (FYM) with humus guano of insectivorous bats in appropriate ratio may help overcome the nutrient deficiency to improve production (Thampan, 1993).

Bat guano stimulates the potassium/silica bacteria in the soil to enable it to work more effectively with the growth forces. Silica makes the plants more inwardly sensitive. Can help increased flowering and frutification. Stimulates the phosphorous process and mobilizes the phosphorus activating bacteria in the soil (Lacki et al., 1994).

2.2, Bat guano as manure:-

Goveas et al. (2006) analyzed bat guano to understand the habitat preference, food habits, and also in developing strategies to conserve bat habitats. The study revealed higher NPK in bolus and guano (3.3:4.3:0.7) vs (2.6:4.2:0.6) of flying fox. As they are frugivorous, phosphorus is fairly high in guano, indicating to meet phosphorus requirements of plants. Phosphorus rich guano is known to induce root growth and flowering in plants. The pH of guano flying fox is close to natural range (7.1 - 7.3). The occurrance of high proportion of bacteria and fungi in guano enhanced decomposition of organic matter in soil on application.

Jackson (1973) and Karen (2003) evaluated that the bat guano and seabrid guano are some of the best organic fertilizers in the world today.

Guano has been used by farmers and growers since a long time. Many civilizations have used guano for soil amendment.

Organic fertilizer is used to improve soil quality and tilth, and to provide nutrients for plant growth. They provide nitrogen, phosphorus, and potassium, as well as other elements essential for plant development and overall good health (Karen, 2003).

Quillen (2001) reported that bat guano was used to make fertilizer.

Dumitras et al. (2003) investigated that calcium phosphates (i.e., hydroxylapatite, carbonate hydroxylapatite, brushite and ardealite) are the most represenative mineral species found in the bat guano piles.

Several organic manures of animal origin are available for use e.g. night soil, bovine dung and urine, sheep manure, poultry manure, bat guano, silkworm wastes and vermicompost. Availability of such manure for crop production is restricted due to many constrains such as geographical region, awarness of manure value, extent of manure production and management. There is ample scope to assess and utilize non-conventional organic manure in agriculture.

Recently, the use of bat guano as composting organic matter has been proposed by Ashwini and Sridhar (2002, 2003). William et al. (2000) stated that natural organic fertilizers are inherently low in available nitrogen levels ranging, between 2 and 8%. However, bat guano contained 10-12% nitrogen levels. These relatively low nitrogen levels made it necessary to apply inordinately high amount of material to achive the desired seasonal

effect since all natural organics depend upon soil microbial activity for nitrogen and mineral release.

There is a long history of bat guano used as an agriculture fertilizer. The nutrient content of guano was based on the diet of the bats, which thrive largely on insects and on fruits. Guano is advertised as being quite safe and non burning to plants (Kuepper, 2000).

Bat guano fertilizes plant growth which created more ground support larger detritivore beetle populations, which in turn supplemented population of their direct predators (Sanchez and Polis, 2000). These effects cascaded along the food web influencing higher consumers such as shrub and canopy invertebrates, reptiles, small mammals and bird populations (Hocking and Reimchen, 2002). They concluded that nutrients from bat guano were significant to the forest invertebrate food web.

Jackson (1973) studied the efficiency of bat guano on plant growth. The pots in which seeds of finger millet and black gram were sowed, watered twice a day upto 3 weeks and 6 weeks respectively. On uprooting the seedlings, shoot and root lengths were measured. Plants were dried, ground and assessed for the total nitrogen, phosphorus and potassium.

2.3, Food habits of bats: -

Rachel (1999) observed that bats are known predators of nocturnal flying insects, and many of these are agricultural pests. The big brown bat, Eptesicus fuscus, in the Midwest consumed cucumber beetles, Diabrotica undecimpunctata; June bugs, stinkbug, pentatomidae; and leafhoppers, cicadellidae. Many species of bats feed on moths whose larval forms are pest caterpillars (Whitaker, 1998). The twenty million Mexican free-

tailed bats, Tadarida brasiliensis, from Bracken Cave, Texas, eat 250 tons of insects each night (McCracken and Gustin, 1987; Tuttle, 1998; Whitaker, 1993). The enormous number of insects consumed by bats has led to the speculation that bats can be a force in agricultural pest control (Murphy, 1993; Whitaker, 1993; Tuttle, 1995). Bats can also chase insect pests away from crops. A number of moths, including cutworms, armyworms, and cotton bollworms are sensitive to bat echolocations up to 131 ft away, and turn away or dive to the ground when exposed to pulsed sounds (Roeder, 1964; Roeder, 1967; Agee, 1969).

Food habits of bats were determined by examining individual bat guano fecal pellets. This approach is reliable for bat work, as all flying insects contain chitin, which is mostly indigestible to bats. As a result, what passes through a bats digestive system are insect parts such as legs, wing and antennae, which can be identified to insect order and sometimes family. (Whitaker and Kunz, 1988 and1994; Long et al., 1996).

Ross (1967) observed that bats chew their food thoroughly before swallowing that everything in the guano pellets can be identified. Whitaker (1988) assesed the frequency with which bats feed on each insect group was determined by counting the number of pellets that contained a particular insect and dividing that number by the total number of pelletts.

Bat guano has been assessed to understand bat diets and food habits (DesMarais et al.,1980; Fenton et al.,1998 and Korine et al.,1999); Nitrogen and mineral budgets (Studier et al.,1994); seed dispersal (Korine et al., 1999); habitat preference, flight activity (Zielinski and Gellman, 1999).

Trees, buildings and caves are the ideal habits of bats and such roosting or swarming sites are of great significance for bat diversity and conservation (Parsons et al., 2003).

The twenty million Mexican bats, Tadarida brasiliensis from Bracken Cave, ate 250 tons of insects (moths including cutworms, armyworms and cotton bollworms) during each night (Belton and Kempster, 1962; Roeder, 1964; McCracken and Gustin, 1987; Tuttle, 1998).

Although several studies have examined the arthropod communities that are associated with bats in Texas caves, more work has been conducted on bat guano communities and its diversity (Palmer and Gunier, 1975; Whitaker, 1995; Jellison and Kohls, 1995; Bernath and Kunz, 1981).

Among the aerial insectivorous, Peropteryx macrotis, Pteronotus parnellii, Natalus stramineus, and Furipterus horrens are the representative Brazilian cave bats. Neotropical bat guano communities are very intresting due to the bat feeding diversity (Wikson et al., 1988; Gnaspini-Netto, 1989; Trajano and Moreira, 1991; Gnaspini, 1992; Campanha and Fowler, 1993; Bredt et al., 1994).

Power and Rainey, (2000) investigated the incorporation of river derived nutrients deposited via bat guano in redwood tree hollow into the terrestrial invertebrate food web and observed that insectivorous bats which fed over the river on aquatic emergent insects translocated river derived nutrients several kilometers into the forest where they roosted in rocks and large trees.

Vanlalnghaka et al. (2005) reported onset and end of activity of the old frugivorous bats, Rousettus leschenaulti roosting in temples of the Lonar crater.

2.4, Bat guano is bacteria rich: -

Tilak et al.(2005) examined a number of bacterial species associated with the bat guano belonging to genera Azospirillum, Alcaligenes, Arthrobacter, Acinetobacter, Bacillus, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Pseudomonas, Rhizobium and Serratia. All this bacteria exert a beneficial effect on plant growth.

Codd et al. (1999) proposed that bat guano aid the soil in proper fermentation, thereby keeping the bacteria and microorganisms within the soil. Bat guano stimulates potassium, silica, selenium activating bacteria and helps combine sulphur with other substances. Aids the soil in connecting with planetary rythyms.

Bat guano helps balance energies to protect against fungal diseases (Kwiecinski et al., 1987; Studier et al.,1991; Barclay, 1994; 1995; Bernard and Davison, 1996).

DesMarais et al. (1980) analyzed molecular composition of the bat guano hydrocarbons which were fully consistent with an insect origin and the feeding habits of the insects that supported the bat.

Ashwini and Sridhar (2002) and Sridhar et al. (2006) assessed the physicochemical characteristics and microflora of guano of an endemic cave and old temple dwelling bat (Hipposideros speoris); and also assessed the feasibility of amending soil with bat guano to increase crop production.

Hutchens et al. (2004) demonstrated that cave atmosphere contained 1-2% methane, although much higher concentration are found in gas bubbles that keep microbial mats float on the water surface was hypothesized aerobic methane-oxidizing bacteria, Methylomonas and Methylococcus.

These organisms possess enzymes capable of degrading a number of substances. Countless microbes are regularly excreted along with waste products and together with soil organisms, they constitute the microbial population of bat guano and play a crucial role in maintaining the delicate ecological balance of the earth.

Chemical analysis of the guano revealed rich in carbon, nitrogen and vital minerals. When the bat guano was added in water, a nutritious broth was formed, supporting the growth of numerous organisms (Murphy, 1989)

An ounce of bat guano contains billions of bacteria, and a single guano deposit, may contain thousands of bacterial species. These bacteria are important to soil composition and enable plants to absorb nutrients and essential elements (Mistry, 1995; Alley and Mary, 1996).

Miranda (2005) investigated the ecological values of flying foxes, with respect to nutrients and microbial composition of guano.

Seena (2005) carried out the nutrient and microbiological analyzes of bat guano. They studied the bacteria, Actinomycetes and fungi from bat guano.

According to Sridhar, et al. (2006), bacteria, actinomycetes and fungi were highest in bolus. Bacteriological examination revealed the presence of Acaligenes and Pseudomonas in guano and Bacillus, Klebsiella

and Proteus in bolus. Among actinimycetes, Streptomyces were common in guano and Micromonospora in bolus. Fusarium and Penicillium were the most common fungi in guano as well as in bolus.

Applications of the biodynamic preparations, bat guano aid the soil in proper fermentation, thereby keeping the bacteria and microorganisms within the soil. Bat guano stimulates potassium, silica, selenium activating bacteria and helps combine sulphur with other substances. Aids the soil in connecting with planetary rythms. It helps in the retaintion of nitrogen and calcium, keeping them in the living realm and prevents loss to the atmosphere, also stimulates boron, as well as azatobacter activity, the best bacteria for making nitrogen in the soil. Helping proper decomposition, the bacteria in guano aids chlorophyll formation and stimulates iron, potassium, calcium, magnesium and sulphur activity in the soil (Lacki et al., 1994). Helps calcium and phosphorus work into the earth in a living form, also helps balance energies to protect against fungal diseases.

Guano acts as a nutrient medium for the growth of Desulfotomaculum nigrificans. Characterisation and sulphate reduction studies revealed that metal sulphides are oxidized chemically and biologically to produce large ammount of dissolved metal, sulphates. The technique involved the development of bacterial sulphate reduction which generated the formation of H2S gas by using simple carbon sources such as ethanol and the precipitation of heavy metal sulphides (Wakao et al., 1979; Weider et al., 1985; Weider and Lang, 1986; Hansen, 1988; Fauque et al., 1991; Martin, 1991; Dvorak et al., 1992; Edenborn and Hedin, 1992; Bechard et al., 1994; Hedin et al., 1994; Hammack et al., 1994; White and Chang et al., 1996;

Christensen et al., 1996; Gladd, 1996; Frank, 2000; Kaksonen et al., 2003; Vallero et al., 2003; Boshoff et al., 2004).

Edwin (1965) isolated dermatophytes Candida sp., Cladosporium sp., C. immitis, C. neoformans, H. capsulatum, M. gypseum, T. mentagrophytes, T. rubrum, T. terrestre and Sporotrichum sp. from fresh guano of Mexican freetail bats.

Takashi et al. (2005) identified 700 yeast like colonies, containing Trichosporon laibachii and T. porosum from caves inhibiting bats. In addition to these yeasts, eight ascomycetes yeast like Candida palmioleophila, C. lusitaniae, Debaryomyces hansenii, Hanseniaspora spp. Saccharomyces cervisiae, S. kluyveri, Williopsis californica and Zygosaccharomyces florentinus and one basidiomycetous yeast, Cryptococcuspodzolicus were isolated from the bat guano samples from 20 caves.

2.5, Bat guano as food:-

Different invertebrate species within guano ecosystems prefer different micro-habitats (Richards, 1971).

Fenolio et al. (2006) during two year population ecology studies in a cave environment observed Eurycea spelaea ingesting bat guano. Eurycea spelaea numbers increased significantly when grey bats Myotis grisescens deposited fresh guano. Nutritional analyses revealed that guano is a comparable food source to potential invertebrate prey items.

Harris, (1973) analyzed sample taken from a heap of bat guano in a small cave chamber. The guano heap was formed over many years of cyclical roosting in the chamber by several thousand bent winged bats, whose

faeces and urine formed the source of energy for the permanent community of organisms in the heap.

Influence of bat guano was studied in forest system, and a relationship between bat guano and invertebrate population were studied by Peck (1980, 1981 and 1982). Bat guano entered the food web of cave invertebrate by detritivores feeding directly on bat guano. This inputs of nutrients supplemented detritivore as well as predator populations. Dipterans are also commonly associated with bat guano cave.

Ferreira and Martins (1999) found predator spider population in caves exibiting more abundance and species richness. Guano inputs increased detritivore density.

Trajano et al. (1991) observed that the guano of bats is an important food source for cavernicoles throughout the world due to the generalized food scarcity in caves.

Gnaspini (1989, 1992 and 2005) showed that some species are totally dependent on guano for its existence, they are called guanobites and may perish if the guano input falls below certain limits.

Elliott and Veni (1994) published that the scientific resources of Texas caves are many hundrads of ancients species, specially adapted to an energy efficient life in permanent darkness are scattered through the karst of Central Texas. Cave adapted salamanders, catfishes, shrimps, isopods, amphipods, snails, spiders, harvestmen, pseudoscorpions, beetles, millipedes, centipedes, and other types have been described (Elliott et al., 1994).

Invertebrate communities associated with bat guano were seen to be increased in density or 'pulse' after the bats appear and deposit fresh guano (Poulson and Lavoie, 2000).

Bat guano is a preferred food of several cave dwelling animals which includes, salamander, cockroach, grasshopper, frogs, beetles etc. (Chippindale, 2005).

Timothy et al. (2004) reported arthropod community in guano of the Inland cave bat (Vespadelus findlaysoni) roosting in the abundoned Eregunda mine, located east of Blinman in the central Flinders Ranges, South Australia. This guano community was seen to be remarkable. Meat ants (Iridomyrmex purpureus) were observed to enter the mine to collect fresh guano. Bat guano is eaten directly by many guanobitic and guanophilic invertebrates as high nitrate food and more readily digested glycogen rich bacteria and fungus.

Collembola and mites are found to make up a large percentage of macroscopic fauna associated with guano in caves (Chapman, 1983) and both showed to respond to pulse inputs of bat guano in cave systems (Martin and Poulson, 1976; 1980 and 1992).

Distinct kinds of bat guano have been recognised in the cave. These include frugivorous guano originating from fruit eating bats and insectivorous guano originating from insectivorous bats. Frugivorous guano is decomposed and consumed via bacteria-nematode food chain while insectivorous guano is broken down through a fungi-mite food chain. The guano of Tamana are heavily populated with cockroaches that feed either directly on the guano or on fungi growing on the guano (Kenny, 1979).

Invertebrates, especially arthropods, make up the majority of all cave organisms. There have been few biospeleological surveys of invertebrates in the southern United States (Barr and Reddell, 1967; Welbourn 1978; Northup et al., 1995; Cokendolpher and Polyak, 1996). In addition, there were surveys of Lava caves in New Maxico and Arizona (Peck, 1982; Northup and Welbourn, 1997). Published studies of Arizona caves invertebrates in the Grand Canyon (Peck, 1980; Dorst and Blinn, 1997), the earth cracks caves Wupatki National Monument (Welbourn, 1979; Muchmore, 1981), and four Lava caves in the vicinity of Flagstaff (Peck, 1982) indicated that invertebrates fed on bat guano.

Kartchner Caverns offered a unique opportunity to establish a baseline survey of the invertebrate cave fauna before the cave was developed, and this survey should allow future studies to assess the effects of commercial development on the fauna (Jagnow, 1999; Tenen , 1999).

The guano piles that were refreshed regularly supported more moul growth and higher nematode and arthropod populations. The first M. velifer guano of the year stimulated visible mold and bacteria growth and this was followed by an increase in nematode and mite populations. The most abundant guano arthropod was Sanassania sp. When fresh guano accumulated at a site, the dormant Sancassania sp. developed rapidly into adults and began their reproductive cycle. Sancassania sp. can be reared on fungus and yeast, but they also feed on insect eggs and larvae (Hughes, 1976) and occasionally nematodes. Hughes (1976) reported that S. berlesei (Michael) completed it’s life cycle in 8-9 days at 220c and 100% relative humidity and that a female could produce over 1000 eggs in 39 days.

After the bats moved to another roost or migrated, the invertebrate fauna of the guano changed when food resources became depleted. Most Sancassania sp. stoped development at the non feeding deutonymphal instar. The deutonymph in many Acaridae can survive long periods without food (Evans, 1992).

Thirty eight invertebrate species were recorded during the study of Kartchner Caverns including 4(11%) troglobites, 19(50%) troglophiles, 1(8%) trogloxene and 12(32%) accidentals. The remaining species, one was an obligate parasite and the other a guanophile. Most of the cave fauna depended upon guano deposited by a summer colony of Myotis velifer. Mites were the most numerous arthopods found in Kartchner Caverns, with at least 14 species restricted to the annual guano cycle.

Hill (1969) and Darlington (1970) reported that bats dominated the caves, particularly the deeper part. Eleven species have been recorded as permanently roosting in the main cave. Pteronotus and Mormoops were confined to the deep part of the cave while Phyllostomus appeared in the upper part of the cave. Other species such as Natalus and Carollia, seemed to be widely distributed throughout the caves. The nectar drinking species, Anoura, was particularly common in the boulder chamber adjacent to the walk-in. Cave guano consisted essentially of arthropods including spiders, cockroaches, crickets, and reduviid bugs.

Cooper (1969, 1975) elucidated the rich aquatic community in Shelta Cave, with three species of extremely long-lived crayfish with low reproductive rates, a shrimp, cavefish, and numerous other troglobites. The

aquatic system was dependent on food from bat guano, and it declined after the bats vacated.

The diet of bat may be haematophagous, insectivorous, frugivorous, or nectarivorous (Gnaspini, 1992; Ferreira and Martins, 1998; Ferreira and Martins, 1999).

Birds and bats are common guano producer in the Northern parts of South America, providing an important energy resource for many cavernicolous animals (Snow, 1975; Trajano and Gnaspini, 2000).

Arthropods in guano communities fed either directly on guano or, more commonly, upon fungus growing on guano deposits. Guano required fungi and bacteria for its partial breakdown before it can be used by the majority of arthropod consumers (Gillieson, 2000).

Low humidity resulted in rapid desication of fresh guano severely reducing fungal growth as many of the opportunistic phycomycetes found on fresh guano were susceptible to drying out (Poulson et al., 1995; Poulson and Lavoie, 2000).

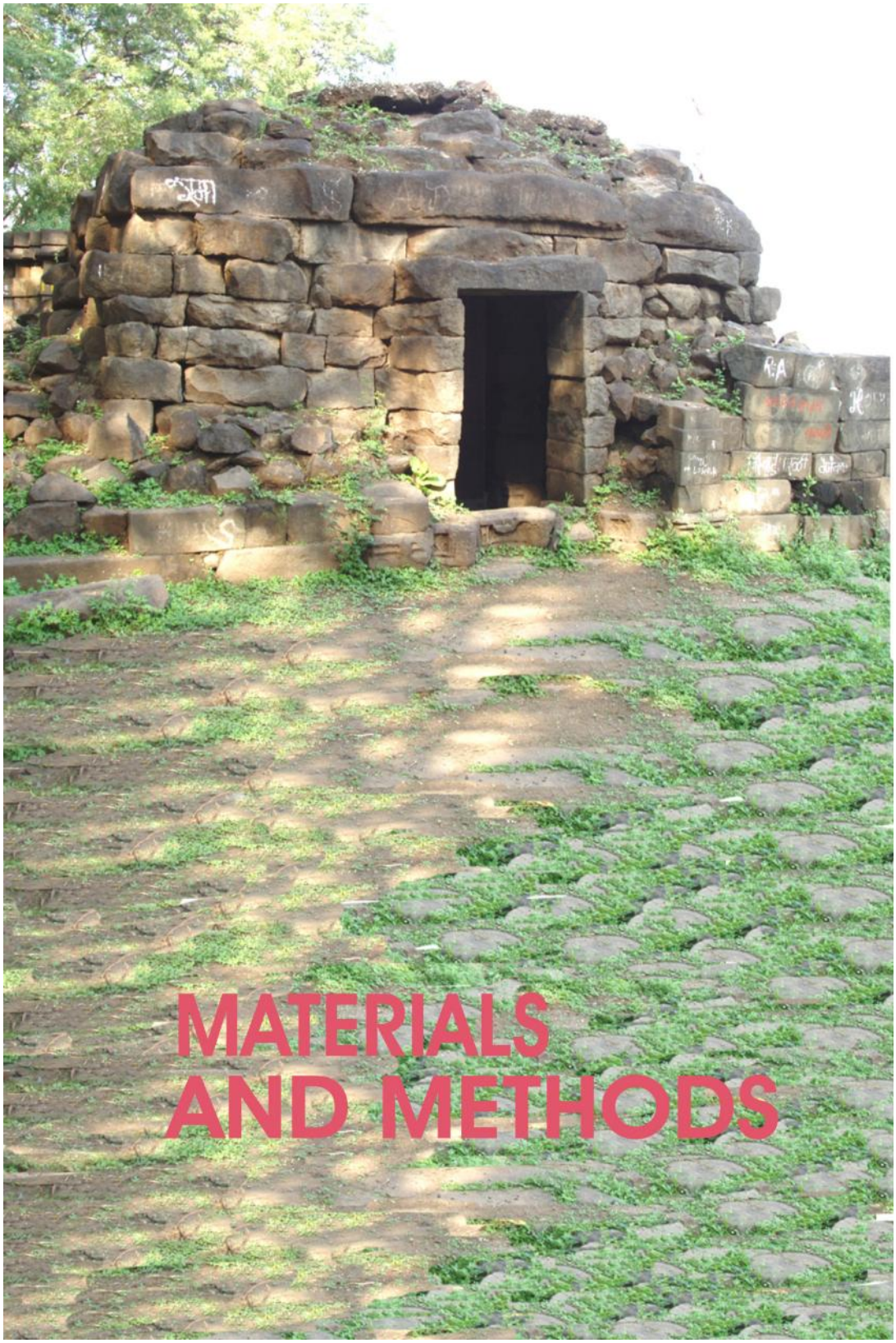
Guano food webs are based upon a guano source and associated fungi and other microorganisms directly supporting guanivores and fungivores including Phoridae (Diptera), Anobilldae (Coeleoptera), Tineidae (Lepidoptera), Collembola and Mesostigmatid mites (Acarina). Specialised parasites and parasitoids are also active in many guano ecosystems. Braconid wasps (Hymenoptera) were found in several Eastern Australian guano caves (Austin and Dangerfield, 1992).

The minute eggs of the guanobite, Derolathrus sp. (Coleoptera: Jacobsoniidae) hidden in guano are parasitised on small Myrmarid wasps

(Naumann, 1991). Some of the most numerous taxa associated with guano deposits are mites, perticularly from the families Gamasidae, Actinedidae, Oribatidae and Arrnadillidae (Womersley, 1963a). Extremely high numbers have been recorded on fresh guano (Bellati, 2001 andBellati et al.,2003). The guano dependent arthropods located within bat caves were identified during the 1950's and 1960's (Womersley, 1963b).

Maher (1997) bat dropping accumulated in caves and the resultant guano contains stratigraphic record of the environment, analogous to the record from lake sediment and peat. In some situation the guano could reached depth of meters in hundreds to thousand of years and it was valuable chronostratigraphycally.

Thus bat guano has been studied extensively in the light of its manuring capacity but there are very few reports from India. Therefore, after a preliminary analysis of bat guano from various habitats, the guano from Lonar crater habitats was selected for the detail study to know its microbial fauna and their impact on the associated ecosystems.



MATERIALS AND METHODS

MATERIALS AND METHODS

Bats inhabit caves, old temples as well as they are mostly roosting on the tall trees in open forest. They may be frugivorous or insectivorous. Depending upon their feeding habits, their faecal matters (guano) differ in chemical contents. The present experimentations were designed to analyse the chemical and biological contents as well as undigested remains in bat guano. The methods adopted are the same for all the guano samples collected from different habitats.

Then several experiments were carried out to know the larvicidal capacity of bat guano. Experiments were also carried out to know its capacity as manure. An attempt is also made to know its use in waste water treatment and bioremediation.

3.1, Collection of bat guano:

The bat guano was collected from three different habitats as follows:-

1) from old temples (Morache temple and Waghache temple) of Lonar crater (District - Buldhana).

2) from Ajantha caves (cave No. 7 and 24) (District - Jalgaon).

3) from Ellora cave (cave no. 16) (District - Aurangabad).

4) from Semadoh (Melghat forest) (District - Amravati).

3.2, Appearance and nature of guano:

The bat guano collected from Lonar crater temples was in the form of flakes and black brown in colour. However, the guano collected from Ajantha and Ellora (Werul) caves was beaded pellets in the form with pale

brown in colour and are light in weight. The guano collected from Semadoh appeared flattened pellets in form with dark cherry brown in colour and light in weight (Plate, 4.1).

3.3, Chemical analysis of bat guano:

For the chemical analysis of bat guano, 10 mg bat guano was dissolved in 100 ml deionized water every time. The undigested substances from the solution were discarded after filtration. Then filterate was analysed for its sodium (Na), potassium (K), calcium (Ca), inorganic phosphorus (iP), magnesium (Mg), manganese (Mn), copper (Cu), Iron (Fe), zinc (Zn), aluminium (Al), selenium (Se), barium (Ba), chloride content, nitrogen, nitrate, nitrite, phosphate and sulphate.

The chemical analysis of bat guano was carried out by using following standard methods (Table: 3.1)

3.3.1, Sodium:

Sodium was determined by the use of flame photometer. When the sample is sprayed into a flame, a characteristic light is produced due to electron excitation whose intensity is directly proportional to the concentration of sodium which can be read at 589 nm using suitable filter.

3.3.1.1, Reagents:

i) Standard NaCl solution: 2.542 g NaCl is dissolved in deionized water. This solution is made to 1 liter by adding deionized water. This solution has sodium concentration of 1000 mg/L (1 ml = 1 mg K).

3.3.1.2, Procedure:

Start the compressor and light the burner of the flame photometer. Air pressure is maintained at 51 lbs. Adjust the gas flow so as to

get a blue conical flame. Filter the sample, feed deionized water and set zero again. Feed standard stock solution and set 100. Recheck both the points. Now feed the sample and read the meter reading. Express the result as mg/L.

3.3.2, Potassium:

Potassium is determined by flame photometer. The intensity of light emission linearly corresponds to the concentration of potassium being spread by the flame which is read by suitable filter for potassium at 768 nm.

3.3.2.1, Reagents:

i) Standard KCl solution:1.907 g NaCl is dissolved in deionized water. This solution is made to 1 liter by adding deionized water. This solution has potassium concentration of 1000 mg/L (1 ml = 1 mg K).

3.3.2.2, Procedure:

Start the compressor and light the burner of the flame photometer. Air pressure is maintained at 51 lbs. Adjust the gas flow so as to get a blue conical flame. Filter the sample, feed deionized water and set zero again. Feed standard stock solution and set 100. Recheck both the points. Now feed the sample and read the meter reading. Express the result as mg/L.

3.3.3, Calcium:

Calcium is determined by the use of flame photometer. The intensity of light emission linearly corresponds to the concentration of calcium being spread by the flame which is read by suitable filter at 540 nm.

3.3.3.1, Reagents:

i) Standard $CaCO_3$ solution: 2.5419 g $CaCO_3$ dissolved in 50 ml of deionized water add dropwise a minimum volume of HCl (about 10 ml) to

complete solution. This solution is made 1 litre by adding deionized water. This solution has calcium concentration of 1000 mg/L (1 ml = 1 mg K).

3.3.3.2, Procedure:

Start the compressor and light the burner of the flame photometer. Air pressure is maintained at 51 lbs. Adjust the gas flow so as to get a blue conical flame. Filter the sample, feed deionized water and set zero again. Feed standard stock solution and set 100. Recheck both the points. Now feed the sample and read the meter reading. Express the result as mg/L (1 ml = 1 mg K).

3.3.4, Nitrogen (Kjeldahl Nesslerization Method):

All the nitrogen is converted into Ammonium sulphate when digested with H2SO4 and K2SO4. The nitrogen in the form of (NH4)2SO4 can be determined by distillation.

3.3.4.1, Reagents:

a) Sulphuric acid: H2SO4 conc. (Sp.gr.1.84).

b) Copper sulphate solution:10 g of copper sulphate is dissolved in 100 ml of distilled water.

c) NaCl solution:10 g of NaCl is dissolved in 100 ml of distilled water to have 10% solution.

d) Potassium sulphate

e) NaOH (10N).

f) NaOH (5N).

g) Hydrochloric acid (0.01N).

h) Phenolphthalein indicator

i) Boric acid and Mixed indicator: 4 g boric acid is dissolved in 100 ml hot distilled water. Alcoholic solution of Bromocresol green (0.5%) and methyl red (0.1%) is mixed in the ratio of 2:1:5 ml of mixed indicator is added in 100 ml of boric acid.

3.3.4.2, Procedure:

3.3.4.2.1, Digestion:

40 ml of sample water is taken in a 100 ml Kjeldahl flask. 4 ml of H_2SO_4, 0.3 ml of $CuSO_4$ solution, 6 g of solid K_2SO_4 and 1 ml of 10% NaCl solution is added in the flask.

Flask is kept on a heater and heating is started. Water boils off and the sample turns from black to pale green. Heat it for further half an hour. Flask is cooled and the volume is made upto 100 ml.

3.3.4.2.2, Distillation:

25 ml of digested sample is taken and distilled with 10N NaOH. Distillate is titrated against 0.01N HCL in the presence of boric acid and mixed indicator. The colour changes from blue to pink or brown. A blank is also run with distilled water using same amount of chemical.

3.3.4.3, Calculation:

$$\text{Nitrogen (mg1-1)} = \frac{(x-y) \times 0.01 \times 100 \times 14 \times Z}{\text{ml of sample distilled}}$$

Where,

x = ml of HCL used with sample.

y = ml of HCL used with blank.

z = dilution factor 2.5.

3.3.5, Inorganic Phosphate (Fiske and Subbarow method):

3.3.5.1, Principle:

The filtrate obtained after removing proteins by means of trichloroacetic acid is treated with an acid-molybdate reagent which reacts with inorganic phosphate to form phosphomolybdate acid. The hexavalent molybdenum of the phosphomolybdate acid is reduced by means of 1,2,4-aminonaphtholsulphonic acid to give blue compound which is estimated colorimetrically.

3.3.5.2, Requirements:

a) Trichloroacetic acid (20%): Dissolve 20 g in water and make up to 100 ml. The quantity of trichloracetic acid is important.

b) Sulphuric acid 10N: Add 450 ml of concentrated sulphuric acid slowly to 1300 ml of distilled water.

c) Molybdate I: 2.5% ammonium molybdate in 5N sulphuric acid. Dissolve 25 g of the salt in about 200 ml of water, transfer to liter flask containing 500 ml of 10N sulphuric acid. Dilute to 1 litre with water.

d) Molybdate II: 2.5% ammonium molybdate in 3N sulphuric acid. Dissolve 25 g of the salt in about 200 ml of water, transfer to litre flask containing 300 ml of 10N sulphuric acid. Dilute to 1 liter with water.

e) Sodium bisulphite solution (15%): Dissolved 150 g of sodium bisulphite in 980 ml of distilled water. Filter from any suspended matter, and keep well stoppered.

f) Sodium sulphite solution (20%): Dissolved 200 g of sodium sulphite in 980 ml of distilled water. Filter from any suspended matter, and keep well stoppered.

g) 1,2,4-aminonaptholsulphonic acid (0.25%): Add 0.5 g of the dry powder of aminonaptholsulphonic acid to 195 ml of 15% sodium bisulphite and 5 ml of the 20% sodium sulphite. Stopper and shake until it is dissolved and keep in brown bottle at cool place .

h) Standard phosphate solution: Dissolved 0.351 g of pure potassium dihydrogen phosphate in deionized water in a litre flask, add 10 ml of 10N sulphuric acid and make to the mark with water. 5 ml contain 0.4 mg phosphorus.

i) Dilute standard for use: Dilute the stock standard 1 to 10.1 ml contain 0.008 mg phosphorus.

3.3.5.3, Procedure:

Dry the faeces, determine their weight, powder well.

1g powder faeces and ash in an electric furnance at a temperature of about 4000 C, rather below red heat.

Dissolve the powder in 25 ml of 20% trichloroacetic acid, filter into a 200 ml flask, washing in with further trichloroacetic acid up to 25 ml and then make up to the mark with water.

Pipette 5 ml of filtrate into a 10 ml stoppered graduated cylinder. Add 1 ml of molybdate II reagent and mix. Then add 0.4 ml of the aminonaphtholsulphonic acid and make to the mark with distilled water.

At the same time put up a standard. Pipette 5 ml of the standard phosphate solution, equivalent to 0.04 mg phosphate, into a similar cylinder, and add 1 ml of molybdate I reagent and 0.4 ml of aminonaptholsulphonic acid. Mix gently after each addition and make to 10 ml. After standing five minutes read, using a red filter or transmission at 680 nm.

3.3.5.4, Calculation:

Since 5 ml of standard contain 0.04 mg phosphorus and 5 ml of filtrate is equivalent to 1 ml of sample.

$$\text{Inorganic phosphorus mg/100 ml} = \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0.04 \times 100/1$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 4$$

3.3.6, Chemical analysis of Bat guano by Atomic Absorption Spectrophotometry :

3.3.6.1, Preparation of standards:

3.3.6.1.1, Magnesium:

Dissolved 10.013 g magnesium sulphate heptahydrate, (MgSO4, 7H2O), in 200 ml deionized water, and 1.5 ml conc. HNO3 and made upto 1000 ml with deionised water; 1 ml = 1 mg.

3.3.6.1.2, Manganese:

Dissolved 3.076 g manganous sulphate (MgSO4, 7H2O), in about 200 ml deionized water, add 1.5 ml conc. HNO3 and diluted to 1000 ml with deionised water; 1 ml = 1 mg Mn.

3.3.6.1.3, Copper:

Dissolved 1.0 g copper metal in 15 ml of 1 + 1 HNO3 and diluted to 1000 ml with deionized water; 1 ml = 1 mg Cu.

3.3.6.1.4, Iron:

Dissolved 1.0 g iron wire in 50 ml of 1 + 1 HNO3 and diluted to 1000 ml deionized water; 1 ml = 1 mg Fe.

3.3.6.1.5, Zinc:

Dissolved 1.0 g zinc metal in 20 ml of 1 +1 HCl and diluted to 1000 ml with deionized water; 1 ml = 1 mg Zn.

3.3.5.1.6, Aluminum:

Dissolved 1 g aluminium metal in 20 ml conc. HCl by heating gently and diluted to 1000 ml with deionized water; 1 ml = 1 mg Al.

3.3.6.1.7, Barium:

Dissolved 1.77 g barium chloride, $BaCl_2, 2H_2O$ in about 200 ml deionized water, added 1.5 ml concentrated HNO_3 and diluted to 1000 ml with deionized water; 1 ml = 1 mg Ba.

3.3.6.2, Procedure:

Atomic Absorption Spectrophotometer was set as per manufacturer's instructions and aspirated the sample and standard solutions directly into an air acetylene.

Copper, iron, magnesium, manganese, zinc are determined using an air acetylene flame. Aluminium, barium, selenium are determined by directly aspirating into a nitrous oxide-acetylene flame.

Using the correct burner and recommended wavelength, lamp current, slit width and gas flow rates, 1 ppm solution of a metal was aspired to maximise the signal by varing the wavelength, aspiration rate, gas mixture and burner position.

3.3.6.3, Calculation:

For soil and biological materials the concentration of metal is calculated by the following formula:

$$C = \frac{(A - B) \times 50}{W}$$

Where,

C = Concentration of metal in the soil mg/kg.

A = Concentration of metal in the 50 ml extract as determined from the calibration curve in mg/L.

B = Blank concentration of the extractant solution or acid digestant as determined from the calibration curve in mg/L.

W = Weight of soil in gram.

3.3.7, Chloride and Salinity:

Silver nitrate reacts with chloride to form very slightly soluble white precipitate of silver chloride. At the end point when all chloride gets precipitated, free silver ions react with chromate to form silver chromate of brick red colour.

3.3.7.1, Chemicals:

a) Silver nitrate titrant (0.0141N)

Dissolved 2.395 g of silver nitrate in distilled water and diluted to 1 liter, stored in dark bottle.

b) Potassium chromate indicator

Dissolved 10 gm potassium chromate in a little distilled water. Added silver nitrate solution to produce red precipitate. Precipitate was allowed to settle overnight then filtered and diluted to 200 ml with distilled water.

3.3.7.2, Procedure:

Chlorides were determined by Mhov's argentometry method (APHA, 1998) 50 ml of water sample was taken in a flask and to it 5 drops of K2CrO4 solution were added, that gave yellow colour to the sample. Then titrated the contents against the AgNO3 (0.0141N silver nitrate titrant) solution until a brick red end point was attained and noted the reading.

3.3.7.3, Calculation:

$$\text{Chloride in mg/L} = \frac{\text{ml. of titrant 't' used} \times N \times 1000 \times 35.5}{\text{ml of sample}}$$

Where, N = Normality of titrant (0.0141N)

3.3.7.4, Calculation of salinity from chloride value:

Salinity (g/L) = 0.03 + 1.805 x chloride in mg/L.

3.3.8, Nitrates:

Nitrate is normally the most common form of combined inorganic nitrogen in aquatic system. The concentration and rate of supply of nitrate is intimatly connected with the land use practices of the surrounding watershed.

Nitrate reacts with phenol disulphonic acid to form a nitroderivative which in alkaline medium develops a yellow colour. The concentration of NO3 can be determined colorimetrically.

3.3.8.1, Requirement:

a) Phenol-disulphonic acid:

Dissolved 25 g pure white phenol in 150 ml conc. H2SO4. Added continuously 75 ml fuming sulphuric acid (15% free SO3); stirred well and heated in water bath for two hours.

b) Potassium hydroxide solution (12N):Dissolved 336.5 g KOH in distilled water and diluted to 500 ml.

c) Standard potassium nitrate solution: Dissolved 7.22 g anhydrous potassium nitrate in nitrate free distilled water and made up to 1 liter (1 ml of this solution contains 1 mg NO3 - N i.e. 4.43 mg NO3 ions).

d) Spectrophotometer

e) Flask.

f) Water bath.

g) Oven.

3.3.8.2, Procedure:

25 ml of water sample was taken in a flask and then evaporated it to dryness on hot pot plate after that the residue was rubbed throughly with 0.5 ml. phenol-disulphonic acid regent as well as added 5 ml of distilled water and 1.5 ml of KOH (12 N) then stirred it well. A yellow colour was developed and then reading was taken at 410 nm against a distilled water blank and found out the value of nitrates with the help of calibration curve.

3.3.9, Nitrite:

Nitrite reacts with sulphanilic acid in an acid medium to form a diazonium salt which on reaction with naphthylamine hydrochloride forms a pinkish dye. The optical density of this colour can be estimated colorimetrically.

3.3.9.1, Requirements:

a) Sulphanilic acid solution : 600 mg of sulphanilic acid is dissolved in warm distilled water. Allow it to cool and then add 20 ml of concentration HCl. This solution is further diluted to 100 ml.

b) Disodium EDTA solution : 500 mg of Na2 EDTA salt is dissolved in distilled water and the volume is made upto 100 ml.

c) a-Napthylamine HCl solution : 600 mg of a-Napthylamine is dissolved in distilled water 1 ml of concentrated HCl was added earlier. This solution was diluted to 100 ml and placed in a cool place.

d) Sodium Acetate solution : 16 g of anhydrous CH3COONa is dissolved in distilled water and the volume is made upto 100 ml.

e) Standard Nitrite solution : 1.232 g of sodium nitrite is dissolved in distilled water and diluted to 1000 ml to obtain concentration of 250 mg/L of NO2-N. Standard solution is prepared by diluting the stock solution to 250 times to yeild solution strength of 1 mg/L of NO2-N.

3.3.9.2, Procedure:

50 ml of sample water is taken in a conical flask. 1 ml each of EDTA solution, sulphanilic acid, napthylamine HCl and sodium acetate solution is added one by one to the above flask. The presence of nitrite is denoted by the appearance of wine red colour. The optical density of the above solution is recorded at 520 nm. The nitrite content is calculated with the help of standard curve. Standard curve is prepared from the standard solution in the range of 0.0 to 1.0 mg of NO2-N at an interval of 0.1 mg.

3.3.10, Phosphate:

All the forms of phosphorus are converted to inorganic forms (orthophosphate) after digestion and oxidation which is then measured spectrophotometrically.

Phosphate reacts with acidified ammonium molybdate solution to from molybdo phosphoric acid which get reduced to complex of blue colour

in presence of SnCl2. This colour is measured by spectrophotometer at 690 nm.

3.3.10.1, Requirement:

a) Perchloric acid (70%).

b) Phenolphthalein indicator: Dissolved 1.25 g phenolphthalein in 125 ml ethyl alcohol and added 125 ml distilled water. Added 1N NaOH dropwise until a faint pink colour appears.

c) Sodium hydroxide solution (1N): Added 4.0 g sodium hydroxide to 100 ml distilled water.

d) Ammonium molybdate strong acid solution: Dissolved 5 g ammonium molybdate in 35 ml distilled water, cautiously added 65 ml conc. H2SO4 to 80 ml. distilled water cooled and then added the molybdate solution and diluted to 200 ml.

e) Stannous chloride solution: Dissolved 0.5 g fresh SnCl2 2H2O in 2 ml conc. HCl, diluted to 20 ml with distilled water freshly prepared stannus chloride solution was used every time.

f) Standard phosphate solution: Dissolved 0.1757 g of potassium dihydrogen phosphate (over dried at 1050 C) in distilled water and diluted to 1 liter (1 ml of this solution contains 40 mg PO4).

g) Spectrophotometer.

h) Flask.

i) Measuring cylinder.

3.3.10.2, Procedure:

25 ml water sample was evaporated and the residue was dissolved in 1 ml perchloric acid (70%) after that fumed off the remaining

perchloric acid and cooled, then added 10 ml of distilled water followed by a drop of phenolphthalein indicator and titrated it with sodium hydroxide (1N) solution to slight pink end point, and made up the volume up to 25 ml, 1 ml ammonium molybdate and 3 drops of freshly prepared stannous chloride were added to it. Blue colour gradually appeared in about 10 minutes. Absorbance was read spectrophotometrically at 690 nm.

3.3.11, Sulphate:

Sulphate ions are precipitated with barium ions in acid solution to form $BaSO_4$ crystals of uniform size which are estimated spectrophotometrically at 420 nm against a standard curve drawn from different known concentrations.

3.3.11.1, Chemicals:

a) Standard H_2SO_4 (0.02 M): Added slowly with stirring 100 ml of conc. H_2SO_4 (18M) to distilled water and made up to 180 ml was added to prepare 2M solution 20 ml 10M H_2SO_4 to 80 ml of distilled water. Diluted 10 ml of 2 M H_2SO_4 to 1 liter and standardized it.

b) NaCl - HCl solution: Dissolved 240 gm of NaCl in 900 ml of distilled water then added 20 ml of HCl (Ar grade) and diluted to 1 liter.

c) Barium chloride.

d) Glycerol and ethanol solution.

e) Spectrophotometer.

f) Magnetic stirrer.

g) Conical flask.

h) Whatmann filter paper no. 1.

3.3.11.2, Procedure:

At first filtered the water sample through Whatmann filter paper no. 1 and then taken 50 ml of sample (containing not more then 10 mg/l sulphate) by adding 10 ml of NaCl - HCl solution followed by 10 ml of Glycerol - ethanol solution and then added 0.015 gm of barium chloride and mixed it for 30 minutes using a magnetic stirrer and measured the absorbance against a distilled water blank at 420 nm by U.V. spectrophotometer and compared with the standard curve.

3.4, Microbial (Bacterial) analysis of bat guano:

To isolation and identification of bacteria and fungi from the bat guano. The suspension of bat guano was prepared by taking 1 gm of bat guano dissolved in 10 ml of sterile distilled water and it was kept for 24 hours (Aneja, 1996; Cappucino and Sherman, 2002). Further its serial dilutions upto 107 of suspension were prepared in sterile distillled water by pour plate method. The 1 ml of each last four dilutions were transfered to sterile Petri plates and then poured 15 to 20 ml sterile nutrient agar in each plate and throughly mixed by rotating the plates. Then plates kept for solidification and after that these plates were incubated in an inverted position for 24 hours at 370 C.

After 24 hours of incubation, the bacterial colonies obtained and selective colonies were picked up and further streaked on a fresh nutrient agar plate for purification and isolation.

The culture was maintained on agar plates for further identification by morphological and biochemical tests. The morphological tests were performed included, Gram staining reaction, motility test, Acid fast

staining reaction and Spore (endospore) staining, where as biochemical tests include Catalase production test, Oxidase test, MRVP test, H2S production test, Nutrient gelatin hydrolysis, Indol production test, Amylase test, Urease test, Citrate utilization test, Oxidation and Fermentation test, Carbohydrate fermentation (sugar utilization) test, Nitrate-Nitrite-Amonia test and Phenylalanine test. All the microbiological tests were carried out with the help of Aneja (1996); Cappucino and Sherman (2002).

According to microbiological tests, medias required and were prepared and sterilized by moist method of sterilization by using autoclave at 15 lbs pressure and 1210 C or as per the requirments was used.

Different species of bacteria show a tremendous range in the metabolic activities they are able to carry out. They vary in their ability to hydrolyze or digest large molecules like carbohydrates (polysaccharides), proteins or fats. These variations are due to the differences in the types of enzymes that bacteria possess, were analysed by various Biochemical tests of bacteria.

The presence of a particular enzyme in a microorganism can be tested by incorporating a specific substrate in a medium, and then detecting the products formed or even checking the disappearance of the substrate from the medium.

3.5.1, Bacterial Motility:

3.4.1.1, Introduction:

Motility is important because it allows microorganisms to move from place to place in order to obtain nutrition for growth and reproduction or to escape from noxious microenvironments.

3.4.1.2, Motility and structure involved:

There are specialized cell structures which are involved with the motility of microorganisms. In some cases cytoskeletal structure play a role in the movement of an organism.

3.4.1.3, Hanging drop technique:

3.4.1.3.1, Requirements:

1) Young culture of a organism.

2) Cavity slide, cover slips and vaseline or grease.

3.4.1.3.2, Procedure:

1) Cleaned a cavity slide, and placed it on the table with the depression uppermost.

2) Using a match stick placed a minute drop of vaseline on each of the four corners of the coverslip.

3) Placed one loopful of culture at the center of the coverslip on the same surface as the vaseline.

4) Inverted the cavity slide over the coverslip in such a way that the drop on the cover slip is exactly under the cavity in center.

5) Pressed the cavity slide lightly and allowed the coverslip to adhere to the ‘vaseline’.

6) Quickly turned the slide up-side-down so that the drop of hay infusion hangs from cover slip into the well of the cavity slide.

7) Examined the preparation using the low power objective to focus on the edge of the droplet.

8) Light intensity into the microscope was reduced by adjusting the iris diaphragm and the condenser.

9) Moved the slide so that the edge of the droplet is in the center of the microscope field. Focused the edge properly.

10) Turned the high-power objective into position and focused the edge of the drop. Best illumination was obtained by adjusting the condenser and diaphragm.

11) Observed the microorganisms and noted down the results.

12) Placed a drop of oil on the coverslip, examine motility of bacteria was under oil immersion objective.

3.4.2, Spore (Endospore) staining :

3.4.2.1, Introduction:

When environmental conditions become unfavorable, certain gram-positive bacteria undergo sporogenesis and give rise to a resistant, metabolically inactive, resting intracellular strcture called endospore. When the favorable conditions return, the spore may revert to metabolically active vegetative cell through germination.

Thus it should be noted that sporogenesis and germination are not means of reproduction, but merely mechanism which ensures the survial of cell under difficult environmental conditions.

3.4.2.2, Bartholomew and Mittwer’s method:

3.4.2.2.1, Principle:

It is a cold method of spore staining and avoids heating. However, spores are stained by,

1) Longer heat treatment during fixation;

2) Prolonged staining period (10 minutes);

3) Higher concentration of stain (7.6% malachite green). Use of tap water as weak decolorizing agent will decolourise the cytoplasm only while the use is unaffected. Cytoplasm is then counter stained with a stain of a different colour froc of primary stain.

3.4.2.2.2, Requirements:

1) Young culture of organism.

2) 7.6% aqueous solution of Malachite green

3) 0.25% aqueous solution of safranin.

3.4.2.2.3, Procedure:

1) Prepared a thick smear and fix it with heat by passing the slide through flame for 20-25 minutes.

2) Allowed the slide to cool, and flooded it with 7.6% malachite green stain for 10 minutes.

3) Rinsed with tap water for 10 seconds.

4) Counter stained with 0.25% safranin solution for 15 seconds.

5) Rinsed in tap water, bloted, air dried and examined. Spores appear green and the cytoplasm is pink in colour.

3.4.3, Carbohydrate fermentation (sugar utilization) test:

3.4.3.1, Introduction:

Microorganisms can catabolize many different types of sugars. Monosaccharides (fructose, glucose, galactose, lactose, mannose, sucrose) can easily enter glycolytic pathway either directly or after phosphorylation. However, disaccharides are first broken down to monosaccharides by hydrolysis or by phosphorolysis.

The monosaccharides so formed then enter glycolytic pathway. Pentose sugars like ribose and xylose are phosphorylated and then are broken down via pentose phosphate pathway.

3.4.3.2, Principle:

Sugars are metabolized through different metabolic pathways to form various acids like pyruvate, lactate, succinate, formate, acetate etc. These acids so formed may further break down to gases.

Due to acid formation the pH of the medium is lowered and Andrade's indicator turns pink. Gas formation can be demonstrated by the use of Durham's tube, which collects the gas.

3.4.3.3, Requirements:

1) Culture of organism.

2) Nutrient sugar broth.

3) Durham's tube.

3.4.3.4, Procedure:

1) Inoculated a loopful of culture into the sugar broth and incubate 370 C for overnight.

2) Observed the tube for acid and gas production.

3.4.3.5, Interpretation:

Acid production changes the colour of the medium to pink and gas produced is collected in Durham's tube as a small bubble.

3.4.4, Oxidation-fermentation (Hugh and Leifson) test:

This is also known as 'oxferm' test or O-F test. Saccharolytic organisms degrade glucose oxidatively or fermentatively. The end products of fermentation are relatively strong acids that can be detected in a conventional

fermentation test medium. however, the acids firmed during the oxidative degradation of glucose are extremely weak, and the more sensitive Hugh and Leifson medium is required for the test.

3.4.4.1, Principle:

Hugh and Leifson medium has peptone and carbohydrate in the ratio of 0.2 : 1, in intrast to the 2 : 1 ratio found in media used for carbohydrate fermentation. The decrease in peptone minimizes the formation of alkaline amines that may be formed from oxidative metabolism. The relatively larger amount of cabohydrate serve to increase the amount of acid that can be formed. The semisolid consistency of agar permits the acids that form on the surface of agar to penetrate throughout the medium. making the interpretation of the pH shift of the indicator bromothymol blue easier to visualize.

Production of gas during the metabolism can also be detected as the gas bubbles are trapped in the medium due to the semisolid consistency of the medium.

Growth of the microorganism in this medium is either by utilizing the tryptone which result in an alkaline reaction (dark blue colour) or by utilizing glucose, which result in the production of acid(turning bromothymolblue to yellow). Those microorganisms which produce acid in both closed and open tubes are described as fermentative while those which produce acid only in the open tube are called oxidative.

Motility can also be observed in this medium.

In order to create the environment for fermentative metabolism, the medium is covered with 2 cm of sterile paraffin oil.

3.4.4.2, Requirements:

1) Hugh and Leifson medium.

2) Test culture.

3) Sterile paraffin oil.

3.4.4.3, Procedure:

1) Heated the tubes of the Hugh and Leifson medium in the boiling water bath for 10 minutes to drive off the oxygen.

2) Cooled and inoculated (stab inoculation) media with the test culture.

3) Overlayed one tube with sterile paraffin oil up to 2 cm above medium.

4) Incubated both the tubes at 370 C for overnight.

5) Observed for the acid and/or gas production in both tubes.

3.4.4.4, Interpretation:

1) Acid and/or gas in aerobic tube only : oxidative metabolism positive.

2) Acid and/or gas in both the tubes: fermentative metabolism positive.

3) Acid and/or gas absent in the tubes: non-saccharolytic organisms.

3.4.5, Gram's staining:

3.4.5.1, Introduction:

The gram stain was developed in 1884 by the Danish bacteriologist Hans Christian Gram. It is a very important differential staining

because it seperates bacteria into two broad categories namely gram-positive and gram-negative.

This differential staining requires the use of at least four reagents that are applied sequentially. The first reagent is called the primary stain- crystal violet (because it is applied first), which impart its colour to all cells in conjuction with the second reagent iodine, the mordant. In order to establish colour contrast the third reagent used is the decolorising agent.It may or may not remove the primary stain from the cell, depending upon its cellular composition.

3.4.5.2, Staining method:

3.4.5.2.1, Principle:

Several theories have been proposed to explain the mechanism of gram's staining, however, the one based on physicochemical nature of cell wall of bacteria is widely accepted. Cell walls of gram-negative bacteria are generally thinner than those of gram-positive bacteria. Gram-negative bacteria possess higher percentage of lipids in their cell wall as compared to gram-positive bacteria. During staining the primary stain crystal violet forms complex with mordant iodine (CV-I) in the cell wall. When gram-positive bacteria are decolorized with ethanol, the alcohol is thought to shrink the pores of the thick peptidoglycan. Thus, the dye-iodine complex, is retained during the short decolorization step and the bacteria remain violet. In contrast, gram-negative peptidoglycan is very thin, not as highly cross-linked and has larger pores. Alcohol treatment also may extract enough lipid from the gram-negative wall to increase its porosity further. For these reasons, alcohol more

readily removes the crystal violet-iodine complex from gram-negative bacteria. These cells subsequently take on the colour of counterstain the safranin.

3.4.5.2.2, Requirements:

1) Young cultures of organism.

2) Crystal violet stain, Gram's iodine, 95% ethanol and safranin stain.

3.4.5.2.3, Procedure (Hucker's modification):

1) Prepared a heat fixed smear of the culture.

2) Covered the smear with crystal violet stain for 1 minute;

3) Added Gram's iodine to wash off crystal violet stain and covered it with iodine till the smear turns coffee brown in colour (approximately 1 minute).

4) Rinsed the slide in running water.

5) Added decolorising solution drop wise at the upper end of slide held in inclind position, till the violet colour failed to come out from the smear; for normal smear 10-15 seconds are enough.

6) Rinsed the smear with water.

7) Counterstained with safrannin for 45-60 seconds.

8) Rinsed with tap water, drain, blot, air dry and examine. Gram positive bacteria are stained purple-violet while gram negative bacteria are stained pink.

3.4.6, Acid-fast staining:

3.4.6.1, Introduction:

Some bacteria have very high lipoidal (waxy) content in the cell wall, hence they are not stained by usual staining methods. however, once

the stain penetrates the cell wall, it can't be removed even with the strong decolorising agent like acid alcohol. Because of this property, these organisms are known as acid-fast. While other organisms which are easily decolorized by acid alcohol are called non acid-fast. This property was first discovered by Ehrlich in 1882 during the staining of tubercle bacilli from sputum. This staining technique is of great importance for the diagnosis of diseases like tuberculosis and leprosy.

3.4.6.2, Staining method:

3.4.6.2.1, Principle:

Explanation of acid fastness lie in the impermeability of bacterial surface to the stain rather than in the ability to bind to a particular bacterial component. Substances involved in preventing removal of the dye by acid alcohol are lipids and mycolic acid esters.

The cell wall of acid fast bacteria have high lipoidal content (25-60%) as compared to gram-positive (0.5%) and gram-negative (3%) bacteria. The principle lipids are high molecular weight mycolic acid esters, mycosides, glycolipids, waxes D, sulpholipids, etc.

Acid fastness property of the intact cell depends on trapping of intracellular mycolate-fuchsin complexes formed in the cell wall during staining. The dye fuchsin is more soluble in phenol than in water or acid alcohol. Phenol in turn, is more soluble in lipids present in the cell wall of acid-fast bacteria. During staining fuchsin enters the cell (phenol and heat acts as intensifiers). On application of acid-alcohol, acid-fast cells resist decolorization reagent. Non acid fast bacteria get decolorized and are counter stained with the dye of different colour like methylene blue or malachite green.

3.4.6.2.2, Requirements:

1) Culture of organism.

2) ZNCF stain, acid-alcohol and malachite green or methylene blue stain.

3.4.6.2.3, Procedure (Ziehl-Neelson Method):

1) Prepared a heat fixed smear from the culture.

2) Placed the slide on staining rack and flooded the smear with carbol fuchsin (ZNCF stain).

3) Gently heated the slide from below till it began to steam; allowed the slide to steam for 3-5 minutes.

4) Allowed the slides to cool, washed well with tap water.

5) Decolorized with acid-alcohol for 10-20 seconds, until no colour comes out of the slide.

6) Rinsed well with tap water.

7) Counter stained with methylene blue or malachite green for 1-2 minutes.

8) Rinsed with water, allowed it to air dry and examined.

3.4.6.2.4, Observations:

Acid-fast organisms are stained red while non acid-fast organisms appeared blue or green as per the counter stain used.

3.4.7, Catalase test:

3.4.7.1, Introduction:

Oxygen is both beneficial as well as toxic to living organisms. It is terminal electron acceptor during aerobic respiration. However, oxygen is also a toxic substance.

Toxicity of oxygen is basically due to formation of toxic derivatives of oxygen. Certain oxidative enzyme system interact with molecular oxygen to produce free superoxide radical (O2)

Superoxide radicals can inactivate vital cell components. Many organisms are protected from the toxic action of superoxide by their ability to produce the enzyme superoxide dismutase (SOD).

Hydrogen peroxide may further interact with superoxide to form Hydroxyl free radical.

Hydroxyl radicals are highly reactive free radicals and can damage almost every kind of molecules found in living cells. Hydrogen peroxide, on the other hand is a very powerful oxidizing agent highly toxic to cells.

Aerobic and facultative organisms have developed protective mechanisms against the toxic forms of oxygen. One is the enzyme SOD which removes superoxide radicals by increasing the rate of reaction leading to formation of hydrogen peroxide. H2O2 so produced can be removed by catalase and peroxidase enzymes.

Since superoxide and hydrogen peroxide are eliminated and the formation of hydroxyl radical is inhibited, because both reactants are required for the reaction.

Most anaerobes and microaerophiles do not have such protective mechanisms , so if they are exposed to oxygen, their growth is inhibited, hence they are found in oxygen free environment only.

3.4.7.2, Principle:

Catalase is an enzyme that splits up hydrogen peroxide into oxygen and water. Chemically catalase is a haemoprotein, similar in structure to haemoglobin, except that the four irons atoms in the molecule are in oxidized (Fe+++) rather than the reduced (Fe++) state.

Catalase is present, often in high concentration in the majority of aerobic organisms. But is absent from most obligate anaerobes. Thus when H2O2 is added externally in a medium, catalase activity results in the production of molecular gaseous oxygen. Catalase activity can be tested either by slide test or tube test.

3.4.7.3, Slide Test:

3.4.7.3.1, Requirements:

1) Microscopic glass slide.

2) 3% Hydrogen peroxide solution.

3) Culture of organism.

3.4.7.3.2, Procedure:

1) Placed one or two drops of hydrogen peroxide solution on a glass slide.

2) With a nicrome wire loop picked up cells from the center of a well isolated colony of the test culture, and transfered them into the drop of hydrogen peroxide.

3) Observed for the production of the gas bubbles or effervescence.

3.4.7.3.3, Interpretation:

Rapid appearance and sustained production of gas bubbles or effervescence constitute a positive test. Since some bacteria may possess enzymes other than catalase that decompose H2O2, few tiny bubbles after 20-30 seconds is not considered as a positive test.

3.4.8, Oxidase test:

3.4.8.1, Introduction:

When carbohydrates are oxidised via a respiratory mechanism under conditions in which oxygen is the final electron or hydrogen acceptor, energy is generated by passage of electrons through a series of electron donars and acceptors. The path through which these electrons flow is called electron transport chain (ETC). The components of ETC include flavoproteins, ubiquinone (coenzyme Q), and cytochromes.

Cytochromes are of three types namely; cytochrome a, cytochrome b, and cytochrome c. Cytochromes act sequentially to transport electrons from ubiquinone to oxygen, thus forming last link in the respiratory chain.

Cytochrome a and a1 together are called cytochrome oxidase. This process of ATP generation during electron transport is known as oxidative phosphorylation.

3.4.8.2, Principle:

As a last link in the respiratory chain of oxidase positive bacteria, cytochrome takes up electrons and passes them to molecular oxygen which being terminal hydrogen acceptor reduced to hydrogen

peroxide. The subsequent regeneration of cytochrome c to the oxidized form is catalysed by the enzyme cytochrome oxidase.

In oxidase test, p-phenylenediamine derivatives used as reagents are oxidized to coloured compounds by oxidized cytochrome c which in turn changes to reduced cytochrome c. The test proceeds only in air, since oxygen is necessary for production oxidized cytochrome c.The oxidase test is useful procedure in the clinical laboratory because some gram-negative pathogenic species of bacteria are oxidase positive, in contrast to species in the family Enterobacteriaceae, which are oxidase negative.

3.4.8.3, Requirements:

1) Nutrient agar plate.

2) Filter paper, Platinum wire loop.

3) Young Culture of organism.

4) 1% tetramethyl-p-phenylenediamine dihydrochloride solution.

3.4.8.4, Procedure (Kovac's method):

1) Grew the test organism freely under aerobic conditions on nutrient agar medium for 18-24 hrs.

2) Took a filter paper strip and moisten it with 3-4 drops of tetramethyl-p-phenylenediamine dihydrochloride solution.

3) With the help of platinum wire picked up a colony and made a compact smear on moistened filter paper.

4) Waited for 10-15 seconds to observe formation of violet colour.

3.4.8.5, Interpretation:

1) Appearance of violet colour on moist filter paper is an indication that the organism possess cytochrome oxidase.

2) The use of platinum wire loop for the test is important because the loop made from other materials lead to false positive reactions.

3) Organisms with less cytochrome oxidase activity can produce colour change after long time.

3.4.9, Methyl- Red and Voges-Proskauer tests :

3.4.9.1, Introduction:

The Methyl-red (M-R) and the Voges-Proskauer (V-P) tests are used to differentiate two major types of facultatively anaerobic enteric bacteria that produce large amount of acid and those that produce the neutral product acetoin as end product. Both these are performed simulteneously because they are physiologically related and are performed on the same medium MR-VP broth. Opposite results are usually obtained for methyl red and Voges-Proakauer tests i.e., MR+, VP-, or MR-, VP+. In these tests, if an organism produce large amount of organic acid: formic, acetic, lactic and succinic (end products) from glucose the medium will remain red (a positive test) after the addition of methyl red a pH indicator (pH remaining below 4.4). In other organism, methyl red will turn yellow (a negative test) due to the elevation of pH above 6.0 because of the enzymatic conversion of the organic acids.

3.4.9.2, Requirements:

1) Young cultures of organism.

2) MR-VP broth tubes (5 ml/tube).

3) Methyl-red pH indicator (dropper bottle).

4) V-P reagent I (napthol solution).

5) V-P reagent II (40% potassium hydroxide).

6) Bunsen burner.

7) Inoculating loop needle.

3.4.9.3, Procedure:

1) Preparation of MR-VP broth (pH 6.9) tubes of the following composition:

a) Peptone.

b) Dextrose/Glucose.

c) Potassium phosphate

d) Distilled water

2) Poured the 5 ml broth in each tube and sterilized by autoclaving at 15 lbs pressure for 15 minutes.

3) Inoculated MRVP tubes with organism and kept one tube as uninoculated (comparative control).

4) Incubated all tubes at 350 C for 48 hours.

5) Added 5 drops of methyl red indicator to each tube.

3.4.9.4, Observations (I):

Observed the change in colour of methyl red for MR test.

1) Added 12 drops of V-P reagent I and 2-3 drops of V-P reagent II to each tubes as well as to uninoculated control tube. .

2) Shaked the tubes gently for 30 seconds with the caps off to expose the media to oxygen.

3) Allowed the reaction to complete for 15-30 minutes.

3.4.9.5, Observations (II):

Observed the tubes for change in colour for the VP test.

4) In the MR test, the methyl red indicator in the pH range of 4 when remain red (throughout tube) indicates of a positive test, while turning of methyl red to yellow is a negative test.

5) In the VP test, the development of a deep Rose colour; may be most intense at the surface, is indicative of positive V-P test while no change in colouration is a negative test.

3.4.10, Indole Production Test:

3.4.10.1, Introduction:

Tryptophan, an essential amino acid is oxidized by some bacteria by the enzyme tryptophanase resulting in the formation of indole, pyruvic acid and ammonia. The indole test is performed by inoculating a bacterium into tryptone broth, the indole produced during the reaction is detected by adding Kovac's reagent (dimethylaminobenzaldehyde) which produces a cherry-red reagent layer.

This exercise deals with the determination of indole production from microbial catabolism of tryptophan.

3.4.10.2, Requirements:

1) Nutrient broth cultures of organism.

2) Tubes containing 1% tryptone broth, 5 ml/tube.

3) Kovac's reagent

4) Dropper bottle/1 ml pipette

5) Bunsen burner

6) Inculating loop needle.

3.4.10.3, Procedure:

1) Preparetion of (1%) tryptone broth : Dissolved 10 g of peptone in one liter of distilled water. Sterilized in the autoclave at 15 lbs (1210 C) for 15 minutes.

2) Inoculated one organism in each tube and kept one tube as an uninoculated comparative control.

3) Incubated inoculated and uninoculated tubes at 350 C for 48 hours.

4) After 48 hours of incubation, added 1 ml of Kovac's reagent to each tube including control.

5) Shaked the tubes gently after intervals for 10 -15 minutes.

6) Allowed the tubes to stand to permit the reagent to come to the top.

3.4.10.4, Observations:

Examined the tubes as to the colour in the reagent at top layer.

3.4.10.4, Interpretation:

Development of a cherry (deep) red colour in the top layer of the tubes is a positive test for indole production. Absence of red colouration in tubes is indole negative.

3.4.11, Citrate utilization test:

3.4.11.1, Introduction:

Citrate test is used to differentiate among enteric bacteria on the basis of their ability to utilize/ferment citrate as the sole carbon source. The utilization of citrate depends on the presence of an enzyme citrase produced by the organism, that breaks down the citrate to oxaloacetic acid and acetic

acid. These products are later converted to pyruvic acid and carbon dioxide enzymatically.

The citrate test is performed by inoculating the microorganisms into an organic synthetic medium, Simmon's citrate agar, where sodium citrate is the only source of carbon and energy. Bromothymol blue is used as an indicator. When the citric acid is metabolized, CO2 generated combines with sodium and water to form sodium carbonate an alkaline product, which changes the colour of the indicator from green to blue and this constitutes a positive test.

Bromothymol blue is green when acidic (pH 6.8 and below) and blue when alkaline (pH 7.6 and higher).

3.4.11.2, Requirements:

1) Nutrient broth cultures of organism.

2) Simmon's citrate agar slants.

3) Bunsen burner

4) Inoculating needle.

3.4.11.3, Procedure:

a) Poured the medium in the culture tubes and sterilized by autoclaving at 15 lb pressure for 15 minutes and prepared the slants.

b) Inoculated Simmon's citrate agar slants, with organism by means of a stabing and streaking inoculation. One tube was kept as an uninoculated comparative control.

c) Incubated all the three slants at 370 C for 48 hours.

3.4.11.4, Observations:

Observe slant cultures for the growth and colouration of the medium.

In inoculated slant, growth is visible on the surface and the medium colour is blue (i.e. citrate positive) while if in those slant there is no growth and there is no change in the colour of the medium (i.e. green) i.e. citrate negative test.

3.4.12, Amylase production test :

3.4.12.1, Introduction:

Amylase is an exoenzyme that hydrolyses (cleaves) starch, a polysaccharide into maltose a disaccharide and some monosaccharides such as glucose. These disaccharides and monosacchrides enter into a cytoplasm of the bacterial cell through the semipermeable membrane and are used by the endoenzymes. Starch is a complex carbohydrate composed of two constituents amylose, a straight chain polymer of 200-300 glucose units, and amylopectin, a larger branched polymer with phosphate groups.

Amylase production is known in some bacteria while well known in fungi. Amylases commercially produced from various aspergilli are used in the initial steps in several food fermentation processes to convert starch to fermentable sugars.

The ability to degrade starch is used a criterion for the determination of amylase production by a microbe. In the laboratory it is tested by performing the starch test to determine the absence or presence of starch in the medium by using iodine solution as an indicator. Starch in the presence of iodine produces a dark blue colouration of the medium , and a

yellow zone around a colony in an otherwise blue medium indicates amylolytic activity.

This exercise deals with testing the hydrolysis of starch for the production of extracellular amylase by three test organism, Bacillus subtilis, Escherichia coli and Aspergillus niger by inoculating these on starch agar medium.

3.4.12.2, Requirements:

1) Nutrient agar slant cultures of organism.

2) Starch agar medium.

3) Gram's iodine solution.

4) Sterile Petri dishes.

5) Dropper.

6) Inoculating loop needle.

7) Bunsen burner.

8) Wax marking pencil.

3.4.12.3, Procedure:

1) Melted the starch agar medium, cool to 450 C and poured into the sterile Petri dishes.

2) Allowed it to solidify.

3) Labelled each of the starch agar plate with the name of the organism to be inoculated.

4) Using sterile technique, made a single streak inoculation of each organism into the center of its appropriately labelled plate.

5) Incubated the bacterial inoculated plates for 48 hours at 370 C in an inverted position.

6) Flooded the surface of the plates with iodne solution with a dropper for 30 seconds.

7) Poured off the excess iodine solution.

Examined the plates for the starch hydrolysis around the line of growth of each organism, i.e. the colour change of the medium.

A typical positive starch hydrolysis reaction shows clear zone surroundings the microbial colonies. A negative reaction is indicated by which showed dark blue colouration of the medium.

3.4.13, Hydrogen sulphide production test:

3.4.13.1, Introduction:

Microorganisms produce H2S by two different ways :

1) By reduction of thiosulphate, and

2) Formation of H2S from sulphur containing amino acids by the enzyme desulphurase.Therefore two kinds of tests exist for detection of H2S production.

3.4.13.2, Lead acetate paper- strip test:

This test is for detection of H2S production from sulphur containing amino acids.

3.4.13.2.1, Principle:

There are about twenty amino acids commonly found in proteins. Out of these methionine and cysteine contain sulphur in their structure. Cysteine often occurs in proteins in its oxidized form called cystine. Organisms possessing the enzyme amino acid desulphurase remove the sulphur found in the amino acid as H2S. Sulphur containing amino acids are first converted to cysteine .

Cysteine so formed can liberate H2S when acted upon by cysteine desulphurase. H2S can be detected by reacting it with salts of certain metals like lead, iron or bismuth; resulting in the formation of dark sulphide of these metals.

3.4.13.2.2, Requirements:

1) 2% peptone broth.

2) Saturated solution of lead acetate.

3) Test culture.

3.4.13.2.3, Procedure:

1) Inoculated a loopful of test culture in 2% peptone broth.

2) Soaked a white filter paper strip (5 mm x 50 mm) in saturated solution of lead acetate.

3) Placed lead acetate filter paper strip in neck of the tube in such a position that 1/4 to 1/2 of the strip projects below the cotton plug. Incubated the medium at 370 C for 24 hours.

4) After incubation, observed for the blackening of the filter paper.

3.4.13.2.3, Interpretation:

Blackening of filter paper strip is due to the formation of lead sulphide, which indicates H2S production by the organism.

3.4.14, Phenylalanine deamination test:

3.4.14.1, Introduction:

Phenylalanine is an amino acid that upon determination forms phenylpyruvic acid (a keto acid).

3.4.14.2, Principle:

Organisms capable of producing the enzyme deaminase can remove the amino group from the amino acid phenylalanine, and forms phenylpyruvic acid. Phenylpyruvic acid so formed react with ferric chloride to form green coloured complex.

3.4.14.3, Requirements:

1) Phenylalanine agar slant.

2) 10% aqueous ferric chloride solution and test culture.

3.4.14.4, Procedure:

1) Streaked the slant with a loopful of test culture, and incubate at 370 C for 18 - 24 hours.

2) After incubation added 4 - 5 drops of ferric chloride reagent on to the surface of the agar.

3) Observed for the appearance of an intense green colour.

3.4.14.5, Interpretation:

Immediate appearance of an intense green colour indicates the presence of phenylpyruvic acid and it is positive test.

3.4.15, Urea hydrolysis test :

3.4.15.1, Introduction:

Urea is a diamide of carbonic acid. Amides are hydrolyzed with the release of ammonia.

3.4.15.2, Principle:

A strongly buffered medium (Stuart’s urea broth), in which urea is the only nitrogen source is used for the test. Urease is an enzyme possessed by many species of microorganisms that can hydrolyze urea.

The ammonia so produced reacts in solution to form ammonium carbonate, resulting in alkalinization and an increase in the pH of the medium.

This is indicated by change in colour of the indicator Phenol red (pH 6.8 - 8.4, yellow-purple red).

Due to high buffering capasity of the medium, only those organisms possessing vigorous urease activity can give the test positive.

3.4.15.3, Requirements:

1) Staurt's urea broth.

2) Test Culture

3.4.15.4, Procedure:

1) Inculated a loopful of test culture in urea broth, and incubated at 370 C for 24 hrs.

2) Observed for the change in colour of the broth after incubation.

3.4.15.5, Interpretation:

Purple red colour throughout the medium indicates alkalinization and urea hydrolysis.

3.4.16, Nitrate Reduction Test:

3.4.16.1, Introduction:

Microorganisms reduce nitrate under two different growth conditions with different purpose.

1) Certain bacteria carry out nitrate respiration under anaerobic/microaerophilic conditions and use nitrate as the terminal electron acceptor, thereby reducing it.

2) In contrast to this, assimilatory nitrates reduction generally occurs under aerobic conditions in absence of sources of nitrogen.

Thus in assimilatory nitrate reduction, nitrate is incorporated into organic material and do not participate in energy production. This process is wide spread among bacteria, fungi and algae which can use nitrate as the sole source of nitrogen.

In the first step nitrate is reduced to nitrite by nitrate reductase. Nitrite is next reduced to ammonia with a series of two electron addition, catalyzed by nitrite reductase. Hydroxylamine may be an intermediate. Ammonia is then assimilated.

3.4.16.2, Principle:

Organisms possessing nitrate reductase when grown in a medium containing nitrate as the sole source of nitrogen will reduce nitrate to nitrite. The formation of nitrite can be detected by adding sulphanilic acid, which forms a diazonium salt, which in turn reacts with a- naphthylamine thereby leading to formation of soluble red azo dye (p-sulphobenzene-azo-a-napthylamine).

3.4.16.3, Requirements:

1) Peptone nitrate broth (PNB).

2) Test culture.

3) Zinc dust.

4) a- napthylamine reagent (reagent A).

5) Sulphanilic acid reagent (reagent B).

3.4.16.4, Procedure:

1) Inoculated PNB with a loopful of test culture and incubated the medium at 370 C.

2) Added 0.5 ml of the reagent A and B each to the test medium in this order.

3) Observed the development of red colour within 30 seconds after adding test reagent.

4) If no colour develops, added a pinch of zinc dust, mixed them well and observed the development of red colour.

3.4.16.5, Interpretation:

The development of red colour within 30 seconds after adding the test reagent indicates the presence of nitrites and its positive nitrite reduction test. If no colour develops after adding test reagents, this may indicate that :

1) Nitrates was not reduced, (a true negative reaction) or,

2) They were reduced to products other than nitrites, such as ammonia, molecular nitrogen (denitrification), nitric oxide (NO), or nitrous oxide (N2O) and hydroxylamine. Since the test reagents detect only nitrites the later process would lead to a false negative reading. Then it is necessary to add a small quantity of zinc dust to all negative reactions. Zinc ions reduce nitrates to nitrites and the development of a red colour after adding zinc dust indicates the presence of residual nitrates and confirms a true negative reaction.

3.4.17, Ammonia Production:

Certain organisms possessing the enzymes nitrate reductase and nitrite reductase can reduce nitrate and nitrite to ammonia. This ammonia can be assimilated to organic nitrogenous compound.

3.4.17.1, Principle:

When nitrates or nitrites are reduced by microorganisms, ammonia is liberated. This ammonia while trying to escape from the tube converts the litmus paper hanging from the neck of the tube from red to blue.

3.4.17.2, Requirements:

1) Peptone nitrate broth.

2) Red litmus paper.

3) Test culture.

3.4.17.3, Procedure:

1) Inoculated a loopful of test culture to peptone nitrate broth.

2) Placed a red litmus paper strip in the mouth of the culture tube in such a position that 1/4 to 1/2 of the strip projects below the cotton plug. Incubated the medium at 370 C for 24 hours.

3) After incubation, observed for the change of red litmus to blue colour.

3.4.17.4, Interpretation:

The change of red litmus to purple or blue indicates the ammonia production and can be read as positive test.

3.4.18, Gelatin Hydrolysis test:

3.4.18.1, Introduction:

Gelatin is a bone protein obtained from bones from which fats, and minerals like phosphates have been removed. Gelatin is an unusual kind of protein which in aqueous solution forms a solid gel at room temperature but changes to liquid above 25 - 280 C. It can tolerate heating at 100 - 1210 C without being coagulated, and therefore, unlike other proteins, it can be sterilized by autoclaving.

Gelatin is also unique in a sense that very few organisms like species of Pseudomonas, and Proteus which possess enzyme gelatinase can hydrolyze it. Production of enzyme gelatinase can be demonstrated by tube test, and plate test.

3.4.18.2, Tube test:

3.4.18.2.1, Principle:

Gelatinase is an extracellular proteolytic enzyme capable of hydrolyzing gelatin. Such hydrolyzed product do not gel at temperature below 25 - 280 c. This is known as liquefaction of gelatin. As the cultures are usually incubated at a temperature above the melting point of gelatin (370 C), it is necessary to cool the medium for 30 minutes prior to reading the results.

3.4.18.2.2, Requirements:

1) Two nutrient gelatin agar tubes

2) Test culture

3) Refrigerator or water bath with ice.

3.4.18.2.3, Procedure:

1) Inoculated a loopful of test culture into one of the tube, and second tube is left uninoculated (control). Incubated both the tubes at 370 C for 24 - 72 hours.

2) After incubation placed both the tubes at 5 -100 C either in refrigerator or in ice water bath, for 30 - 60 minutes.

3) After refrigeration, slightly tilted tubes, so as to check the liquefaction of gelatin.

3.4.18.2.4, Interpretation:

The test is positive (indicating gelatinase production) if the inoculated medium remains in a liquid state even after refrigeration; while the control medium solidifies.

3.4.19, Dehydrogenase test:

3.4.19.1, Introduction:

The biological oxidation of organic metabolites is the removal of electrons. In most cases it involves the removal of two electrons and thus simultaneous loss of two protons. This is equivalent to the removal of two hydrogen atoms and is called dehydrogenation.

When a pair of electrons from an oxidizable substrate is coupled with the reduction of an ultimate electron acceptor, such as oxygen, there is a generation of energy.

Certain enzymes which removes electrons and hydrogen ions from reduced substrates are reffered to as dehydrogenases. These enzymes have NAD+ or NADP+ as their coenzymes. The vitamin niacin (nicotinic acid) forms the part of the structure of NAD and NADP.

Another class of dehydrogenases are known as flavoproteins and they contain either FAD or FMN as prosthetic group. One of the basic part of their coenzyme structure is the vitamin riboflavin. These enzymes are present in organisms which carry out aerobic respiration.

3.4.19.2, Principle:

The test involves the detection of the dehydrogenase enzyme by using methylene blue as the compound which accepts the hydrogen released in the respiratory electron transport chain, and gets reduced (colourless form).

3.4.19.3, Requirements:

1) Nutrient broth

2) Test culture

3) Methylene blue solution (1% solution)

3.4.19.4, Procedure:

1) Prepared a dense culture (10 organisms/ml) of the test organism, or alternatively used the overnight incubated culture.

2) Inoculated 0.5 -1.0 ml of the above culture in a sterile nutrient broth and mixed it well.

3) Added 1 ml of sterile methylene blue solution (1%) in nutrient broth.

4) Incubate the nutrient broth at 370 C for 24 hours.

5) After incubation observed for the disappearance of blue colour and recorded the results.

3.4.19.5, Interpretation:

Disappearance of the blue colour indicates the presence of enzyme dehydrogenase and the test is considered positive. The methylene

blue remains blue in colour at the top surface of the broth due to the oxidation of the methylene blue by air present at the surface.

3.5, Water and soil pH studies:

To study the impact of bat guano on the pH of water, 10 mg bat guano was dissolved in 100 ml of water for every time.

After addition of bat guano in water, the water was analysed for the change in its pH.

The changes in water pH was noted after one hour upto 24 hours. Thereafter, the analyses were carried out for 30 days at an interval of 5 days.

The water samples were analyzed by using standard methods for water analysis suggested by APHA (1998).

To study the impact of bat guano on the pH of soil, 10 mg bat guano and 100 g of fine soil was mixed. 100 ml distilled water (pH - 7) was added into it. After addition of water in the mixture of bat guano and soil, the soil was analyzed for the change in its pH.

The changes in soil pH was noted after 1 hour up to 24 hours. Thereafter, the analysis were carried out for 5 days at an interval of 1 day.

The soil samples were analyzed by using standard methods for soil analysis suggested by APHA (1998).

3.5.1, pH:

pH is the negative logarithm of the concentration of free hyrogen ions in a solution. It can be measured by electrometric method.

Activity of Hydrogen ions in a solution is measured as the difference in potential (e.m.v.) of glass electrode with that of calomel reference electrode over a scale calibrated directly in pH units.

There are a number of models available for pH meters. Some pH meters employ two electrodes, an indicator glass electrode and a conbined glass and reference electrode.

3.5.1.1, Procedure:

pH was recorded in the laboratory by electrical pH meter (systronics make) maintained at the room temperature.

The pH meter was set with a buffer solution of pH 4.0 and 9.2. Then washed the electrode in distilled water and taken a suitable size of water and soil sample in a beaker, then dipped the electrode in sample and operated the pH meter accordingly and then noted the pH value of the sample.

3.6, Water analysis experiments (Bioremediatation):

To study the impact of bat guano on water, 10 mg bat guano was dissolved in 100 ml of distilled water (10:100 perportion) for every time. After addition of bat guano in water, then the water was analysed for the change in its chloride, salinity, nitrate (NO2), phosphate (PO4) and suphate (SO4) contents.

The changes in water parameters were noted after every one hour upto 24 hours. Thereafter, the analyses were carried out for 30 days at an interval of 5 days.

The water were analyzed by using standard methods for water analysis suggested by APHA (1998) as described in 3.3.

3.7, Larvicidal activity:

3.7.1, Determination of 96hLC50 of bat guano:

As the guano samples were of unknown toxicity, first experiments were conducted to determine toxicity range (Exploratory tests). The aqueous solution of bat guano containing 10 mg/L to 100 mg/L were prepared. 10 mosquito larvae were transferred to each of the containers containing 1 liter of bat guano solution. Observation for 24 hours indicated test concentration for full scale experiments from which different 9 concentrations were selected for the final experiment. Final experiments were conducted with 5 liters of bat guano treated water containing 100 mosquito larvae. Mortality was recorded after 1, 6, 12,24,48 and 96 hours and the data was used to calculate 96hLC50 value for different bat guano by graphical interpolation method (Kamleshwar, Shukla and Trivedi, 2005). Dose mortality curves were also drawn.

3.8, Plant growth studies:

Efficiency of bat guano as an organic manure was assessed by amending bat guano with soil. Five treatments (5 replicates each) consisting of black soil (collected at 1 feet depth) mixed with bat guano from Lonar crater (Morache temple) were tested. 6 plastic pots were taken and they were filled with the black soil. All the pots were watered equally to set the soil in each pot. The pots were labeled as P1 to P6. P1 was a control of which the soil was not mixed with bat guano. P2 to P6 pots received 500 ml water in which different quantities of bat guano was dissolved e.g. P2 received 10 mg bat guano, P3 20 mg, P4 30 mg, P5 40 mg and P6 50 mg of bat guano. The control pot received only 500 ml solvent water.

Then healthy 200 seeds of Triticum aestivum were sowed in each pot and the pots were observed daily initially for germination and then after the height of the plantlets were measured upto 13 days. All pots were watered with equal amount of water (100 ml) at 8:30 am, daily once upto 13 days.

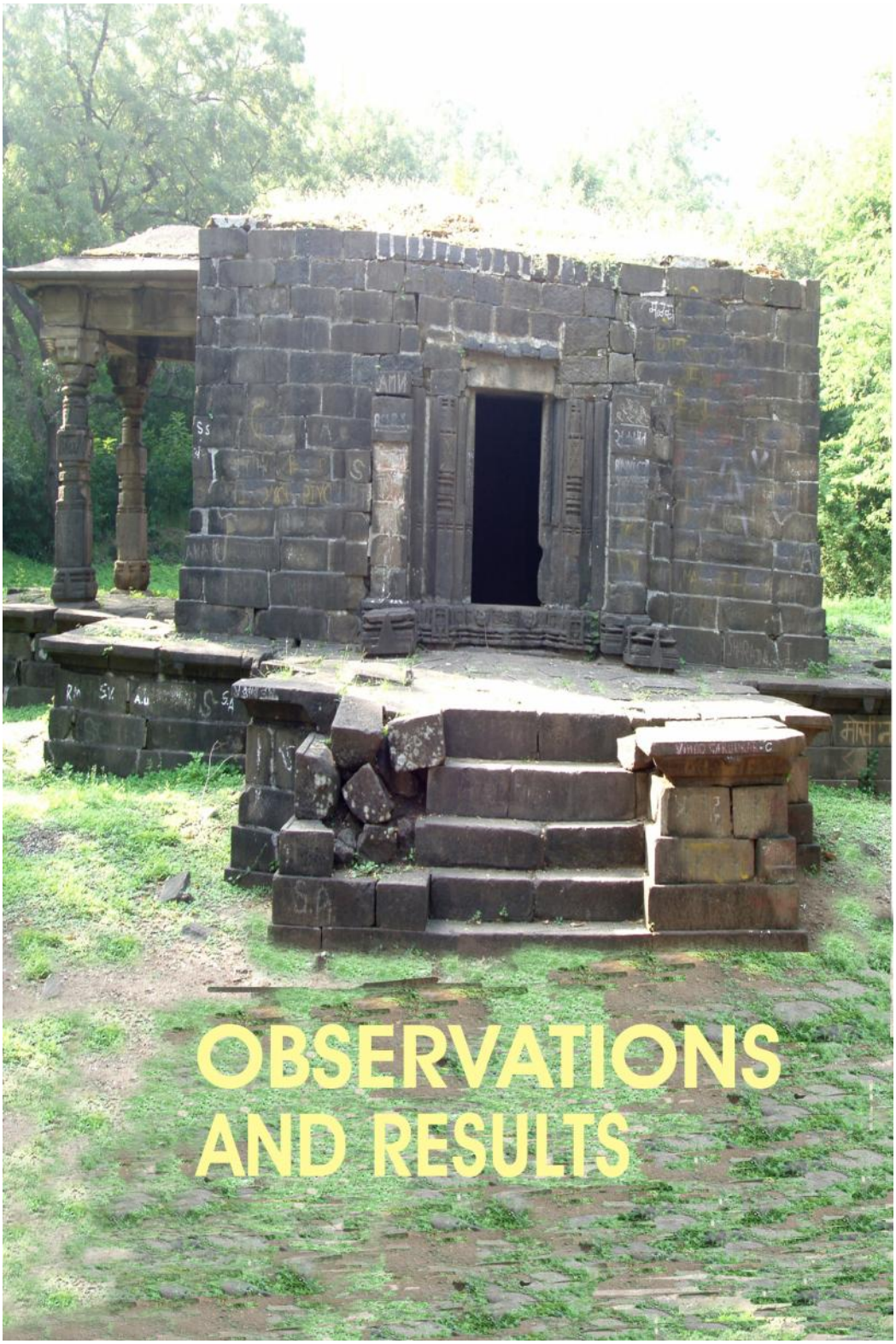
Similarly the plant growth studies were also carried out for Vinca rosea, Tagels elens and Amaranthus brazilansis. All experimental pots received 100 mg of bat guano from Morache temple of Lonar crater. In this study the seeds of Vinca rosea were sowed and the germination time and growth studies were recorded with respect to height and branching intensity.

For Tagels elens and Amaranthus brazilansis healthy plantlets of 2 cm height were selected and were planted. The growth of plantlets were recorded on 5 and 13 days and 8 and 13 days respectively.

3.9, Faecal analysis for undigested remains:

Bat guano samples were collected from different habitats used for observation of undigested remains. Bat guano was dissolved in water and kept for 4 hours and then filtered in fine sieve. The undigested food fragments which remained in the sieve (Insect fragments and others) were observed under dissecting microscope and then through the compound microscope, for faecal analysis.

The undigested food fragments in the faecal matter of bat guano were seperated with needle and identified. Whole mounts of undigested remains were prepared and photographs were taken.


OBSERVATIONS
AND RESULTS

OBSERVATIONS AND RESULTS

Bat guano from different habitats like Lonar crater temples, Ajanta caves, Ellora caves and Semadoh (Melghat forest) were collected to assess their chemical contents, to know how they alter the pH, salanity and soil parameters and to study their role in plant growth and as larvicidal activity if any. An attempt is also made to analyse the bat guano for undigested remains. Absolutely no work has been carried out in India on the bat guano till date. This is probably the first attempt.

4.1, General Observations:-

4.1.1, Bat guano from Lonar crater-

The bat guano collected from Lonar crater temples was in the form of flakes and black brown in colour (Plate, 4.1).

4.1.2, Bat guano from Ajanta and Ellora caves-

The guano collected from Ajantha and Ellora (Werul) caves is in the form of beaded pellets with pale brown colour and is light in weight. (Plate, 4.1).

4.1.3, Bat guano from Semadoh (Melghat forest)-

The guano collected from Semadoh appeared flattened flakes in form with dark cherry brown in colour and had light in weight.

The fresh and old guano from all the habitats showed acidic and alkaline nature respectively. When the guano was dissolved in water it developed alkaline pH in general within few hours.

During collection of bat guano in old temples of Lonar crater it is observed that various invertebrates and even vertebrates were feeding on the bat guano (Table, 4.1).

4.2, Chemical Analysis of bat guano:-

The chemical analysis of fresh bat guano collected from different habitats was carried out for Na, K, Ca, N, iP, Mg, Mn, Cu, Fe, Zn, Al, Se, Ba, Cl, NO2, NO3, PO4, and SO4 and the results are given in Table -4.4. With respect to Na, N, Mg, Mn, Cu, Fe, Zn, Cl, NO2, NO3, and SO4 contents, their quantity (mg/L) was found to be in the order of LCMT > LCWT > AC7 > AC24 > EC16 > SD. However, the Ca content (mg/L) present in guano from different habitats was in the order of LCMT > LCWT > AC7 > SD > EC16 > AC24. K, Ca and iP were seen slightly in larger quantity in bat guano from forest habitat as compared that in the guano collected from Ellora cave (Table, 4.4 and Fig. 4.1 to 4.6; 4.7 to 4.12; 4.13 to 4.18).

4.3, Microbial analysis of bat guano:-

The microbial diversity of bat guano collected from Morache temple of Lonar crater was studied. Initially 10 mg bat guano was dissolved in 100 ml sterilized distilled water and then from that 1 ml of bat guano sample was diluted to various dilutions and they were inoculated on culture medium to obtain the bacterial colonies. The mixed colonies so obtained were subjected to pure cultures. The process was repeated to get pure cultures. Then the colonies were maintained in the laboratories as pure cultures.

The bacteria isolated from these colonies were tested for several standard bacteriological tests as described in materials and methods. The results are given in tables, 4.5.1, 4.5.2 and 4.5.3 and Fig, 4.19 to 4.21; 4.22 to 4.24; 4.25 to 4.27; 4.28 to 4.30; 4.31 to 4.33; 4.34 to 4.36; 4.37 to 4.39. The results yielded 11 different pure colonies and their slides are prepared (Plate, 4.2; 4.3; 4.4; 4.5 to 4.6). The slides are sent for identification to Institute of Microbial Technology, Chandigrah. They reported the following bacteria in the cultures (Table, 4.2).

Besides several tests for bacteria, bacterial count in bat guano also undertaken. It is observed that the bat guano from Lonar crater temples exhibited highest number of bacterial count (Table, 4.3).

4.4, Alterations in water parameters due to bat guano:-

4.4.1, Impact of bat guano on the pH of Water:-

To study the impact of bat guano on pH of water, 10 mg bat guano was dissolved in 100 ml of distilled water (pH-7) and the change in pH of water was noted after every one hour upto 24 hours. Such, observations were carried upto 30 days at an interval of 5 days.

When known quantity of bat guano collected from different habitats were dissolved in distilled water, changes in the pH of each sample were noted immediately.

Bat guano from Morache temple at Lonar crater was dissolved in distilled water, the pH changed to 7.4 and then gradually after one hour it was noted to be 8.42. After 2 hours interval upto 13 hours, the pH was increased gradually and it reached to maximum 9.23. After 13 hours the pH started declining and it remained static (8.30) after 24 hours later (Table, 4.6).

The sample was kept under observation till 30 days and the pH was noted after an interval of 5 days upto 30 days. After 5 days the pH was seen to be decreasing upto 15 days and thereafter it remained constant during 25 to 30 days of observations (Table, 4.7 and Fig. 4.40).

When bat guano from Waghache temple of Lonar crater was dissolved in distilled water, the pH initially changed to 6.50 and then after 1 hour, it was reached to 7.81. After 2 hours upto 13 hours, the pH was observed to be increased gradually and it reached to 8.74 after 13 hours. Thereafter, the pH showed declining trend. It was 7.94 after 24 hours (Table, 4.6).

The sample was kept undisturbed till 30 days and the pH was noted after every 5 days upto 30 days. After 5 days the pH was seen to be lowered down upto 15 days and then it remained constant till 30 days of observations (Table, 4.7 and Fig. 4.41).

When bat guano from Ajanta cave - 7 was dissolved in distilled water, the pH noted was 6.61 and then after 1 hour it was changed to be 7.30. After 2 hours upto 13 hours, the pH was increased gradually and it reached to 8.86 after 13 hours. Thereafter, the pH started declining. It was 7.60 after 24 hours (Table, 4.6).

The sample was kept undisturbed till 30 days and the pH was noted after every 5 days upto 30 days. After 5 days the pH was increased to 8.18 ±0.31 and then after 10 days it was slightly lowered and then it remained constant during 25 to 30 days of observations (Table, 4.7 and Fig. 4.42).

When bat guano from Ajanta cave - 24 was dissolved in distilled water, the pH changed to 5.82 and then after 1 hour it was 6.40. After 2 hours, the

pH was increased gradually and it reached to 7.90 after 13 hours. Thereafter, the pH started declining. It was 7.05 after 24 hours (Table,4.6).

Then sample was kept undisturbed till 30 days and the pH was noted after every 5 days upto 30 days. After 5 days the pH was elevated to 7.76 ±0.27 and showed slight fluctuations till 20 days and remained constant after 25 days (Table, 4.7 and Fig. 4.43).

When bat guano from Ellora cave - 16 was dissolved in distilled water, the pH changed to 6.21 and then after 1 hour it was 6.71. After 2 hours upto 13 hours, the pH was increased gradually and it reached to 8.10 after 13 hours. Thereafter, the pH started declining. It was 7.21 after 24 hours (Table, 4.6).

The sample was kept undisturbed till 30 days and the pH was noted after every 5 days upto 30 days. After 5 days the pH was 7.48 ±0.29 then it started lowering and remained constant at 6.30 ±0.43 after 25 days till 30 days (Table, 4.7 and Fig. 4.44).

When bat guano from Semadoh (Melghat forest) was dissolved in distilled water, the pH became acidic (6.00) and then after 1 hour it was found to be 6.49. After 2 hours upto 12 hours, the pH was increased gradually and it reached to 7.67 after 12 hours. Thereafter, the pH started declining. It was 6.91 after 24 hours (Table, 4.6).

The sample was kept undisturbed till 30 days and the pH was noted after every 5 days upto 30 days. After 5 days the pH was 7.42 ±0.28 and thereafter it lowered down upto 20 days and then it remained constant till 30 days of observations (Table, 4.7 and Fig. 4.45).

When bat guano was dissolved in experimental water with pH 5.00, the pH changed to 6.15 after 1 hour. After 2 hours upto 16 hours, the pH was found to be increased gradually and it reached to 7.95 after 16 hours. Thereafter, the pH started declining. It was 7.25 after 24 hours (Table, 4.6).

The water was kept undisturbed till 30 days and the pH was noted after every 5 days upto 30 days. After 5 days the pH was seen to be lowered down upto 10 days and increased gradually after 15 and 20 days then it remained constant till 30 days of observations (Table, 4.7 and Fig, 4.46).

When bat guano was dissolved in water of Wadali Lake with pH 7.19, the pH was found to be changed to 7.30 after 1 hour. After 2 hours upto 15 hours, the pH was found to be increased gradually and it reached to 8.30 after 15 hours. Thereafter, the pH started declining. It was 7.53 after 24 hours (Table, 4.6).

The water was kept undisturbed till 30 days and the pH was noted after every 5 days upto 30 days. After 5 days the pH was seen to be lowered down upto 15 days and then it remained constant during 25 to 30 days of observations (Table, 4.7 and Fig. 4.47).

4.4.2, Impact of bat guano on the pH of soil:-

To study the impact of bat guano on soil, 10 mg bat guano and 100 g of fine soil was mixed. 100 ml distilled water (pH-7) was added into it uniformly. The change in pH of soil was noted after 1, 6, 12, 18 and 24 hours. Thereafter, the observations were carried out for 5 days. Appropriate control was maintained. 100 ml double distilled water having pH 7 and 100 ml water with 10 mg bat guano dissolved in it were added in control and experimental

soil daily at 8 am respectively. The results are shown in table, 4.8 and Fig. 4.48 to 4.51; 4.52 to 4.55.

In all the experimental soil samples the alkalinity of soil was found to be increased. Soil (sample No. 8 and 9) was added with 100 ml of water each from industrial waste and eutrophic lake respectively. These soils represented a pH of 5.00 and 7.50 respectively. After adding bat guano dissolved water (10 mg/100 ml) in both these soil samples, the pH was increased to 7.40 and 8.08 respectively after 5 days (Table, 4.8).

4.4.3, Effect of bat guano on chloride, salinity, nitrate, phosphate and sulphate content of water:

Known quantity of bat guano from different habitats were added to experimental water (Wadali lake) and then alteration in respective parameters were noted for 24 hours initially and then upto 30 days with 5 days interval. The results are shown in tables, 4.9; 4.10; 4.11; 4.12; 4.13; 4.14; 4.15; 4.16; 4.17 and 4.18 and Fig. 4.56 to 4.59; 4.60 to 4.63; 4.64 to 4.67; 4.68 to 4.71; 4.72 to 4.75; 4.76 to 4.79; 4.80 to 4.83; 4.84 to 4.87; 4.88 to91; 4.92 to 4.95.

All the water parameters (chloride, salinity, nitrate, phosphate and sulphate) studied were declined significantly after 25 days of treatment.

4.5, Larvicidal activity of bat guano from different ecosystems:-

To study the effect of bat guano as larvicidal activity, various concentrations 40 to 120 mg of bat guano were dissolved in 1 litre of distilled water separately. 100 mosquito larvae were introduced in each of the concentrations. Mortality data upto 96 hours recorded to calculate the LC50. The observations are given in tables, 4.19; 4.20; 4.21; 4.22; 4.23 and 4.24 and Fig. 4.96 and 4.97; 4.98 and 4.99; 4.100 and 4.101.

The 96 hours LC50 values for bat guano from different habitats was in the order of LCMT < LCWT < SD < AC-7 < EC-16 < AC-24. The 96 hours LC50 values for bat guano collected from LCMT was 84.0 mg/L (Table, 4.19 and Fig. 4.96). Similarly 96hLC50 of bat guano collected from AC-24 was 97.0 mg/L (Table, 4.22 and Fig. 4.99). The 96 hours LC50 values of rest of the bat guano lie between the two above values.

4.6, Impact of bat guano on the plant growth:-

To study the impact of bat guano on plant growth, 10 to 50 or 100 mg bat guano was dissolved in 500 ml tap water and was sprinkled uniformly over 1 Kg soil.

Five replicates were kept ready. The control was maintained in which 500 ml of ordinary tap water was sprinkled on 1 kg soil .

Wheat (Triticum aestivum), Sadafuly (Vinca rosea), Zendu (Tagels elens) and Welvet (Amaranthus brazilansis) were selected to know the effect of bat guano on their growth with respect to height, branching and and flowering capacity of these plants. The results are shown in tables, 4.25; 4.26; 4.27 and 4.28 and Fig. 4.102; 4.103; 4.104 and 4.105.

Wheat (*Triticum aestivum*):

The healthy certified seed were brought from the Mahabeej seed corporation Ltd and 200 seeds were sowed in each pot. This is treated as day 0 (zero). Observations were made every day and it was found that pot No. 4, 5 and 6 showed the germination on 2nd day while germination was seen on 3rd day in pots 1, 2 and 3. The seedlings were watered with 100 ml tap water every day at 8:30 am. Then height of the growing seedlings was recorded on

every day upto 13 days and the results are shown in table, 4.25 and Fig. 4.102. After 13 days the long blades of seedlings were found to be bent down and they appear yellowish in colour.

Sadafuly (*Vinca rosea*):

The healthy mature seeds were selected and 2 seeds were sowed in each pot. This is treated as day 0 (zero). Observations were made every day and it was found that pot No. 2 showed the germination on 3rd day while germination was seen on 4th day in pot No.1 (control). The seedlings were watered with 100 ml tap water every day at 8:30 am. Then height of the growing seedling was recorded on every day upto 13 days and the results are shown in table, 4.26 and Fig. 4.103. After 13 days the height and flowering of seedlings were found to be increased than control.

Zendu (*Tagels elens*):

The 2 cm young plants were selected and 2 young plants were Planted in each pot. This is treated as day 0 (zero). Observations were made every day and the seedlings were watered with 100 ml tap water every day at 8:30 am. Then height of the growing seedling was recorded on every day upto 13 days and the results are shown in table, 4.27 and Fig. 4.104. After 13 days the height and flowering of seedlings were found to be increased than control.

Welvet (*Cealocea argentia*):

The 2 cm young plants were selected and 2 young plants were Planted in each pot. This is treated as day 0 (zero). Observations were made every day and the seedlings were watered with 100 ml tap water every day at 8:30 am. Then height of the growing seedling was recorded on every day upto 13

days and the results are shown in table, 4.28 and Fig. 4.105. After 13 days the height and flowering of seedlings were found to be increased than control.

4.7, Bat guano analysis for undigested remains:-

The bat guano collected from various habitats were 100 mg dissolved in water separately. The contents were allow to settle down. Supernatant was poured out and the undissolved remains with 5ml to solution were centrifuged at 3000 rpm. The supernatant was rejected and the settled remains were observed under light microscope. Slides were prepared and photomicrographs were takens. Identification reveals the undigested chitinous parts of mosquitoes and insects (Fig. 4.106; 4.107; 4.108; 4.09 and 4.110). Similarly the bat guano of frugivorous bats collected from forest habitat showed undigested epidermal remains of fruits (Fig. 4.111).

Most of the bat guano showed undigested remains of Trichoptera, Lepidoptera, Diptera, Hymenoptera, Orthoptera and Odonata etc. The actual remain in bat guano included antennae, mandible, wings, scales hairs turga, elitra and legs etc (Fig. Fig. 4.106; 4.107; 4.108; 4.09 and 4.110).


DISCUSSION

DISCUSSION

The present study was aimed to assess the physico-chemical characteristics and microflora of bat guano from 3 different habitats like old temples of in deep saline Lonar crater, world fame Ajanta and Ellora caves and the Melghat reserve forest. The impact of bat guano on plant growth and its larvicidal activity was also assessed.

Bat guano deposits have been found in several natural caves of the world. It is being commercially exploited as organic manure (Bhat and Shreenivasan,1990; Korine et al., 1999; Sridhar et al.,2006). In the present investigation the guano collected from different habitats was of the bats, Rousettus leschenaulti which are endemic to these habitats and are habitual of roosting in the old temples in Lonar crater and on tall trees from the Melghat forest. The roosting bats usually drop their droppings on the floor of temples and forest land respectively. The guano collected from Ajanta and Ellora caves was of the bats, Hipposidoros fulves fulves, which are insectivorous. The guano was collected carefully from the hips.

Organic fertilizers are used to improve soil quality and tilth and to provide nutrients for plant growth. They provide nitrogen (N), phosphorus (P) and potassium (K), as well as other elements essential for plant development and overall good health. Nutrient values vary greatly among organic fertilizers. Differences reflect variations in the age of organic material, its decomposition rate, application method and timing, incorporation time, the percentage of organic matter, carbon to nitrogen ratio, microbe population and

soil type (Ross, 2003). He compared the N, P, K content in different animal wastes which are used as manures (Table, 5.1).

Sridhar et al. (2006) analyzed contents in bat guano pellets and bat guano (humus) of cave bats, Hipposideros speoris. He recorded N, P and K as 7.7 to 8.5%, 2.0 to 3.0% and 0.4 to 1.2% respectively in the bat guano. In another study Goveas et al. (2006) revealed NPK in bat guano as 2.6:4.2:0.6. These bats (Pteropus giganteus) are frugivorous and phosphorus is fairly high in their guano. Phosphorus in bat guano is higher than in cow and sheep manure. Mathur et al., (1990) reported NPK in bat guano of frugivorous bats as 1.14:16.3:0.94. Young and Holt (1977) analyzed the NPK in guano of two bats, desert bat (8:4:1) and Dry Bar Cave bat (3:10:1). Similarly NPK content in bat gauno of Jamacian bat is claimed by the Nitron Industries (2000) as 3:8:1. All the above studies revealed that insectivorous bat guano contains more nitrogen than phosphorus and the frugivorous bat guano has more phosphorus than nitrogen. Bat guano deposits have been found in several natural caves of the world and commercially exploited as natural manure (Bhat and Shreenivasan, 1990; Korine, et al.,1999). Thus among the bat guano two broad categories have been identified based on NPK ratios: high phosphorus guano from frugivorous bats and high nitrogen guano from insectivorous bats.

In the present investigation (which is probably a first report), the bat guano analyzed from different habitats showed more phosphorus than nitrogen indicating that it is of frugivorous bats. But when it is compared with the earlier studies, it appears that the nitrogen and potassium contents in the bat guano under study, contain comparatively more amount except the bat

guano collected from forest habitat. This may be due to the secondary feeding habits adapted by the bats roosting in old temples. These bats feed on insects also. When the guano was analyzed for undigested remains, undigested chitinous remains like parts of legs, mandibles, wing scales were observed (Fig. Fig. Fig. 4.106; 4.107; 4.108; 4.09; 4.110 and 4.111).

These results are very important as, (looking towards the NPK content in the bat guano) it can be used as manure. “Wealth from Waste” can be achieved by its use in agriculture. It will also help in improvement of soil’s nutritive value. However, it appears that bat guano’s composition varies according to the bat’s feeding habits and the type and form of caves/temples where they live, among other factors like age and the biological composition of bat guano.

When the guano collected from different habitats was analyzed for different trace metals like Mg, Mn, Cu, Fe, Zn, Al, Se, Ba etc., it is observed that the guano collected from old temples of Lonar crater and that collected from Ajanta and Ellora caves as well as from forest vary greatly in above element contents. All the trace metals studied were appreciably higher in bat guano from old temples of Lonar crater. This may be because of safe sites of roosting and the guano settled on floors of temples is not washed out. These ionic contents in the bat guano are of high significance with respect to its nutritive value and as such they are of great biological significance. The presence of these trace metals in variable quantities provide useful data for calculating doses of micro-nutrients and analyzing the product’s physical properties for agricultural uses.

The bat guano from Lonar crater is carrying higher amount of trace metals because the frugivorous bats feed also on the insects of the habitat which extract ions from the basaltic rocks of the crater, during feeding. When the bats feed on these insects, the ions enter in the alimentary canal of bats and unabsorbed ions are excreted out in bat guano and/or through the bolus and urine. It is important to note the meteriorite impact in basaltic rocks of Lonar crater which had resulted in high proportion of ferrous, nickel, cobalt, zinc, aluminium, selenium, magnesium, barium and manganese, which provided an unique environment rich in heavy metal ions (Wani, et al., 2006).

The bat excrement and remains might be the main source of the elements nitrogen, phosphorus and potassium in guano. The organic compounds in the guano contain sulphur, phosphorus and nitrogen, which might be forming sulphuric, phosphoric and nitric acids after decomposition and oxidation. These acids may react with elements in the rocks of temples/caves to form their corresponding salts, sulphates, phosphates and nitrates and the sodium and potassium compounds are washed out, while the insoluble phosphates and sulphates are deposited and accumulated in the guano hips.

Metals like arsenic, barium, beryllium, cadmium, cobalt, chronium, copper, lead, selenium, zinc were reported by (Graening, 2005) in cave stream sediment having bat guano. Martin (1992) also reported similar elemental composition of bat guano and he opined that the elemental residues present in the guano are the undigested portions of ingested prey species. Thus the bat guano is a dominant energy resource which can be recirculated in the agroecosystem for energy transfer. The present

observations are in agreement with that of Mitzutani et al. (1992) who reported that bat guano is rich in calories and nutrients. The rich calories in bat guano may be because of comparatively less digestive efficiency of bats resulting in the expulsion of unabsorbed nutrients in guano and urine or because of the extremely short digestive tracts and rapid food transit times (Mitzutani et al., 1992).

Bat guano from Lonar crater was also analyzed for microorganisms present in it. Few actinomycetes and fungi are also reported (Table, 4.5.1, 4.5.2 and 4.5.3).

The present results with respect to the the bacterial species are important because bacteria in bat guano may be helping in the breakdown of the organic material. Tilak et al. (2005) reported a number of bacterial species associated with the bat guano belonging to genera, Azospirillum, Alcaligens, Arthrobacter, Acinetobacter, Bacillus, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Pseudomonas, Rhizobium and Serratia. He also suggested that these bacteria aid the plants to grow faster and healthy having better flowering capacity. Hutchens et al. (2004) had demonstrated aerobic methane oxidizing bacteria, Methylomonas and Methylococcus in bat guano. The bacterial enzymes capable of degrading a number of substances (Martin, 1991; Dvorak et al., 1992; Edenborn et al., 1992; Bechard et al., 1994; White and Chang, 1996; Frank, 2000; Kaksonen, et al., 2003; Vallero et al., 2003; Boshoff, et al., 2004; Miranda, 2005; Seena, 2005; Tilak et al., 2005).

Murphy (1989) demonstrated a nutritious broth formation when the bat guano was added in water and further he proved that this broth supported the growth of numerous microbes. Alley and Mary (1996) stated

that an ounce of bat guano contains billions of bacteria and thousands of bacterial species and these bacteria are important to soil composition and enable plants to absorb nutrients and essential elements. Sridhar, et al., (2006) have examined the bacterial and fungal fauna of bat guano. Their results revealed the presence of Acaligens, Pseudomonas, Fusarium and Penicillium in the bat guano. In the present investigation also, Acaligenes and Pseudomonas as well as Fusarium and Penicillium are observed in the bat guano of Lonar crater in addition to many other bacteria and fungi (Table, 4.5.1, 4.5.2 and 4.5.3). These results are also in consistence with those of Edwin (1965) who isolated Cladosporium sp., Sporotrichum sp., H. capsulatym and M. gypseum from fresh guano of Mexican freetail bats.

In the present investigation, when the bat guano in solution was added to soil and when seeds or plantlets were sowed/enrooted in these soils, better growth of plants was noted along with more branching and flowering to these experimental plants (Fig. 4.102; 4.103; 4.104 and 4.105). Applications of biodynamic preparations of bat guano, aid the soil in proper fermentation, thereby keeping the bacteria and microorganisms within the soil. Bat guano is known to stimulate potassium, silica, selenium activating bacteria and helps combine sulphur with other substances. It helps in the retaintion of nitrogen and calcium, keeping them in the living realm and prevents loss to the atmosphere. Also stimulates boron as well as azatobactor activity. It helps proper decomposition and aids chlorophyll formation. Stimulates iron, potassium, calcium, magnesium and sulphur activity in the soil (Lacki et al., 1994). Mistry (1995) and Alley and Marry (1996) were also of the opinion that the bacteria in bat guano are important to soil composition and enable plants

to absorb nutrients and essential elements. Bernard and Davison (1996) stated that bat guano helped balanced energies to protect aganist fungal diseases.

In the present studies, early germination of Triticum aestivum was observed when 30 mg, 40 mg and 50 mg of bat guano was added to the soil (Table, 4.25). In this experiment faster growth of the seedlings was also noted. Similar results were seen when 100 mg of bat guano was added to soil in which Vinca rosea seeds were sowed. The experimental seedlings also exhibited more branching (Fig, 103). All these results are due to high NPK and mineral contents in the bat guano. The bacterial flora in bat guano might have also supported the growth of plants by decomposing the organic contents in the soil. William (1999) suggested that bat guano can be used as good fertilizer soil builder and compost activator. Bat guano might have stimulated the potassium/silica bacteria in the soil to enable it to work more effectively with the growth factors. Ca and Mg can help in increasing flowering and filling out of fruit out to the tips. They also stimulate the phosphorus process and mobilizes the phosphorus activating bacteria in the soil. They can assist with the flowering process (Lacki et al., 1994).

Phosphorus rich guano as is reported in the present studies, is known to induce root growth, multiple branches in shoot and flowering in plants. In this respect, it can be said that the bat guano of frugivorous bats from Lonar crater is one of the best organic manures in the world today and hence a special urgent need is focussed to conserve these bats and their habitats.

Several organic manures of animal origin are available today for use e.g. night soil, bovine dung and urine, sheep manure, poultry manure, bat guano, silkworm wastes and vermicompost. Availability of such manure for crop production is restricted due to many constrains such as geographical region, awareness of manure value, extent of manure production and management. Recently the use of bat guano as composting organic matter has been proposed by Ashwini and Sridhar (2002, 2003). Guano is advertized as being quite safe and non-burning to plants (Kuepper, 2000). The present results regarding plant growth (height, branching, flowering, early germination) are very promising and they may only be because of the chemical ingradients and microbial fauna present in the bat guano of the bats inhabiting old temples in Lonar crater. The bats, though they are truly frugivorous (Rousettus leschenaulti) also feed on the insect fauna of the crater which are dependent on the elements in the rocks of the crater. It is observed that during feeding they secrete saliva on the rocks due to which the rock contents might have dissolved which are then sucked by the insects. These elements in the insects in turn enter in the bat's alimentary canal from where the unabsorbed ions escape in bat guano making it rich in various nutritive elements. Hence unlike other bat guanos reported (Martin, 1992; Mathur et al., 1990; Hedin et al., 1994; Vallero et al., 2003; Boshoff et al., 2004; Tilak et al., 2005), the bat guano on which the present results are obtained, contain not only more phosphorus but also it has comparatively more nitrogen and potassium also. The results clearly indicated that addition of moderate amount (100 mg/kg) of bat guano into soil enhances crop production. The amount tested in present investigation is quite low when compared with other organic

manures in practice. All experimental plants that received bat guano, produced plants that were greener, shiner, with broader stems, more branching than in the control (Pot No.1). This confirms that bat guano can be used as manure.

In one experiment, where different quantities of bat guano were used, better results were obtained at a dose 100 mg/kg soil. This indicates that bat guano can be used in low doses for better and satisfactory results.Hence it can be said that bat guano from Lonar crater “works wonders” as an organic manure.

Thus, bat guano is an ecological product that can be used in agriculture as it is an efficient soil regenerator producing quality organic food. It is prooved that the bat guano has a wide range of chelates (natural, organo mineral compounds with a high molecular weight), giving it greater structural stability and an extended residual effect on the soil on which it is applied (Studier et al., 1991; Sridhar et al., 2006)

Bat guano was also examined for its capacity to alter chemical properties of industrial waste water and water from eutrophic lake. However, before testing its effect on the experimental water, several experiments were performed to test the effect of different bat guanos on the chemical properties of experimental water.

When 10 mg of bat guano from Morache temple was dissolved in 100 ml distilled water, the pH of water was changed to 7.4 and after 24 hours to 8.3 (Table, 4.6) . After 25 days onwards, it remained constant as 8.65 showing 16.89% rise. These results indicated that bat guano has a capacity to change the pH. This property was examined for bat guano

collected from other habitats and was found that the bat guano make the water more alkaline. Even the acidic pH was made alkaline close to neutral.

This unique property of bat guano was then examined for experimental acidic water like that of industrial waste water with pH 5.00. 100 ml of eutrophic lake water was taken to which 10 mg bat guano from Morache temple from Lonar crater was added and dissolved properly. After one hour the pH was measured and it was 6.15 and after 24 hours it was increased gradually to 7.25 and ultimately it gave constant reading of pH after 25 days (7.55). Thus a pH was improved by addition of bat guano (Table, 4.6and 4.7). This change in pH of water may be because of the chemical contents in bat guano (Table, 4.4) or may be because of microbial fauna present in the bat guano. Harries (1970), reported that fresh guano is commonly basic, with the pH varying according to the volume of urine deposited with faeces. Fresh guano commonly had pH of 8.5 - 9.0 that rapidly became 5 to 5.5 with age and depth, although the centre of guano piles has a stable pH of around 4. This age related variation in pH is due to the biodegradation of guano with the help of hundreds of different types of bacteria associated with the guano (Coyane, 1999). Tilak et al. (2005) examined a number of bacterial species associated with the bat guano.

Addition of bat guano resulted into improvement in pH of soil also (Table, 4.8). Decline in chloride, nitrate, phosphate and sulphate contents of water from industrial waste and eutrophic lakes is observed in the present studies (Table, 4.9; 4.10; 4.11; 4.12; 4.13; 4.14; 4.15; 4.16; 4.17 and 4.18). This could also be due to the microbial flora present in the bat guano. Bat guano was used as a nutrient medium for the growth of Desulfotomaculum

nigrificans and sulphate reduction studies and metal sulphides are oxidized chemically and biologically to produce large amount of dissolved metals and sulphates. The technique involved, the development of bacterial sulphate reduction generating the H2S gas (Frank, 2000; Kaksonen et al., 2003; Vallero et al., 2003; Boshoff et al., 2004). Bat guano dissolved in water acts as a culture medium for bacteria. These bacteria bring about biodegradation of toxic compounds in favourable environmental conditions (Coyane, 1999). Pawar and Deshmukh (2004) successfully carried out the bioremediation of lake soil by using bat guano. They reported increase in soil microbial population from 65x104 bacteria to 278x105 within 12 days. Keleher (1996) suggested that bat guanos are rich in “bioremediation microbes” that assist in cleaning up soil toxins. Molecular analysis of microbial diversity associated with the Lonar crater were carried out by Wani et al. (2006) and they reported that these bacteria produced enzymes that can be used to detoxify industrial waste. Thus bat guano can be used for bioremediation of sick lakes and also for pre-treatment of industrial waste water before it is released in the environment. It is suggested that bat guano can also be used in intensive fish management practices to improve pH of water. At present liming practices are followed in fish and aquaculture.

During collection of bat guano, in old temples of Lonar crater it is observed that various invertebrates and even vertebrates were feeding on the bat guano (Table, 4.1). Gnaspini (1992) showed that some animal species are totally dependent on guano for their existence. They are called guanobites. Trajano et al. (2000) observed that the guano of bats is an important food source for cavernicoles throughout the world. Elliott et al.(1994) also reported

that the species like salamanders, catsfishes, shrimps, isopods, amphipods, snails, spiders, pseudoscorpions, beetles, millipedes, centipeds depend on the bat guano. Chippindale (2005) reported that bat guano is a preferred food of several cave dwelling animals like cockroaches, grasshoppers, frogs, toads, beetles etc. Invertebrate communities associated with bat guano were seen to be increased in density or pulse, after the bats appear and deposit bat guano (Poulson and Lavoie, 2000).

Fenolio et al. (2006) during population ecology studies in a cave habitat, observed 15 Eurycea spelaea ingesting bat guano. He analyzed the nutritional status of bat guano and stated that bat guano is a comparable food source to potential invertebrate prey items. Dipterans, nematodes and mites are also commonly associated with bat guano in caves (Peck, 1981). Ferreira and Martins (1999) recorded predator spiders feeding on bat guano. Specialized parasites and parasitoids are also active in many guanosystems. Braconid wasps (Hymenoptera) were found in several Eastern Australian guano caves (Austin and Dangerfield, 1992). Arthropods in guano communities fed either directly on guano or upon fungus growing on guano deposits. Guano required fungi and bacteria for its partial breakdown before it can be used by the majority of arthropod consumers (Gillieson, 2000). The present observations and the review made with respect to bat guano as food clearly indicates that the bat guano is no doubt a preferred food of detritivore animals inhabiting the caves. This is because the bat guano is very rich in several nutritive essentials elements (Table, 4.4). The guano is also known to provide the primary nutrients for entire cave ecosystem. The guano contains digested but unabsorbed nutritive food. It escapes from the gut of bat as the

length of alimentary canal of bat is short, (a “secondary morphological flying adaptation”). It is because of this, bats feed voraciously, digest it to absorb maximum but most of the undigested and unabsorbed nutritive food passes out unutilized and that is used by large number of invertebrates in cave ecosystem. Because of this the humus bat guano forms a sort of niche in cave ecosystem to which, I have described as umbrella ecosystem (Fig 5.1). Through the evolution, the bat guano has become a separate exclusive source food for many animals. Therefore, to save the race of such group of animals, bat conservation is essential. Some animals can not lay eggs until the fresh guano is available to it and accordingly they have changed their reproductive cycle. Others migrate to cave to feed on bat guano during pre-reproductive phase.

Bat guano from Lonar crater temple was also studied for testing its larvicidal property. The results are given in tables, 4.19; 4.20; 4.21; 4.22; 4.23 and 4.24. As the bat guano is nitrogen rich and also contains high amount of chlorine and also liberates NH3 on dissolving in water, it was thought to study larvicidal activity. The 96hLC50 values of bat guanos were calculated for mosquito larvae. The values are in the order of LCMT < LCWT < SD < AC7 < EC16 < AC24. The toxicity of bat guano is thus important so as to control the mosquitoes. The pungent smell and the suffocation in caves harboring the bats is because of the evaporating gases from the bat guano. When the guano is dissolved in water these gases get dissolved changing the pH of water. Thus it appears that the larvicidal activity shown by the bat guano is not only because of the high N and Cl contents but also due to change in pH. It may also be because of histoplasmosis as bat guano contains the

fungus Histoplasm capsulatum. Further bat guano is known to contain chitinase producing organisms. It is this chitinase which acts on the insect larvae and kill them, and then the decomposing microbes help in the further process.

Thus if bat swarming and roosting sites are preserved, their guano may become low cost natural manure for pot mixtures, gardens, nurseries, green house and landscapes. Astonishingly the bat guano from Lonar crater temples is not only rich in phosphorus but also in nitrogen and potassium. It is the presence of different bacteria in guano which helps in rapid plant growth by developing more branched root system, enabling the plants to absorb more nutrients from soil. However, it is predicted that continuous use of bat guano or use of bat guano in excess doses may acidify soils (Kinsey, 1994).

Therefore, it can be said that bat guano is a concentrate of highly nutritive minerals and also a live manure having benificial bacteria forming a natural biofertilizer of varied properties. It is just different from the existing bio-manures having protective functions for plants of agricultural importance. Hence, it is suggested that the bat must be conserved. They are the sources of several benificial microbes and thus form the live organic manure.


SUMMARY
& CONCLUSIONS

SUMMARY AND CONCLUSION

The present work was undertaken to analyze the chemical and microbial composition of bat guano and also to assess its use by other cave dwelling animals. Possibility of a role of bat guano in bioremediation, agriculture and as larvicide is also assessed.

The salient findings are as below.

1) Bat guano is rich in NPK content. Guano of frugivorous bats (from forest ecosystem) was rich in phosphorus content and that of insectivorous bats (from cave ecosystem) is rich in nitrogent content.

2) Bat guano of frugivorous bats from Lonar crater temples was rich in both phosphorus and nitrogen contents. Potassium was also at higher concentration when compared with the potassium contents of other bat guanos. This is probably the first record with respect to guano of bats from Lonar crater temples. The NPK richness of guano bats from Lonar crater is because the bats Rousettus leschenaulti also feed on the insects during night. The insects of this habitat make use of the ions in rocks of the crater which probably are the actual remains of meteriorite.

3) The faecal analysis of bat guano from Lonar crater temples was carried out. Though the bats roosting in these temples are frugivorous (Rousettus leschenaulti) their faecal contents contain undigested remains of chitinus parts of insects like legs, antennae, butterfly scales, mandibles, insect wings etc.

4) The economic value of the bat guano in terms of NPK richness indicates its possible importance in agriculture as cheaper organic manure and can be instrumental in third agriculture revolution.

5) The elemental analysis of bat guano indicated that they are rich in iron, nickel, cobalt, zinc, aluminium, selenium, manganese, magnesium, barium and sulphur.

6) The microbial assay of bat guano indicates that it contains several bacterial and fungal species. The microbial fauna decomposes the undigested part in fecal contents and convert it into humus guano.

7) These bacteria on humus guano were identified to some extent by carrying out several microbial tests. The identified bacteria in Lonar crater bat guano sample are Parabacterium spelei, Crenothrix putealis, Macromonas bipuntata, Nitrosomonas and Nitrobacter.

8) The bacterial count was the highest in the bat guano sample from Lonar crater temples. It was in the order of LCMT> LCWT > AC > AC> EC > SD.

9) The gunao was also found to contain fungal species like Cunnighamella, Fusarium, Penicillium cyclopium, Penicillium cirreonigrum, Aspergillus wenti, Aspergillus niger, Aspergillus flavus, Aspergillus tamari, Chysosporium, Rhizopus stolonifer, Chaetomium sp.,

10) The use of bat guano as manure indicated that it helps the plants to grow in height, to develop more branching and more flowering. Application of guano in soil also resulted into early germination of seeds. The guano kept the plant healthy during the period of investigation. Bat guano exhibited better results at low doses. At higher dose (500 mg/kg soil). The plant exhibited yellowness in colour of leaf blades. Thus bat guano can be used in low doses for better results and higher yields.

11) The application of bat gunao from Lonar crater temples resulted into increase in pH of industrial waste water and an eutrophic lake. It is also resulted into improvement in pH of soil. Decline in chloride, nitrate, phosphate and sulphate contents of water from industrial and eutrophic lake was also observed after addition of bat guano. These results indicated that bat guano from Lonar crater can be used for bioremediation of sick lakes and also for waste water treatments. This important property of bat guano may be because of the associated bacteria in it. If these bacteria are isolated as pure cultures, they can be used for production at commercial level. Their genome can be isolated and the multiple copies of then can be obtained by using modern biotechnological methods.

12) Various invertebrates and even vertebrates were seen feeding on bat guano in the old temples of Lonar. The humus bat guano in the temples has formed almost a separate ecosystem which is described as “Umbrella Ecosystem”, as the bat is supporting several animals by providing nutrient rich food. The dependents are ranging from prokaryotes and unicellular animals to

multicellular animals. Thus, through the microevolution, the today's, humus bat guano, has become a separate exclusive source of essential energetic food for many animals. Therefore, to save this much specialized micro system, bat conservation is essential. More studies are required in this direction as the animals who have become dependent on bat guano might have genetically evolved for the new micro-environment. In other sense, the humus bat guano is a representative of the evolutionary processes that took place since million of years.

13) The guano being a homogeneous mixture of several elements and evaporating compounds like NH3 and Cl2, it exhibited larvicidal property. This can be encashed commercially, for formulation of larvicidal products. Bat guano contains microbes which start multiplying when added to water. If the stagnant water bodies are treated with bat guano, the bacteria can be multiplied automatically and liberate enough gases like H2S, NH3 and Cl2 that help in killing the mosquito larvae. The chitinase liberating bacteria in bat guano can act on the chitinous body wall making it soft and delicate. The decomposing bacteria can then decompose them. Thus the larvicidal property of bat guano is because of the elemental as well as microbial population present in it. The bat guano from Lonar crater temples is one of the best organic manures in the world having larvicidal property and capacity to biremeditate the sick lakes. It is useful in treatment of industrial waste water. Therefore, an urgent need is focused to conserve these bats along with their microhabitat. Genome studies of these bacteria present in and growing on bat guano are essential.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