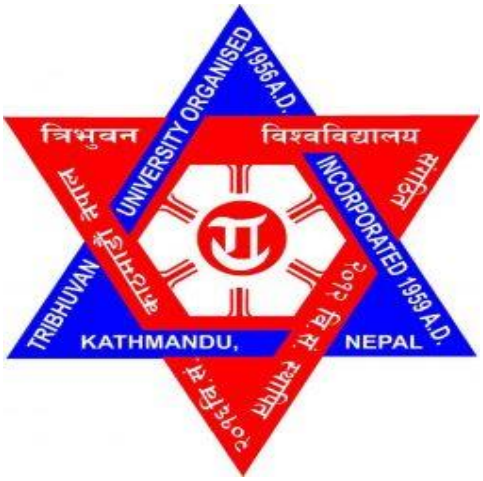
STUDY OF PHYSICO-CHEMICAL PROPERTIES OF SOIL AND CHARACTERIZATION OF *Azotobacter sp.* FROM PANCHAKANYA FOREST AND IT'S SURROUNDING AREA.



A

Dissertation Submitted to the Department of Microbiology,

Central Campus of Technology,

Tribhuvan University, Dharan, Nepal

In the Partial Fulfillment of the Requirements for the Award of Degree

Of Masters of Science in Agriculture Microbiology

Submitted by

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RECCOMENDATION

This is to certify that **Ms. Susmita Phattepuri** has completed this dissertation work entitled **"Study of Physico-chemical Properties of soil and Characterization of *Azotobacter spp*. from Panchakanya Forest and its Surrounding Area"** as a partial fulfillment of the requirements of M.Sc. degree in Microbiology (Agriculture) under my supervision. To my knowledge, this work has not been submitted for any other degree.

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CERTIFICATE OF APPROVAL

On the recommendation of **Associate Prof. Dr. Dil Kumar Limbu** this dissertation work of Susmita Phattepuri entitled **"Study of Physico-chemical Properties of soil and Characterization of *Azotobacter spp*. from Panchakanya Forest and its Surrounding Area"** has been approved for the examination and is submitted to the Tribhuvan University in partial fulfillment of the requirements for M.Sc. degree in Microbiology (Agriculture).

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ABSTRACT

The forest ecosystems provide critical and diverse services and values to human society. As primary habitat for wide range of species, forests support biodiversity maintenance and conservation. We cannot understand about forest without the knowledge of forest soil. Therefore, the main objective of this study is to study about the physical, chemical and microbiological properties of soil of Panchakanya forest and its surrounding area. 30 soil samples from 5 different locations of Panchakanya forest and 5 different locations surrounding Panchakanya forest were collected and analyzed for physical properties (moisture content, water holding capacity, pH, bulk density, electric conductivity), soil texture (sand, silt and clay) and chemical properties (carbon, nitrogen, potassium, phosphorus, soil microbial biomass carbon and soil microbial biomass nitrogen). Total plate count was done to study about the microbial load of each sample. Different species of Azotobacter was isolated and identified according to Bergey's Manual of Systematic Bacteriology. To establish the correlation between the physico-chemical variables and organism, the canonical analysis (CA) was performed.

Most of the soil samples were loamy sand having mean moisture content of 24.33%, mean water holding capacity of 60.5% mean bulk density of 1.24 gm/cm^3 and mean electric conductivity of 0.246mS/cm. Most of the soil samples were slightly acidic to neutral. The value of mean soil nitrogen was 0.1016%, organic carbon was 1.99%, mean phosphorus content was 2.278mg/l, mean potassium content was 44.85ppm, mean SMBC was 808.75 mg/kg and mean SMBN was 16.108 mg/kg. The value of total plate count ranged from 9×10^5 cfu/ml to 79×10^7 cfu/ml. After the study of colonial characteristics and microscopic examination, 27 isolates of *Azotobacter* were found. Further biochemical tests confirmed 20 isolates of *Azotobacter sp*. And among them 10 species were *A. chroococcum*, 6 species were *A. vinelandii* and 4 species were *A. beijerinckii*. The canonical analysis suggested that the physico-chemical variables such as soil texture (sand, silt and clay), organic carbon, pH, bulk density, potassium, nitrogen and electric conductivity are highly associated with organism (TPC) but negatively related to soil microbial

biomass carbon. In the contrary, variables of gravel, soil microbial biomass nitrogen and moisture content are not associated with organisms.

Keywords: Forest, physical properties, chemical properties, *Azotobacter spp*., canonical analysis

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LIST OF ABBREVIATIONS

UN= United Nations

FAO= Food and Agriculture Organization

GDP= Gross Domestic Product

PCA= Plate count agar

NA= Nutrient agar

NPK= Nitrogen, phosphorus, potassium

SMBC= Soil microbial biomass carbon

SMBN= Soil microbial biomass nitrogen

EC= Electric conductivity

BD= Bulk density

SOC= Soil organic carbon

SOM= Soil organic matter

TPC= Total plate count

CFU= Colony forming unit

CHAPTER-I

INTRODUCTION OBJECTIVES

1.1 Background of the study

According to the U.N. FAO, 25.4% or about 3,636,000 ha of Nepal is forested. Of this 14.5% (526,000 ha) is classified as primary forest, the most bio diverse and carbon-dense form of forest. Nepal has 43,000 ha of planted forest. Nepal's forests contain 485 million metric tons of carbon in living forest biomass. For a mountain country like Nepal altitudinal limits are most convenient to define ecological zones or life zones. On the basis of altitude, the forest of Nepal is classified into six categories. Tropical forest up to an elevation of 1000 m altitude, the sub-tropical forest of altitude 1000-2000m, the temperate forest having an altitude of 2000-3000 m, the sub-alpine forest of 3000-4000 m, the alpine forest of 4000-5000m altitude and the nival forest having height above 5000 meters (Shrestha 2008).

There are 35 major forest types and 118 ecosystems found in Nepal. Forests are an integral part of the farming system in Nepal. Farmers must have access to forest products such as leafy biomass for fodder and animal bedding, wood for energy and timber for agricultural implements and buildings (Acharya and Dangi 2009; Gilmour and Fisher 1991). Non-wood forest products have become the source of income for the rural poor, medicine for primary health care and revenue for the government. Forestry sector has a significant role in the economic development of country. FAO has estimated that Nepal's forestry sector has contributed 3.5% to the GDP of the country in 2000 and 4.4% for the period in 1990 to 2000. But it is estimated that the forestry sector alone contributes 15% to the GDP of the country (Sasatani 2009).

Forests are important carbon pools which continuously exchange carbon dioxide with the atmosphere, due to both natural processes and human action. At the global level, 19% of the carbon in earth's biosphere is stored in plants, and 81% in the soil. In all forests, tropical, temperate and boreal together,

approximately 31% of the carbon is stored in the biomass and 69% in the soil. In tropical forests, approximately 50% of the carbon is stored in the biomass and 50% in the soil (Dolman et al. 2010). Organic matter contains carbon which is oxidized and returned to atmosphere in the form of carbon dioxide. Carbon dioxide is the principal greenhouse gas (McKinley et al. 2011; Winjum et al. 1992). Forest's role in climate change is two-fold. They act as both cause and solution for greenhouse gas emissions.

In Nepal, information and study on carbon stock density in different forest ecosystem is still insufficient. Inventory of forest and soil has been paid little attention regarding the carbon that it sequestrated, hence amount of soil and biomass carbon sequestrated is unknown (Shrestha and Singh 2008) for many forests. But the study of microbial biomass carbon and total carbon in soil has been done by plenty of researchers. The constitution of Nepal, 2015 envisioned that carbon is a service. After amendment of prevailing Forest Act, 1993 (Gautam et al. 2004), carbon is recognized as an ecosystem service which is the first legalized document that emphasized that carbon has some economic value.

Forest ecosystem is an important field of research due to the role of forest as carbon sinks and the site of different biogeochemical cycles. In the forest soils, bacteria in habitat multiple habitats with specific properties, including bulk soil, rhizosphere, litter, and dead wood habitats, where their communities are shaped by nutrient availability and biotic interactions. Bacterial community takes part in essential biogeochemical cycles like carbon, nitrogen and phosphorus. They involve in decomposition of dead plant and animal biomass and are highly important in decomposition of dead fungal mycelia. Bacteria in forest soils respond to the effects of global change, such as climate warming, increased levels of carbon dioxide, or anthropogenic nitrogen deposition (Herrero et al. 2021).

Soil characteristics are studied on the basis of two properties: physical and chemical, and the nature of soil are determined according to the proportion and organization of these particles (Chinevu et al. 2013). Soil consists of four basic components: minerals, air, water and organic matter. In most soils,

minerals represent around 45% of the total volume, water and air about 25% each, and from 2% to 5% organic matter (Retallack 2008). Physical properties of soil include color, texture, structure, density, porosity, consistency, temperature, air and resistivity.

Colors of soil vary widely and indicate properties as organic matter, water and redox conditions. Soil texture, structure, porosity, density and consistence are related with types of soil particles and their arrangement. There are two types of soil particles- primary and secondary. Sand, silt and clay are the primary particles which are categorized on the basis of their diameter. Their relative proportion in soil is called soil texture (Osman 2012). Soil density, particularly bulk density, is a measure of soil compaction. Soil porosity consists of the part of the soil volume occupied by air and water. Soil consistency is the ability of soil to stick together. Soil temperature is self-defining. Resistivity refers to the resistance to conduction of electric currents and affects the rate of corrosion of metal and concrete structures (Santos et al. 2011).

The chemistry of soil determines the availability of nutrients, the health of microbial populations, and its physical properties. Soil chemistry also determines its corrosivity, stability and ability to absorb pollutants and to filter water. The surface chemistry of clay and humus colloids determines the chemical properties of soil. The high surface area of colloids gives soils its great ability to hold and release cations in what is referred to as cation exchange. Most soils contain organic colloidal particles as well as inorganic colloidal particles of clays (Li et al. 2021). There are about 72 mineral elements present in the soil which are referred to as plant nutrients and are classified as major and minor. They are very important for plant growth and development. The major elements include carbon, nitrogen, phosphorus, potassium, calcium, magnesium, sulphur, manganese, iron, zinc, copper, boron and molybdenum (Donahue et al. 1977).

Carbon is one of the main elements in soil which have many origins and sources of occurrence. Each source may have its own specialty and capability in giving the soil better properties (Munghate et al. 2020). Soil inorganic

carbon is mineralized forms of carbon, such as calcium carbonate, or caliche. It is more stable than most organic carbon because it does not provide food or fuel for microorganisms (Quilchano et al. 2008). Inorganic carbon, while it does not possess the water holding and soil enhancing properties of organic carbon, is nevertheless a significant store for atmospheric carbon (Liu et al. 2010). Soil nitrogen (N), phosphorus (P), and potassium are important sources of micronutrients for plant growth and productivity and they play an important role in terrestrial functions by influencing soil properties, plant growth and soil activities (Hati et al. 2008).

Forest ecosystem provide a broad range of habitats for bacteria, including soil and plant tissues, surfaces and rocks, but bacteria seem to be especially abundant on the forest floor, in soil and litter (Hardoim et al. 2015). Bacteria regulate soil ecosystem function (e.g. nutrient cycling, decomposition of organic matter, soil structure, and greenhouse gases (Rousk and Bengtson 2014) . Bacteria belonging to phyla *Acidobacteria, Actinobacteria, Proteobacteria, Bacteroidetes,* and *Firmicutes* appear to be abundant in forest soil (Lauber et al. 2009).

Fungi are the most well studied microbes in forest soils that harbor abundant and diverse communities of saprophytic and mycorrhizal fungal taxa (Peršoh 2015). Fungi are considered as the main decomposers in forest because of their ability to produce a wide range of extracellular enzymes that allow them to efficiently degrade the recalcitrant fraction of dead plant biomass (Lladó et al. 2017). Most of the forest trees are associated with mycorrhizal fungi which play a pivotal role in the mobilization and sequestration of nitrogen and phosphorus in the forest soil and are also responsible for significant soil transport of carbon (Clemmensen et al. 2015; van der Heijden et al. 2015).

The bacteria associated with fungal biomass decomposition like *Pseudomonas*, *Ewingella*, *Pedobacter*, *Variovorax*, *Stenotrophomonas*, *Chitinophaga* etc. are found in forest soil which are responsible for the secretion of chitinolytic enzyme (Brabcová et al. 2016). *Sphingomonas*, *Burkholderia*, *Enterobacter*, *Ochrobacterium*, *pseudomonas*, *Bacillus*,

Paenibacillus, Rhodococcus, Mycobacterium, Microbacterium and *Streptomyces* are the ligninolytic bacteria which are abundant in soil (Bandounas et al. 2011 and Tian et al. 2014). Cellulose-C is accumulated mainly by *Betaproteobacteria, Bacteroidetes* and *Acidobacteria*. Members of the *Proteobacteria* (*Burkholderiales, Caulobacteriales, Rhizobiales* and *Xanthomonadales*), Bacteroidetes (*Sphingobacteriales*) are responsible for cellulose derived-C accumulation (Eichorst and Kuske 2012). Species of *Azospirillum, Clostridium*, and *Azotobacter* etc. are found in the rhizosphere of different plants and are responsible for non-symbiotic nitrogen fixation. pH, organic matter content, nutrient availability, climate conditions and biotic interactions (especially the effect of vegetation) affect the composition of bacterial communities (Fierer and Jackson 2006; Lauber et al. 2009 and Urbanová et al. 2015).

The genus *Azotobacter*, which belongs to the family Pseudomonadaceae from the subclass γ-Proteobacteria comprises seven species: *Azotobacter vinelandii*, *A. paspali*, *A. chroococcum*, *A. nigricans*, *A. beijerinckii*, *A. armeniacus*, *A. salinestris* (Rubio et al.). The species of *Azotobacter* are usually motile by means of peritrichous flagella, oval or spherical that forms thick-walled cysts (means of asexual reproduction under favorable condition) and may produce large quantities of capsular slime. They are typically polymorphic and their size ranges from 2-10μm long and 1-2μm wide (Wani et al. 2016). *Azotobacter* species are aerobic, Gram negative bacteria found in neutral or alkaline soils, in water or in association with some plant or plant parts (R. Kumar et al. 2007; Tejera et al. 2005). *Azotobacters* are heterotrophic and their main property is the ability to fix nitrogen non-symbiotically, with a genomic content of G-C of 63-67.5% (Becking 1992; Setubal et al. 2009).

Azotobacter utilizes atmospheric nitrogen gas for their cell protein synthesis. The cell protein is mineralized in soil after the death of Azotobacter cells, contributing towards the nitrogen availability of the crop plants (Brakel and Hilger 1965; Jnawali et al. 2015). Beside nitrogen fixation, Azotobacter has beneficial effect on crop growth and yield through, biosynthesis of biologically active substances (like: thiamin, riboflavin, nicotine, indole acetic

acid, auxin, gibberellin, cytokinin etc.), and stimulation of rhizospheric microbes. When *Azotobacter* is applied to seeds, seed germination is improved to a considerable extent (Brakel and Hilger 1965).Hence, the main objective of this research is to study the physical, chemical and microbiological properties of soil sample of Panchakanya forest and its surrounding area and to study the association between them.

1.2 Objectives of the study

1.2.1 General objective

To study the physico-chemical and microbiological properties of soil of Panchakanya forest and its surrounding area, to study the association between them and to study different species of *Azotobacter sp.* Isolated from the samples.

1.2.2 Specific objectives

a. To study the physico-chemical properties of soil samples.
b. To isolate and characterize *Azotobacter species* from soil samples.
c. To study the relationship between physico-chemical properties and total no. of bacteria in soil samples.
d. To compare the physical, chemical and microbiological properties of forest soil and non-forest soil.

CHAPTER-II

LITERATURE REVIEW

2.1 Forest ecosystem

The growth and reproduction of forest cannot be understood without the knowledge of soil. Forest soil is the medium that produces the nature's most significant association of plants and animals, distinguished by immense practical usefulness and indefinite richness of pattern. The soil and vegetation are interrelated because they develop together over a long period of time. Different tree and plant species selectively absorbs nutrient elements and return them to the soil which brings changes in physical properties (Singh and Malhi 2006). The vegetation influences the physical properties of soil to a great extent. The vegetation improves the soil structure, infiltration rate, water holding capacity, hydraulic conductivity and aeration (Sharma et al. 2010b).

The composition of forest soil changes constantly by the growth of trees and ground cover vegetation, activity of organisms and effect of climatic agents. Under the influence of these factors, mineral and organic matter undergoes gradual decomposition and disintegration (Sharma et al. 2010a). Different tree species can differ significantly in their influence on soil properties as well as soil fertility (Augusto et al. 2002). Therefore, the adequate understanding of theoretical and practical knowledge of various forest soils and the complex relationship between the life of various trees and other plants of forest is necessary to study.

In tropical forest ecosystems, soil nutrients play an important role in the formation of plant communities, their species and structural diversity (Karpachevsky 1977). Change in diversity may be related to initial nutrient condition of soil. According to Theresa and Bowman (Theodose and Bowman 1997)nutrient enrichment increases the biodiversity in poor soils. There is positive correlation between soil variables and tree diversity (Homeier et al. 2010; A. Kumar et al. 2006). The growth of *Shorea robusta* (Sal) and other tree species, such as *Terminalia alata* and *Syzygium cumini* in tropical forests

are highly influenced by nitrogen, phosphorus, potassium and soil pH (Bhatnagar 1965).

2.2 Study of forest soil

Physiochemical characteristics of soil vary in space and time due to variations in topography, climate, physical and chemical weathering processes, vegetation cover, microbial activities and several other biotic and abiotic variables. Vegetation plays an important role in soil formation (Chapman and Reiss 1992) through decomposition of plant tissues for the main source of soil organic matter, which controls the physical and chemical properties of soil such as pH, water holding capacity, texture and nutrient availability (Adams et al. 1986).

Knowledge of physical properties of soil is important for determining soils suitability for agricultural, environmental and engineering uses. The supporting capability; movement, retention and availability of water and nutrients to plants; ease in penetration of roots, and flow of the heat and air are directly associated with physical properties of the soil. Physical properties also influence the chemical and biological properties of soil (Phogat et al. 2015). The most important and pertinent physical properties of soil relevant to its use as a medium for plant growth are discussed in following topics:

2.3 physical properties of soil

2.3.1 Moisture content of soil

The moisture content of soil is the quantity of water it contains. Soil moisture is important to know because it determines the yield of plants, soil water helps in chemical and biological activities of soil and microorganisms require moisture for their metabolic activities (SU et al. 2014). A research done by Shishir Paudel and Jay P Shah (Paudel and Sah 2003) in Sal Forest of Triyuga municipality in Udayapur district of eastern Nepal (86°9'-87°10' E and 26°39'-27°11' N and altitude ranging from 210 to 250m), moisture content of soil forest was 7.34% ± 1.47%. According to Tilak Prasad Gautam and Tej Narayan Mandal (Gautam and Mandal 2013)moisture content of tropical moist

forest (charkoshe jhari) in Sunsari district of Eastern Nepal (86°53'E to 87°21'E and 26°24' N to 26°52'N) was 47.0±2.8 in 0-15cm, 39.0±2.3 at the depth of 15-30cm and 28.4±1.7 at the depth of 30-45 cm. Whereas in Siwalik Forest around Letang Raja-Rani wetland Morang (26°24.9'22" N latitude and 87°28.9'10" E longitude with 470 m altitude and 1700 ha area) a research conducted by Momita Chetry, Rijan Ojha and Bhabindra Niroula (Chettry et al. 2021) shows 12.5% ± 0.9% of soil moisture.

2.3.2 Soil water holding capacity

Soil water holding capacity is the amount of water that a given soil can hold for crop use. Soil organic matter has a natural magnetism to water. Therefore increase in soil organic matter increases soil water holding capacity (Curell 2011).

The vaue of WHC for forest soil ranged from 43.03 to 49.80% in a study conducted by Shishir Paudel and Jay P shah (2003) in Sal Forest of eastern Nepal (86°9'-87°10' E and 26°39'-27°11' N and altitude ranging from 210 to 250m). A study conducted by Momita Chettry, Rijan Ojha and Bhabindra Niroula (2021) in Siwalik Forest around Letang Raja-Rani wetland Morang (26°24.9'22" N latitude and 87°28.9'10" E longitude with 470 m altitude and 1700 ha area) showed 50.5 ± 1.43% water holding capacity. Krishna Prasad Bhattarai and Tej Narayan Mandal (2020) reported 59.6±1.3% water holding capacity in 0-15cm soil depth and 54.58±1.2% of WHC in 15-30 cm depth in tarai sal forest of tropical region in Jhapa district of eastern Nepal (87°55' and 88°03'E and 26°27' and 26°32' N having altitude in the range of 62-129 m and area of 6300 ha).

2.3.3 Bulk density of soil

Bulk density of a soil is a dynamic property which varies with the soil structural conditions. The bulk density of a soil sample of known volume is the mass (or weight) of that sample divided by bulk volume (Chaudhari et al. 2013). It is regarded as a key factor that is correlated with soil compaction and many physical, chemical and biological properties of soil (Ahmed et al. 2021).

The value of bulk density of forest soil sample was 1.28 gm/cm^3 in a study conducted by Gautam TP and Mandal TN (2013) which was done in Sal bearing tropical forest located in the Bhabar belt of Sunsari district, Nepal (longitude 86°53' E to 87°21' E and latitude 26°24'N to 26°52'N). Similarly in the research conducted by Naghdi, Labelle and Solgi (Naghdi et al. 2016) in Sorkhekolah forest, Mazandaran province, northern Iran (36°21' N and 36°25'N and 53°5'E and 53°6'E with elevation of 1520 m) the average bulk density was 0.74 gm/cm^3. The bulk density of siwalik forest soil around Letang Raja-Rani wetland Morang (26°24.9'22" N latitude and 87°28.9'10" E longitude with 470 m altitude and 1700 ha area) was reported as 1.17± 0.07 gm/cm^3 (Chettry et al. 2021).

2.3.4 Soil texture

The relative proportion of sand, silt and clay in soil which make up the mineral component of soil is called soil texture. Soil texture is the fundamental property of soil which is not easily altered (Osman 2012). Different size groups of soil particles were chosen by International Society of Soil Science (ISSS) as clay, fine sand, coarse sand and gravel. The United States Department of Agriculture (USDA) has further subdivided the sand fraction. The two systems of textural classification are given in table 2.1.

Table 2. 1: USDA and ISSS soil textural classification

USDA classification		ISSS classification	
Size (mm)	Name	Size (mm)	Name
More than 2.0	Gravel	More than 2.0	Gravel
1.0-2.0	Very coarse sand		
0.5-1.0	Coarse sand	0.2-2.0	Coarse sand
0.25-0.5	Medium sand		
0.10-0.25	Fine sand	0.02-0.2	Fine sand
0.05-0.10	Very fine sand		
0.002-0.05	Silt	0.002-0.02	Silt
Less than 0.002	Clay	Less than 0.002	Clay

There are 12 textural classes of soil based on the USDA which are; clay, sandy clay, silty clay, sandy clay loam, clay loam, silty clay loam, sand, loamy sand, sandy loam, loam, silt loam, and silt. The soil texture triangle is used by scientists for the visualization and the understanding of the meaning of soil texture names. This triangle shows how each of these 12 textures is classified based on the percentage of sand, silt and clay in each other (figure 1).

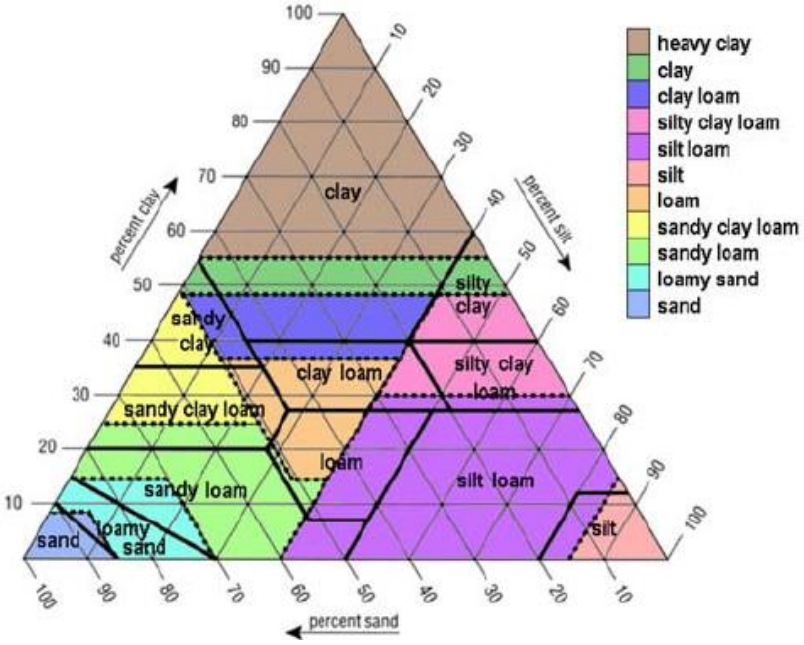


Figure 1: Soil texture triangle

In a research conducted by Shishir Paudel and Jay P (2003) Shah in two types of Sal Forest (pure *shorea robusta* and mixed *shorea robusta*) of eastern Nepal 86°9'-87°10' E and 26°39'-27°11' N and altitude ranging from 210 to 250m), soils were found to be sandy loam in both types of forest with sand content of 60.12% and 50.58%, silt content of 28.59% and 35.24% and silt fraction of 11.12% and 22.41%. Similarly, a study conducted by Tilak Prasad Gautam and Tej Narayan Mandal (2013) in tropical moist forest of Bhabar belt of Sunsari district (longitude 86°53' E to 87°21' E and latitude 26°24'N to 26°52'N) reported loamy soil in 0-15 cm depth of soil with 45.0±2.7 % of sand, 37.9±2.3% of silt and 17.1± 1.0% of clay, loamy soil in the depth of 15-30 cm of soil with 51.2 ± 2.6 sand %, 32.9 ±1.6% of silt and 15.9 ±0.8 clay % and sandy loam type of soil was observed in the depth of 30-45 cm with 66.7 ± 3.3 sand %, 26.5 silt % and 6.8 % of clay.

2.4 Chemical properties of soil

2.4.1 Soil pH

Soil pH or soil reaction is an indication of the acidity or alkalinity of soil. The effect of the soil pH is great on the solubility and availability of minerals or nutrients. Fourteen of the seventeen essential plant nutrients are obtained from the soil before a nutrient can be used by plants it must be dissolved in soil solution. pH influences the choice of crops grown and the type of soil organism that are present in the soil (Walworth et al. 2011). The majority of plant species prefer approximately neutral pH level. Acidic or alkaline nature of soil has low productivity (Adhikari et al. 2014).

A study carried out by Krishna Prasad Bhattarai and Tej Narayan Mandal (Bhattarai et al. 2020) in Sal Forest of tarai region situated at Jalthal near Kechana (extreme lowland of Nepal) of Jhapa district (87°55' and 88°03'E and 26°27' and 26°32' N having altitude in the range of 62-129 m and area of 6300 ha) reported acidic forest soil with pH of 5.35 ± 0.03 in 0-15cm of soil depth and 5.59±0.02 pH at the depth of 15-30cm. In the same research, Sal Forest of hill region located at Kiteni of Kolbung, Ilam district (elevation in the range of 500 to 850 m msl, 88°02' and 88°04' E longitude and 26°44' to 26°47' N latitude) was also studied which showed 6.42 ± 0.03 pH in 0-15cm depth of soil and 6.58 ± 0.03pH in the depth of 15-30cm. Similarly, a study of soil of Siwalik Forest around Letang Raja-Rani wetland Morang also showed acidic soil with pH value of 4.8±0.17 which was conducted by Momita Chetry, Rijan Ojha and Bhabindra Niroula in 2021. From a study done by Anup KC, Govinda Bhandari, Subigya Prabhat Wagle and Yubraj Banjade (Anup et al. 2013) in community forest in Mid Hill region of Nepal the soil pH was found in the range of 5.7 to 7.18.

2.4.2 Soil electrical conductivity

Soil electrical conductivity (EC) is a measure of the ability of the soil to conduct an electrical current. Most importantly to fertility, EC is an indication of the availability of nutrients in the soil. Soil electrical conductivity is regulated by several soil fertility attributes, such as pH, P, K, Ca, Mg, organic

matter, cation exchange capacity and by the contents of other soluble salts and organic ligands (Guo et al. 2012; Kitchen et al. 2005). The higher the EC, the more negatively charged sites (clay and organic particles) there must be in soil, and therefore the more cations (which have positive charge) that are being held in soil (Aimrun et al. 2009).

EC value of soil should not be too high, as too many of these nutrients especially Na and Mg, can be detrimental to soil health. Too low EC levels indicate low available nutrients, and too high EC level indicates an excess of nutrients. Optimal level of EC of soil ranges from 110-570 MilliSiemens per meter (mS/m). Low EC's are often found in sandy soils with low organic matter levels. Whereas, high EC levels are usually found in soils with high clay content (Aimrun et al. 2009). Noof A El Khamas (2016) reported 1.25 mS/cm to 1.39 mS/cm of electric conductivity in his research. Whereas a study done by (Shrestha and Lal 2011) showed the value of EC from 0.043 mS/cm to 0.154 mS/cm which was conducted in eight counties of Eastern Ohio (elevation ranging from 140m to 457m with slope gradient from 8 to 25%).

2.4.3 Soil nitrogen

Nitrogen, the most intensively used element, is available in virtually unlimited quantities in the atmosphere and is continually recycled among plants, soil, water and air. However, it is often unavailable in the correct form for proper absorption and synthesis by the plant (Gruhn et al. 2000). Nitrogen in soil is present in different forms such as organic, ammonium, nitrates and nitrites. Most of the nitrogen in soil is present in organic form. Small amount of nitrogen ordinarily occurs in ammonium and nitrate form (Mengel and Kirby 1987). It is a substrate needed for the synthesis of amino acids and proteins which are constituent of protoplasm and chloroplast (Sainju et al. 1998). Although total forest soil nitrogen pools can be quite large, nitrogen availability often limits forest growth and productivity (Keeney 1980).

Tilak Prasad Gautam and Tej Narayan Mandal (2013) reported 0.24%±0.01% of nitrogen in soil sample of 0-15 cm of depth, 0.12%±0.02% of nitrogen in the sample of 15-30 cm depth and 0.09%±0.01% of nitrogen in the depth of 30-45 cm in a study conducted in tropical moist forest (charkoshe jhari) in

Sunsari district of eastern Nepal (longitude 86°53' E to 87°21' E and latitude 26°24'N to 26°52'N). A study carried out by Krishna Prasad Bhattarai and Tej Narayan Mandal (2020) in tarai sal forest, Kechana, Jhapa (87°55' and 88°03'E and 26°27' and 26°32' N having altitude in the range of 62-129 m and area of 6300 ha) concluded 0.129 ±0.003% N in 0-15cm of soil and 0.106± 0.002% of N at the depth of 15-30cm and in hill sal forest, Ilam, reported 0.173± 0.005% N in the depth of 0-15cm and 0.124± 0.004 nitrogen percentage at the depth of 15-30cm. Gandhiv Kafle (Kafle 2019) reported 0.10% of nitrogen concentration in average in a research conducted in Kankali community forest, Chitwan, Nepal (27.65°N and 84.57° E with area of 749.18 ha and height of 220 to 580 meters from the sea level).

2.4.4 Soil carbon

Carbon accumulations in forest ecosystem involve numerous components including biomass carbon and soil carbon. The world's forest and forests soil currently store more than one billion tons of carbon which is twice the amount present in atmosphere (Oli and Shrestha 2009). The tree store carbon by sequestrating atmospheric carbon in the growth of wood biomass through the process of photosynthesis and thereby increasing the soil organic carbon (Brown and Pearce 1994). In recent years, soil organic carbon has received worldwide attention in the context of international policy agendas of CO_2 emission (Dahal and Bajracharya 2013).

After carbon enters the soil in the form of organic material from soil flora and fauna, it can persist in soil for decades, centuries or even millennia. Soil organic carbon (SOC), as a main component of soil organic matter (SOM), helps give soil its water retention capacity, its structure and its fertility. A high SOM content also provides nutrients to plants and improves water availability both of which enhances soil fertility and ultimately improves the food productivity (Sparling 1992). Moreover, SOC improves soil structural stability by promoting aggregate formation which together with porosity, ensure sufficient aeration and water infiltration to support plant growth. Carbon levels are commonly higher in surface soil but wide variations from almost zero to above 15% C are possible (Whitehead 2011).

Krishna Prasad Bhattarai and Tej Narayan Mandal (2020) reported 1.6 ± 0.09% soil organic carbon in 0-15cm of soil and 0.908± 0.05% of SOC at 15-30cm depth of soil in tarai sal forest of Kechana, Jhapa district of Nepal (87°55' and 88°03'E and 26°27' and 26°32' N having altitude in the range of 62-129 m and area of 6300 ha) and 2.09±0.12% of SOC in 0-15cm soil sample and 1.53±0.11% of SOC in soil collected from 15-30 cm depth from hill sal forest of Kolbung, Ilam district of Nepal (elevation in the range of 500 to 850 m msl, 88°02' and 88°04' E longitude and 26°44' to 26°47' N latitude). According to a study carried out by Tilak Prasad Gautam and Tej Narayan Mandal (2013) in tropical moist forest of Sunsari, Nepal (longitude 86°53' E to 87°21' E and latitude 26°24'N to 26°52'N) soil organic carbon was 3.07±0.15% in a sample collected from 0-15cm, 1.34±0.12 % in 15-30 cm and 1.17±0.10% in the soil sample from the depth of 30-45cm. Momita Chetry, Rijan Ojha and Bhanbindra Niroula reported 0.52 % ± 0.09% of organic carbon in study of Siwalik Forest around Letang Raja-Rani wetland, Morang, eastern Nepal.

2.4.4 Soil phosphorus

Phosphorus constitutes about 0.2% of a plants dry weight, where it is primarily a component of tissue molecule such as nucleic acids, phospholipids, and adenosine triphosphate (ATP). After nitrogen, phosphorus is the second most limiting nutrient (Chakraborty and Prasad 2021). Soil generally contains between 0.1 to 0.3 g Phosphorus kg^{-1} soil. (Perks et al 2015).

The phosphorus nutrition is critical to plants because it occupies a key position in metabolism. Carbohydrate metabolism proceeds when organic compounds are esterifies with phosphoric acids. It also participates in fat and protein metabolism. It highly affects the plant growth. It is essential for root development, seed formation and disease resistance to plants. Plants absorb phosphorus from the soil solution mostly in the form of orthophosphate ($H_2PO_4^-$) ions (Pattanayak et al. 2011).

Anup KC, Govinda Bhandari, Subigya Prabhat Wagle and Yubraj Banjare (2013) found soil phosphorus in the range of 73.71 kg/ha to 93.23 kg/ha in a

study conducted in Ghwangkhola Sapaude Babiyabhir Community Forest in Putali Bazzar Municipality-8, Syangja, Nepal. Sudarshan Kharal, Babu Ram Khanal and Dinesh Pandey (2018) reported available soil phosphorus value as 4.15 mg/kg in forest land and 41.07 mg/kg in vegetable farm, which shows lower phosphorus level in forest compared to farm land in Nuwakot and Chitwan district of Nepal (coordinates 84°54.7'E to 84°50.5'E and 27°48.3'N to 27°55.3'N with elevation of 575m in lowland to 760m in forestland).

2.4.5 Soil potassium

Potassium is one of primary nutrients for plant, which encourages normal cell division in young meristematic tissues. Plants absorb potassium in the form of K^+ ions. The plant requirement for available potassium is quite high. The plant roots take up potassium ion actively from soil solution. It is not coordinated with biomolecules in the plant (Brady and Weil 1990).

According to a study conducted by KC Anup et al (2013) the concentration of potassium on soil varied from 2.54 kg/ha to 4.23 kg/ha in Ghwangkhola Sapaude Babiyabhir Community Forest in Putali Bazzar Municipality-8, Syangja, Nepal. Kharal S et al (2018) reported 77.5 mg/kg of potassium in forest land and 130.2kg/ha of potassium in vegetable land in a study which was conducted in Rampur, Chitwan, Nepal (altitude 575 m in lowlands to 760 m in forestland with 84°54.7'E to 84°50.5'E and 27°48.3'N to 27°55.3'N). Siwalik Forest around Letang Raja-Rani wetland Morang (26°24.9'22" N latitude and 87°28.9'10" E longitude with 470 m altitude and 1700 ha area) a research conducted by Momita Chetry, Rijan Ojha and Bhabindra Niroula shows 300±9.38 kg/ha.

2.5 Soil Microbial Biomass

Soil microbial biomass (SMB) is the living portion of soil organic matter, constituted by archaea, bacteria and eukaryotes, excluding roots and animals smaller than 5×103 μm^3 (BRooKEs et al. 1984). It plays an efficient part in the formation of organic pool by decomposing organic matter and by controlling the nutrient dynamics which ultimately affect the primary

productivity in various biogeochemical progressions in terrestrial ecosystems (Kara et al. 2008). Changing microbial biomass may affect the cycling of soil organic matter (Shahriari et al. 2011). Generally, up to 5% of the total organic carbon and nitrogen in soil are in the microbial biomass. When microorganisms die these nutrients are released in such forms that can be taken up by plants. Microbial biomass is also an early indicator of changes in total soil organic carbon (Wiesmeier et al. 2019).

Foresters have always relied on knowledge of physical and chemical properties of soils to access capacity of sites to support productive forests. Assessment of physical and chemical properties of forest soil is also important for determining the consequences of management practices on the quality of soil relative to sustainability of forest ecosystem functions in addition to plant productivity. The concept of soil quality includes assessment of soil properties and processes as they are relative to the ability of soil to function effectively as a component of a healthy ecosystem (Schoenholtz et al. 2000).

2.5.2 Soil Microbial Biomass Carbon

According to a study conducted by Tilak Prasad Gautam and Tej Narayan Mandal (2013) in tropical moist forest (charkoshe jungle) in Sunsari district of Eastern Nepal (longitude 86°53'E to 87°21' E and latitude 26°24' N to 26°52' N) soil microbial biomass carbon was found to be 676.6±37 µg/g. Similarly, Krishna Prasad Bhattarai and Tej Narayan Mandal (2020), in a comparative study on soil microbial biomass in Tarai and Hill Sal forests of tropical region in Eastern Nepal (87°55' and 88°03'E and 26°27' and 26°32' N having altitude in the range of 62-129 m and area of 6300 ha) found 216.2 ± 11.8 µg/g of SMBC in Tarai Sal forest and 359.08 ± 13.94 µg/g of SMBC in Hill Sal Forest (elevation in the range of 500 to 850 m msl, 88°02' and 88°04' E longitude and 26°44' to 26°47' N latitude). According to a research conducted by Dil Kumar Limbu, Madan Koirala and Shang ZhanHuan (KumarA et al. 2020) in Tinjure Mike Jaljale (TMJ) mountain ridge-political border of three districts i.e, Taplejung, Tehrathum and Sankhuwasabha of Eastern Himalaya, Nepal (27°6'57" to 27°30'28" Northern latitude and 87°19'46" to 87°38'14" Eastern longitude with altitude ranging from 2400m to 3000 m asl), the soil microbial

biomass carbon (SMBC) of the study area was in the range of 219.84±1.6 mg/kg to 987.5 ± 1.93 mg/kg.

2.5.2 Soil Microbial Biomass Nitrogen

Tilak Prasad Gautam and Tej Narayan Mandal (2013) reported 59.0±38 µg/g of soil microbial biomass nitrogen in a study conducted in Sal bearing tropical moist forest (Charkoshe Jhari), located in bhabar belt of Sunsari district, Nepal (longitude 86°53'E to 87°21' E and latitude 26°24' N to 26°52' N). In a research carried out by Krishna Prasad Bhattarai and Tej Narayan Mandal (2020) in Sal Forest of Kechana of Jhapa district, Nepal (87°55' and 88°03'E and 26°27' and 26°32' N having altitude in the range of 62-129 m and area of 6300 ha) the value of SMBN was 29.67 ± 1.35 µg/g and the value of SMBN was 38.72 ± 1.62 µg/g in Hill Sal Forest of Kiteni of Kolbung, Ilam district, Nepal (elevation in the range of 500 to 850 m msl, 88°02' and 88°04' E longitude and 26°44' to 26°47' N latitude). Whereas the value of soil microbial biomass nitrogen was in the range of 15.11 ± 0.89 mg/kg to 44.1 ± 1.99 mg/kg in a study conducted by Dil Kumar Limbu, Madan Koirala and Shang ZhanHuan (2020) in Tinjure-Milke-Jaljale, Eastern Himalaya, Nepal.

2.6 *Azotobacter sp.* in soil

The family *Azotobactereceae* is a coherent group of aerobic, free-living, heterotrophic bacteria whose main characteristic is the ability to fix atmospheric nitrogen in a nitrogen-free medium with organic carbon compounds as energy source. Members of this group are generally called nonsymbiotic nitrogen fixers because, they fix N without the involvement of other plants or organisms. The genus *Azotobacter* was recognized in 1901 by Dutch microbiologist and his co-workers as the first aerobic free-living mitrogen fixer. These bacteria are known to exploit atmospheric nitrogen atmospheric nitrogen for their cellular protein synthesis which is mineralized in the soil, imparting the plants a considerable part of nitrogen available from the soil source (Aquilanti et al. 2004).

The population of *Azotobacter* in soil is influenced by soil physic chemical factors such as organic matter, pH, temperature, soil moisture, depth and

microbiological interactions (Kizilkaya 2009). It has been reported that *Azotobacter* are much available in the rhizosphere of plants than in surrounding soil, and this abundance mostly depends on crop species (Kuchekar and Pawar 2019).

2.7 Role of *Azotobacter* in soil

The abundance of *Azotobacter sp* in soil could improve the availability not only of N through BNF process but also phosphorus as well (Velmourougane et al. 2019). A study by Kizilkaya (2009) showed that soil carbon and sulphur contents increased in response to inoculation with Azotobacter sp. By accelerating mineralization of soil organic residues, this subsequently reduced heavy metal absorption by roots. Besides BNF, Azotobacter sp. are to influence directly plant growth by secreting plant-growth hormones (Example: Indole acetic acid 'IAA', gibberellins and cytokinin). These hormones enhance plant growth and nutrient uptake, as well as indirectly protect host plants from phytopathogens stimulates other beneficial rhizospheric microorganisms ((Bhardwaj et al. 2014). Azotobacter sp. is also important as plant growth promoting N_2-fixing rhizobacterium (PGPR) (Choudhury and Kennedy 2004).

Several strains of *Azotobacter* are found to produce amino acids when grown in culture media supplemented with various carbon and nitrogen sources (González-López et al. 2005). Such substances produced by these rhizobacteria are implicated in several processes thus leading to plant-growth promotion (Jnawali et al. 2015). *Azotobacter sp* has positive effects on plant growth, crop yield and plant N requirement of several economically important cereal and pulse crops reaching significant yield improvement up to 40% (Kannan and Ponmurugan 2010).

2.8 Biological nitrogen fixation

Biological nitrogen fixation refers to a microbial mediated process based upon an enzymatic nitrogenase conversion of atmospheric nitrogen into ammonium readily absorbable by roots. This process is also called biological nitrogen

fixation (BNF). N_2-fixing microorganisms also known as “diazotrophs” are able to fix N_2 biologically in association with plant roots (Aasfar et al. 2021) or without the association. BNF can provide an ecologically acceptable complement for mineral N fertilizers. This process is controlled by the availability of phosphorus (P), molybdenum (Mo), and water (Schulze and Drevon 2005). Published estimates of N derived from BNF ranges from 1.95×10^{11} kg of N-NH_3 (Galloway et al. 2004) to 2.5×10^{11} kg of N-NH_3 annually (Cheng 2008). *Azotobacter* is extremely resistant to oxygen during N_2-fixation due to respiration protection of nitrogenase (Wani et al. 2016). In addition to the respiratory protection there also exist hydrogenase uptake as well as switch on-off mechanism for the protection of nitrogenase enzyme from oxygen (Wani et al. 2016).

2.8.1 Mechanism of N_2-fixation

Azotobacter species play an important role in balancing soil nitrogen status. The estimated contribution of non-symbiotic BNF rates is subject to variations due to several factors including environmental variability, management and cropping practices, genotypic differences, and technical aspects related to methods used to estimate BNF (Peoples and Herridge 2000). BNF is an energetically expensive process because 16 ATP molecules are needed to break down N_2 molecule. Twelve additional ATP molecules are required for NH_4^+ assimilation and transport, totaling 28 ATP molecules (Buscot and Varma 2005). This BNF process under aerobic condition is the principal characteristic of the genus, and the process is catalyzed by nitrogenase.

Nitrogenase is an enzyme complex with two metal components: dinitrogenase MoFe (molybdenum-iron protein) serving as the catalytic component and dinitrogenase reductase (Fe protein). These two metal components are encoded by nif genes, the nifD and nifK genes coding for MoFe dinitrogenase and nifH gene coding for Fe dinitrogenase reductase (Buren and Rubio 2018). These two proteins act together to catalyze the reduction of dinitrogen in a complex reaction stoichiometry shown as follows (1) (Kirn and Rees 1992):

$$N_2 + 8e^- + 16MgATP + 8H^+ \longrightarrow 2\,NH_3 + H_2 + 16MgADP + 16Pi \qquad (1)$$

During the catalytic cycle, a Fe protein binds to one MoFe protein αβ unit. During this encounter, one electron is transferred from the cluster 4Fe-4S to the MoFe protein. This electron transfer step is coupled to the hydrolysis of a minimum of two MgATP molecules. Following electron transfer and ATP hydrolysis, the Fe protein disengages from the MoFe protein and a new Fe protein binds in its place to repeat the cycle (Kirn and Rees 1992). Only one electron is transferred per cycle, a minimum of 8 encounters must occur to reduce N as demonstrated by the eq 1.

CHAPTER-III

MATERIALS AND METHODOLOGY

3.1 Materials

The materials required for this work are listed in Appendix I.

3.2 Methodology

3.2.1 Study Design

This study was conducted at the Central Campus of Technology Hattisar, Dharan from August 22, 2019 to April 25, 2021.

3.2.2 Study Area

The study was conducted in Panchakanya forest and its surrounding area in Dharan. The sample area lies between 26º47.743' to 26º50.087' Northern latitude and 87º17.430' to 87º18.067' Eastern longitude. The altitude of the study area ranges from 460 m to 646 m. The average soil temperature at the time of sample collection was 26.52±3ºC and the average air temperature was 27.61ºC.

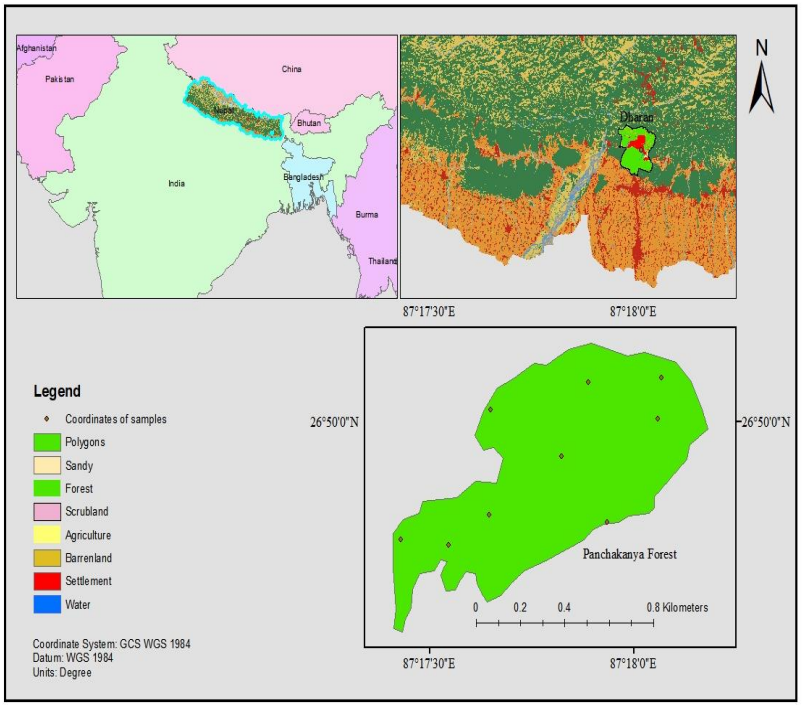


Figure 2: Panchakanya forest study area map

3.2.3 Sample collection:

Soil sampling was done in Bhadra of 2076. One quadrate (1m×1m) was established at each sampling point. Within each quadrate, four soil samples were collected at the depths of 0-5cm (upper soil profile), 5-10cm (second soil profile), 10-15cm (third soil profile) and 15-20cm (fourth soil profile). Then the samples of same height were mixed (composite soil sampling). Determination of physical and chemical parameters of soil was done from 0cm to 15 cm and microbial study was done from the samples collected from the height of 0 cm to 20cm. Samples were collected from five different locations inside the forest and from five different locations surrounding the forest. The latitude, longitude and elevation of these locations are listed in Appendix II. Soil borer having diameter 4cm and length 15 cm was used. Soil core from the borer was separated into three sections viz. 0-5, 5-10, and 10-15 cm slice with 5 cm length. Sample of each layer was packed in separate zipped polythene bag, placed in ice box and brought to laboratory of Central Campus of Technology. Samples for microbiological analysis were stored at 4ºC until analysis.

3.3 Laboratory analysis

3.3.1 Moisture content:

Gravimetric method was used for the calculation of moisture content of soil sample. For this, 100 g of soil sample was taken in a pre-weighted petridish and placed it in an oven at 70℃ for 24 hours. The petridish with dried soil samples were weighted (Yerima and Van Ranst 2005). The soil moisture content was then calculated by using given formula:

Moisture content (%) = (moisture lost/dry weight of soil) ×100

3.3.2 Water holding capacity:

The soil sample was dried in oven for about an hour. 10ml of water was sprinkled uniformly in a filter paper placed in a funnel. The oven dried and crushed soil sample (20 gm) was placed in funnel. Measured amount of water was uniformly poured with the help of glass rod until a drop of water was seen

passing from the tip of funnel. The volume of water retained by the dry soil sample was noted (Priha and Smolander 1999). Water holding capacity was calculated by using given formula,

Water holding capacity = (volume of water retained/mass of dry soil) ×100

3.3.3 Soil pH:

20 gm of soil sample was taken in a beaker. 100 ml of distilled water was added to stand for 30 minutes. Then the pH of the solution was noted using calibrated electronic pH meter (Yerima and Van Ranst 2005).

3.3.4 Bulk density

During sample collection, the cylindrical core sampler (borer, having 4cm diameter and 15cm tall core) was dipped up to 15 cm of soil below ground level. Soil from the borer was separated into 3 sections viz. 0-5, 5-10 and 10-15 cm slices with 5cm length. Sample of each layer was packed in separate zipped polythene bag and brought to the lab. The samples were dried at 105℃ for 24 hours and then the weight of the oven dried sample was taken (Al-Shammary et al. 2018). Bulk density of the sample was calculated by using given formula;

Bulk density (gm/cc) = weight of dry soil (gm)/volume of dry soil (cc)

3.3.5 Electric conductivity:

Soil sample (40 g) was taken in a 250 ml Erlenmeyer flask, to which 80 m l of distilled water was added (Tejada et al. 2006). The flask was sealed with stopper and shaken for an hour. Then, it was filtered through Whatmann no. 1 filter paper. The electrode of the conductivity meter was washed, and then dipped into the soil extract. The EC value displayed in the conductivity meter was recorded and noted.

3.3.6 Soil texture:

100 g of dried soil sample was taken in a box containing sieve of different diameters. Each component (gravel, coarse sand, silt etc.) of soil were separated and weighed separately. The percentage of each component was calculated separately (Alexander 1978) using given formula:

Percentage of gravel = (weight of gravel/total weight of soil) ×100

3.3.7 Soil Nitrogen by Kjeldahl method:

Soil nitrogen was determined using Kjeldahl method (Rai 2016). For this, 2gm of soil sample was taken in 250ml digestion flask. 2gm of catalyst mixture and 25ml of conc. H_2SO_4 was added. Blank sample was prepared likewise without soil sample. The sample and blank were digested till green color was seen. After cooling, the content was transferred quantitatively into 100 ml volumetric flask. 5ml of sample and blank was taken, one at a time into distillation assembly with 10 ml of 30% NaOH. The solution was distilled for 5 mins. The released ammonia was trapped in 5 ml of 2% boric acid containing 4 drops of mixed indicator until its color was changed. Then the boric acid mixture in the flask was titrated against standard (0.01N) HCL until its color disappeared. The soil nitrogen content was calculated by using given formula,

$$\text{Nitrogen (\%, wet basis)} = \frac{(\boldsymbol{sample\ titer - blank\ titer}) \times \boldsymbol{Nof\ Hcl} \times 14 \times 100 \times 100}{\boldsymbol{aliquot\ ml} \times \boldsymbol{weight\ of\ sample} \times 1000}$$

3.3.8 Soil carbon:

Estimation of soil organic carbon was done by Walkley-Black chromic acid wet oxidation method (Heanes 1984). The soil sample was dried, grinded and sieved through 2mm sieve. It was again sieved through 0.5mm sieve and 0.5 g sample was taken in 250ml conical flask. 10ml of 1N potassium dichromate was added to the sample and then 20ml of conc. H_2SO_4 was added with swirling to disperse the soil. A 200℃ graduated thermometer was inserted inside the flask and heated in gas burner over asbestos gauze. When the temperature was 135℃, the flask was kept aside to cool slowly. After 30 minutes, 200ml of deionized water was added and it was titrated with $FeSO_4$ using ferroin indicator. Blank titration was performed in similar manner without sample. The organic carbon content was calculated using given formula:

Organic carbon % = 0.003×N×10×1-TS×100/ODW

Where, ODW = oven dried weight

N = Normality of $K_2Cr_2O_7$

T = Volume of $FeSO_4$ used in sample titration (ml)

S = volume of $FeSO_4$ used in blank titration (ml)

3.3.9 Soil potassium:

Soil potassium was estimated by following protocol: P05-001A. The air-dried soil sample was passed through 2mm sieve. 10gm of sieved soil was transferred to a 100ml volumetric flask together with 50ml of the ammonium acetate/ acetic acid solution. The flask was transferred to a shaker and the sample solution was shaken for 30 minutes. The flask was removed from the shaker, allowed to stand for several minutes and then decanted the supernatant liquid through a dry Whatmann no. 2 filter paper. Potassium standard solution was prepared using potassium chloride and ammonium acetate/acetic acid solution. And then, standard solutions of 20, 40, 60, 80 and 100 ppm were prepared. Calibration of flame photometer was done by aspirating these five-potassium standard solutions. After calibration, potassium content of sample was estimated by aspirating the sample solution (Tellen and Yerima 2018).

3.3.10 Soil phosphorus

Ammonium molybdate blue method was used for the estimation of soil phosphorus (Tellen and Yerima 2018). For this, 0.5 gm finely ground soil sample was taken in a beaker and 5 ml of conc. Sulfuric acid was added and swirled gently. 3 ml of 30% hydrogen peroxide was added in 0.5 ml portions and swirled vigorously. After that, `1 ml of conc. Hydrofluoric acid was added to the beaker and gently swirled. The beaker was placed on a hot plate at 150℃ for 10-20 min to eliminate excess H_2O_2. After slight cooling, the sides of the beaker we washed down with approx. 15ml distilled water and mixed. The content of the beaker was transferred to a 50 ml volumetric flask, passing it through a filter paper. Two additional washings of beaker were done, filtered and the volume was adjusted. Blank was prepared in the same way but without sample. Phosphorus standard solution was prepared by dissolving potassium dihydrogen orthophosphate with deionized water and conc. H_2SO_4. Working solution of concentration 10, 20, 30, 40, and 50mg/L were prepared. Blank was prepared without sample. Absorbance of working solution and sample

were observed using blank in spectrophotometer. Phosphorus content in sample was calculated by using excel.

3.3.11 Soil microbial biomass carbon and nitrogen

Microbial biomass carbon was determined using the fumigation extraction methods (Brookes et al. 1985). The filtered soil extracts of both fumigated and non-fumigated samples were analyzed for organics carbon using the acid dichromate method (Vance et al. 1987). Total nitrogen in K_2SO_4 soil extract was determined by acid digestion and Kjeldahl distillation (Brookes et al. 1985).

Fumigation

3 samples of 50gm oven soil sieved through 2mm sieve was weighed and placed in screw top jars. The jars were placed in desiccator having moistened tissue paper at bottom. 25 ml vial of soda lime and 30 ml $CHCl_3$ with anti-bumping granules in a beaker were also placed in the desiccator. The desiccator was evacuated using vacuum pump until $CHCl_3$ boils vigorously. It was continued for 2 minutes after that the valve was closed and the pump was detached. The desiccator was placed in room temperature and left in dark for 24 hours. Fumigated as well as non-fumigated soil samples were transferred separately to volumetric flask and 200 ml of 0.5M K_2SO_4 was added. The flasks were placed in shaker for 30 mins. The soil extract was filtered through Whatmann filter paper no. 42. The blanks were prepared in similar way without sample.

3.3.12 Biomass carbon measurement

8 ml of filtered extract was placed with 2ml of 66.7mM $K_2Cr_2O_7$, 70 mg HgO and 15 ml of a mixture of 2 parts H_2SO_4 and 1-part H_3PO_4 in a round bottom flask. The mixture was boiled gently under reflux for 30 mins. Cold blank was not heated. It was then cooled and diluted with 20ml water. The residual dichromate was measured by back titration with 0.4M ferrous ammonium sulphate solution using 25 mM 1,10 phenanthrone ferrous sulphate complex as an indicator. Extractable carbon was calculated using following formula:

$C\ (\mu g ml^{-1}) = (Hbl\text{-}S)/Cbl \times N \times Q/A \times B \times 1000$

Where: Hbl= titration solution consumed by hot blank

S= titration solution consumed by sample

Cbl= titration solution consumed by cold blank

N= normality of $K_2Cr_2O_7$ =0.4

Q=Quantity of $K_2Cr_2O_7$ =2ml

A= Aliquot quantity =8ml

B= 3 = conversion of Cr VI to Cr III

1000= to change into μg

3.3.13 Microbial biomass nitrogen measurement

30 ml of K_2SO_4extracts (both fumigated and non-fumigated) were placed into digestion tubes containing anti-bumping granules. 0.6 ml of $CuSO_4$ (0.19M) and 10 ml of conc. H_2SO_4 were added and refluxed for 3 hours. It was then cooled and diluted with 20 ml of water. To these tubes, 25 ml of 10 M NaOH was added and mixed. Then the sample was placed in steam-distillation and distilled into a conical flask containing 5 ml 2% boric acid which absorbed evolved NH_3. The solution was titrated with 50mM H_2SO_4 using a standard burette. Total N was determined using following relation,

$N\ (\mu g\ g^{-1}) = Vs\text{-}Vb \times M \times At \times 100 \times 0.5W$

Where; Vs = Volume of H_2SO_4 used to titrate the sample

Vb = Volume of H_2SO_4 used to titrate the blank

M = Molarity of H_2SO_4 = 0.05

At = Atomic weight of Nitrogen

1000 = to convert into microgram

0.15 = the fraction of extract used for the titration

W = K2SO4 extract soil moisture content oven dried weight of soil

3. 4 Microbial analysis

3.4.1 Isolation of *Azotobacter spp.*

Isolation, identification and characterization of *Azotobacter* were done according to Bergey's manual of Bacteriology (Bergey 1994). For the isolation of Azotobacter spp. 1gm of soil sample was subjected to serial dilution up to 10^{-6} dilution in sterile water. Nitrogen free media (Ashby's and Jensen's media) were prepared, autoclaved at 121ºC for 15 minutes for sterilization and then poured in sterile plates. 0.1ml suspension from dilution 10^{-3}, 10^{-5} and 10^{-6} were spread on Ashby's and Jensen's media plates uniformly with the help of sterile dolly rod. Then, the inoculated plates were incubated at 32ºC for 2 to 5 days. After incubation the plates were observed and colonial characteristics like color, shape, margin, opacity, elevation, consistency etc. were noted. Creamy and dark brown colored colonies from Ashby's media and water droplet type of colonies from Jensen's media were selected and sub-cultured on Nutrient Agar (NA).

3.4.2 Identification and characterization of *Azotobacter*

After obtaining the pure culture, the organism was identified by using standard microbiological techniques as described in Bergey's Manual of Systematic Bacterilogy-1986 (Sneath et al.). For the identification of Azotobacter form pure culture, colonies from NA plates were taken and Gram stained. The colonies having rod shaped bacteria were again sub-cultured in NA. Then the further identification was done by performing biochemical tests like motility test, catalase test, starch hydrolysis test, citrate utilization test, urease test, indole test and MR-VP test. Carbohydrate fermentation (glucose, sucrose and fructose) tests were performed for further confirmation of Azotobacter. To perform biochemical test; biochemical medias were prepared in test tubes, autoclaved and slants or broth were made. Colonies to be tested were stabbed or inoculated with the help of sterile inoculating needle/loop and incubated at 32ºC for 24 hours. After incubation, the result was noted by observing change in color (without or after adding reagents).

3.4.3 Total plate count

Total plate count was carried out by pour plate technique. Plate Count Agar (PCA) media was used. After performing serial dilution, 1 ml aliquot from each dilution were added to sterile petriplates in which molten and cooled (40-45ºC) PCA was poured. The plates were gently rotated for uniform distribution throughout the medium. The plates were allowed to solidify and then incubated at 32ºC for 24-48 hours (Aneja 2007). The plates were screened for the presence of discreet colonies after 24 hours and the actual number of bacteria was estimated as colony forming unit in per ml (cfu/ml).

Number of cells/ml= no. of colonies × dilution factor/volume of sample

3.5 Data analysis

For and foremost, all the obtained raw data were arranged in Excel 2010. To establish the correlation between the physico-chemical variables and organism, the canonical analysis (CA) was performed. To find the determining factors on the population of organisms, analysis of variance (ANOVA) was performed. All the statistical analysis was performed in Past 3 software.

3.6 Methodology design

Chart 1: Laboratory analysis of physico-chemical parameters of soil

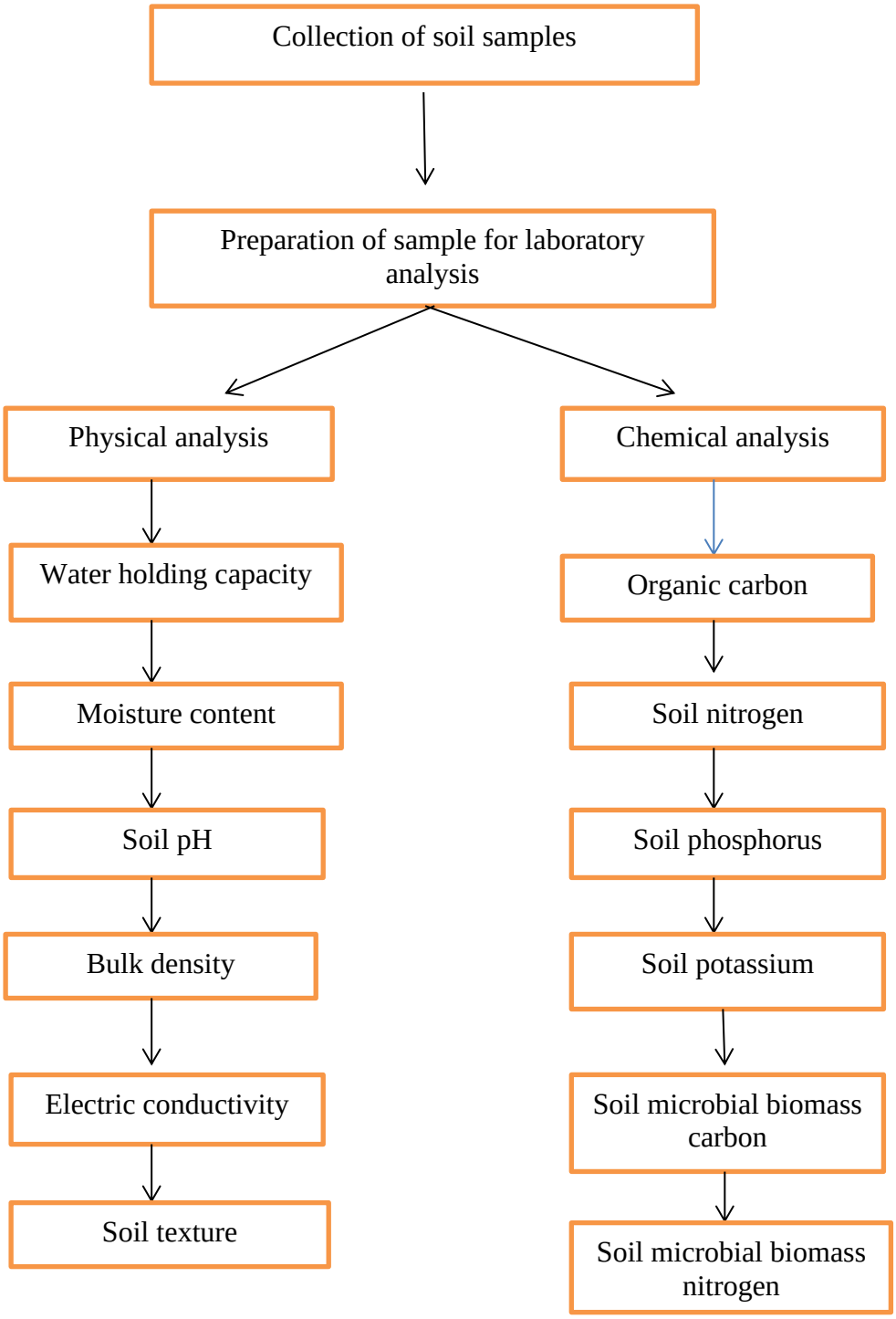
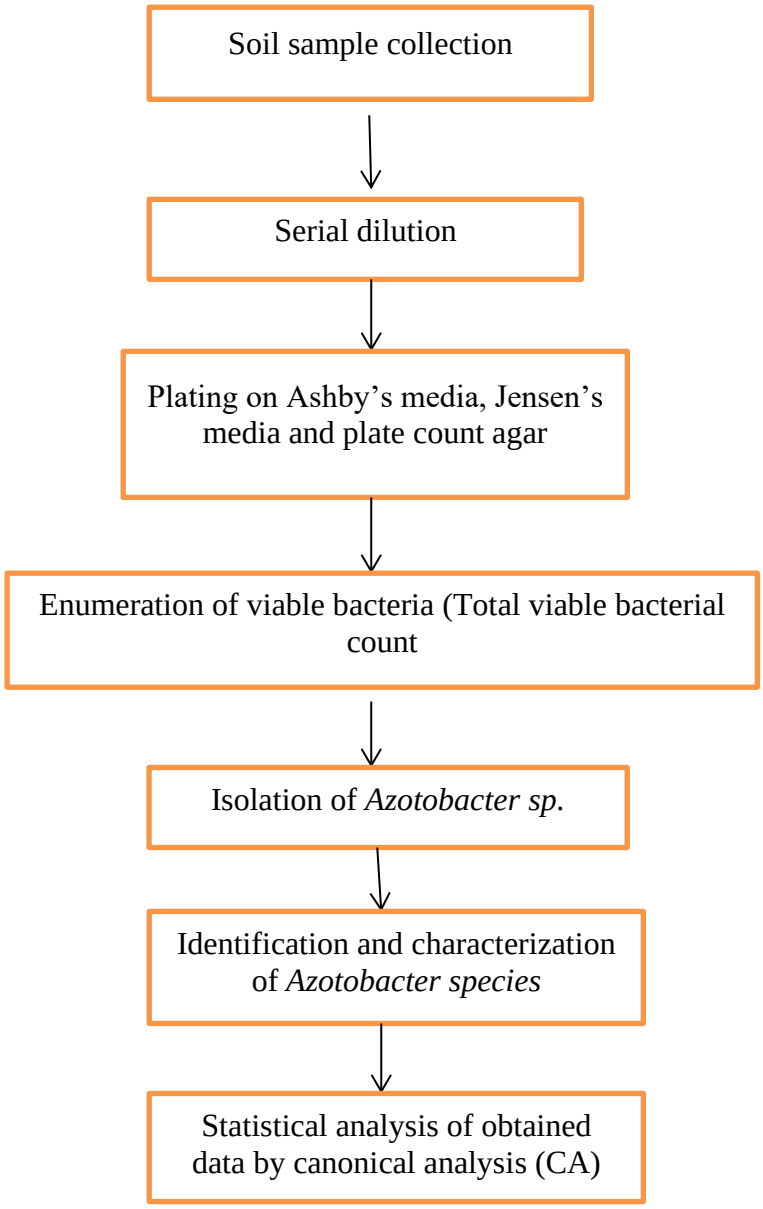


Chart 2: microbiological and statistical analysis of soil sample



CHAPTER IV

RESULTS

4.1 Physical properties of soil

Descriptive statistics of some physical and chemical properties of the soil samples studied in this research are given in tables below. The water holding capacity of all 30 soil samples (10 samples each having 3 layers of soil) ranged from 40.5% to 78.5% with an average value of 60.5%. The soil moisture content ranged from 0.08% to 128.62% for all soil samples in 10 different locations with an average value of 23.33%. The value of the moisture content varied largely according to the type and texture of soil. The pH of most of the soil samples were acidic ranging from 5.17 to 6.6 except sample S5 (pH value 7.6 to 9.2). The bulk density increased with the depth. The bulk density of all samples of surface levels (0-5cm) ranged from 0.66gm/cm^3 to 1.46gm/cm^3 with an average value of 1.13gm/cm^3. The bulk density of the samples from depth 5 cm to 10 cm ranged from 0.86gm/cm^3 to 1.45gm/cm^3 with average value of 1.24gm/cm^3. Similarly, the bulk density value of the samples from depth 10cm to 15cm ranged from 0.98gm/cm^3 to 2.05gm/cm^3 with an average value of 1.34gm/cm^3. Values of electrical conductivity of all samples are given in table no.1. The electrical conductivity value ranged from 0.15 mS/cm to 0.52 mS/cm.

Different physical properties viz. water holding capacity, moisture content, soil pH, bulk density, electric conductivity and soil texture of the soil samples were analyzed and the results are shown in table 4.1 and 4.2.

Table 4. 1: Physical properties of soil

Sample no.	Depth (cm)	Water holding capacity (%)	Moisture content (%)	pH	Bulk density (gm/cm^3)	Electric conductivity (mS/cm)
S1	0 to 5	63.5	23.21	5.42	1.2	0.22
	5 to 10	67.5	23.3	5.78	1.38	0.24
	10 to 15	53.5	128.62	5.84	1.14	0.2
S2	0 to 5	53	13.37	6.3	1.25	0.23
	5 to 10	65	18.57	6.2	1.4	0.25
	10 to 15	63	20.38	5.9	1.8	0.18
S3	0 to 5	61	14.52	6.5	1.27	0.24
	5 to 10	56	9.9	6.2	1.42	0.22
	10 to 15	50	10.68	6.6	1.5	0.19
S4	0 to 5	52.5	2.88	6	1.46	0.18
	5 to 10	55	6.45	5.8	1.45	0.18
	10 to 15	52	6.08	5.9	1.53	0.24
S5	0 to 5	45	2.84	9.2	1.35	0.52
	5 to 10	40.5	5.43	7.7	1.31	0.25
	10 to 15	43.5	5.52	7.6	2.05	0.3
F1	0 to 5	46.5	46.93	5.46	0.66	0.41
	5 to 10	78.5	40.19	5.49	0.99	0.2
	10 to 15	74.5	35.65	5.62	0.98	0.21
F2	0 to 5	61	0.08	5.49	1.27	0.29
	5 to 10	52.5	23.67	5.48	1.29	0.21
	10 to 15	48.5	21.21	5.53	1.17	0.18
F3	0 to 5	70.5	35.57	5.34	1.2	0.36
	5 to 10	67	22.04	5.5	1.05	0.17
	10 to 15	62	19.8	5.21	0.98	0.18
F4	0 to 5	77.5	30.53	5.17	0.81	0.29
	5 to 10	75	32.89	5.27	1.24	0.17
	10 to 15	73.5	33.62	5.35	1.23	0.15
F5	0 to 5	63.5	31.73	5.32	0.91	0.43
	5 to 10	72.5	33.87	5.31	0.86	0.29
	10 to 15	71	30.53	5.25	1.07	0.22

The percentage of gravel, coarse sand, fine sand and silt are depicted in table no.2. The nature of soil collected from surrounding area of forest (S1, S2, S3, and S4) was mainly loamy sand. According to the percentage of soil particles, forest soil samples were found to be loamy sand, sand and silt loam. The

percentage of sand was relatively more on the surface (0-5 cm) than in the depth (10-15 cm) of the soil.

Table 4. 2: Soil texture

Sample no.	Depth (cm)	Gravel (%)	Sand (%)	Silt (%)	Clay (%)	textural class
	0-5	34.21	53.03	11.17	1.16	loamy sand
S1	5 to 10	41.21	43.47	13.58	1.66	loamy sand
	10 to 15	19.88	60.23	17.85	1.64	loamy sand
	0-5	50.04	40.4	7.2	2.22	sand
S2	5 to 10	38.4	48.46	11	1.79	loamy sand
	10 to 15	61.24	30.86	6.73	1.04	sand
	0-5	47.79	40.28	9.2	2.28	sand
S3	5 to 10	44.63	44.49	7.93	2.47	sand
	10 to 15	34.62	48.83	13.04	3.29	loamy sand
	0-5	75.12	17.28	6.06	1.33	loamy sand
S4	5 to 10	40.86	40.32	15.52	3.07	loamy sand
	10 to 15	59.33	26.32	11.8	2.28	loamy sand
	0-5	6.48	16.12	63.88	12.97	silt loam
S5	5 to 10	68.31	17.22	10.3	3.98	loamy sand
	10 to 15	61.44	21.22	12.64	4.04	loamy sand
	0-5	20.49	59.52	14.58	5.02	loamy sand
F1	5 to 10	21.58	57	17.48	3.13	loamy sand
	10 to 15	17.69	55.92	23.49	9.34	loamy sand
	0-5	33.37	52.93	13.32	0.18	loamy sand
F2	5 to 10	36.44	51.93	9.95	1.39	loamy sand
	10 to 15	29.58	57.58	12.64	0.19	loamy sand
	0-5	27.59	54.53	15.48	1.5	loamy sand
F3	5 to 10	43.09	46.36	8.93	1.41	loamy sand
	10 to 15	51.92	40.62	6.04	1.2	sand
	0-5	19.03	66.33	11.11	3.28	loamy sand
F4	5 to 10	17.1	70.33	10.3	2.03	loamy sand
	10 to 15	18.56	65.89	12.52	2.8	loamy sand
	0-5	39.36	51.25	7.61	1.5	sand
F5	5 to 10	22.84	61.45	13.84	1.67	loamy sand
	10 to 15	22.54	62.35	12.65	2.12	loamy sand

Table 4. 3: Descriptive statistical value of physical properties of the soil sample.

Parameters	Maximum	Minimum	Mean	Standard deviation	Variance
Water holding capacity	78.5	40.5	60.5	10.85	117.81
Moisture content	128.62	0.08	24.33	23.37	546.45
pH	9.2	5.17	5.92	0.88	0.78
Bulk density	2.05	0.66	1.24	0.285	0.082
Electric conductivity	0.52	0.15	0.246	0.085	0.007
Sand	70.33	16.12	46.75	15.29	234.013
Silt	63.88	6.04	13.59	10.238	104.832
Clay	12.97	0.18	2.73	2.58	6.65

4.2 Chemical Properties of soil

Soil organic matter content is the rich mineral constituents in the soil that allows development and growth of plants. It contributes immensely to soil nitrogen, phosphorus, potassium, carbon, sulfur, cation capacity and exchangeable cation (Opeyemi Dr et al. 2020). The soil nitrogen of studied samples ranged from 0.01% to 0.27% with average value of 0.101%. The value of available phosphorus in soil was 0.66 mg/kg to 21.87mg/kg whereas soil potassium was observed from 6.30ppm to 83.40ppm with an average value of 30.68ppm. The mean value of organic carbon was 1.99%, values ranging from 0.67% to 4.35% (Table no. 4.4).

Table 4. 4: Chemical properties of soil

Sample no.	Depth (cm)	Nitrogen (%)	Phosphorus (mg/kg)	Potassium (ppm)	Organic carbon (%)
S1	0 to 5	0.27	5.699	29.8	2.734
	5 to 10	0.18	21.873	24	1.469
	10 to 15	0.17	9.997	21.6	2.02
S2	0 to 5	0.1	0.755	18	2.612
	5 to 10	0.1	1.078	12.3	1.244
	10 to 15	0.07	0.986	11.5	2.142
S3	0 to 5	0.18	1.263	42.4	0.979
	5 to 10	0.11	0.986	37.8	1.469
	10 to 15	0.08	0.801	60.5	1.469
S4	0 to 5	0.15	0.847	49.2	2.142
	5 to 10	0.14	1.032	35.7	1.346
	10 to 15	0.13	1.956	35	1.285
S5	0 to 5	0.18	1.402	29.4	3.244
	5 to 10	0.18	0.986	60.4	2.591
	10 to 15	0.1	1.54	19.5	1.285
F1	0 to 5	0.14	0.893	9.7	1.897
	5 to 10	0.07	2.141	12.2	1.714
	10 to 15	0.08	1.217	11.1	1.591
F2	0 to 5	0.14	0.662	27.8	2.204
	5 to 10	0.13	0.893	83.4	3.061
	10 to 15	0.06	1.448	54.5	1.591
F3	0 to 5	0.06	1.124	10.8	2.326
	5 to 10	0.04	0.708	7.1	0.979
	10 to 15	0.03	0.801	6.3	0.674
F4	0 to 5	0.03	0.986	27.8	4.346
	5 to 10	0.014	1.448	16.6	2.857
	10 to 15	0.014	1.032	10.6	1.183
F5	0 to 5	0.03	0.986	25.5	3.122
	5 to 10	0.03	1.078	79.4	2.51
	10 to 15	0.04	1.725	50.6	1.897

The value of soil microbial biomass carbon (SMBC) of samples surrounding the forest and samples from the forest was found to be different. The average SMBC value of samples surrounding the forest area was 482.5 mg/kg and its value ranged from 225 mg/kg to 1087.5 mg/kg. Whereas, the average value of SMBC of forest soil sample was 808.75 mg/kg, ranging from 487.5 mg/kg to 1312.5 mg/kg. With the increment of depth, the value of both soil microbial biomass carbon and nitrogen was found to be decreasing (table no.4).

Soil microbial biomass nitrogen of samples surrounding forest area was observed from 2.539 to 17.135 mg/kg with an average value of 9.65. The SMBN value of forest sample ranged from 6.712 to 24.032 mg/kg with an average value of 16.108 mg/kg. From this data, we can say that the soil microbial biomass nitrogen of forest soil can be higher than non-forest soil.

Table 4. 5: Soil microbial biomass carbon and nitrogen

Sample no.	Depth (cm)	SMBC (mg/kg)	SMBN (mg/kg)
S1	0 to 5	1087.5	12.722
	5 to 10	881.25	6.346
	10 to 15	843.75	2.539
S2	0 to 5	337.5	17.135
	5 to 10	300	14.481
	10 to 15	225	6.870
S3	0 to 5	637.5	8.234
	5 to 10	600	9.761
	10 to 15	506.25	4.732
S4	0 to 5	337.5	13.594
	5 to 10	337.5	11.331
	10 to 15	300	11.530
S5	0 to 5	337.5	13.624
	5 to 10	281.25	5.948
	10 to 15	225	5.922
F1	0 to 5	843.75	15.699
	5 to 10	712.5	13.212
	10 to 15	600	9.597
F2	0 to 5	731.25	16.118
	5 to 10	562.5	12.571
	10 to 15	487.5	6.712
F3	0 to 5	1312.5	24.032
	5 to 10	1237.5	19.686
	10 to 15	1050	20.953
F4	0 to 5	1181.25	21.353
	5 to 10	712.5	20.301
	10 to 15	693.75	14.997
F5	0 to 5	731.25	20.805
	5 to 10	693.75	14.921
	10 to 15	581.25	10.677

Table 4. 6: Descriptive analysis of chemical properties of soil

Parameters	Mean	Std. deviation	Variance	Minimum	Maximum
Nitrogen %	0.1	0.06	0.004	0.01	0.27
Phosphorus (mg/kg)	2.27	4.12	17.02	0.66	21.87
Potassium (ppm)	30.68	21.12	446.24	6.3	83.4
Organic carbon(%)	1.99	0.82	0.679	0.67	4.35
SMBC (mg/kg)	645.62	308.94	95447.6	225	1312.5
SMBN(mg/kg)	12.8801	5.60907	31.462	2.54	24.03

4.3 Total plate count (TPC or TVBC)

The total viable bacterial count obtained from all the 40 soil samples are listed in table 5. The highest total viable bacterial count was found in sample F2 (7.9×10^8) from the depth 5 to 10 cm and the lowest bacterial count was found in sample F5 (1.5×10^6) from the depth of 10 to 15 cm.

Table 4. 7: Total viable bacterial count

Samples	depth (cm)	no. of cells/ml (cfu/ml)
	0 to 5	176×10^6
S1	5 to 10	156×10^6
	10 to 15	108×10^6
	15 to 20	167×10^6
	0 to 5	68×10^5
S2	5 to 10	47×10^5
	10 to 15	17×10^5
	15 to 20	49×10^5
	0 to 5	117×10^6
S3	5 to 10	196×10^6
	10 to 15	175×10^6
	15 to 20	9×10^5
	0 to 5	32×10^6
S4	5 to 10	13×10^6
	10 to 15	41×10^6
	15 to 20	24×10^6
	0 to 5	82×10^6
S5	5 to 10	79×10^6
	10 to 15	38×10^6
	15 to 20	32×10^6
	0 to 5	19×10^7
F1	5 to 10	82×10^6
	10 to 15	6×10^6
	15 to 20	45×10^6
	0 to 5	9×10^6
F2	5 to 10	77×10^7
	10 to 15	79×10^7
	15 to 20	28×10^6
	0 to 5	63×10^6
F3	5 to 10	57×10^7
	10 to 15	6×10^7
	15 to 20	41×10^6
	0 to 5	44×10^6
F4	5 to 10	82×10^6
	10 to 15	8×10^6
	15 to 20	3.4×10^7
	0 to 5	3.9×10^7
F5	5 to 10	1.7×10^7
	10 to 15	1.5×10^6
	15 to 20	4.2×10^7

4.4 Isolation and identification of *Azotobacter spp*.

In the present study, out of 40 soil samples (4 soil surfaces from 10 different locations) only 15 samples were observed to have *Azotobacter spp*. From the bacteria that grown on N_2-free Ashby's and Jensen's medium, well-spaced, circular, creamy, dark brown colored and watery type of colonies were isolated. Then, pure cultures were prepared and depending on cultural characteristics and microscopic tests, 27 isolates were selected for detailed study.

The isolates were studied microscopically. Simple staining and Gram staining were performed (table no. 5). The morphological characters of *Azotobacter* isolates showed that among 27 isolates, 14 isolates were rod shaped (large and medium) and Gram negative, 8 isolates were small rods and Gram negative and 5 isolates were observed to be oval or coccoid and were Gram negative. The colonies developed on Ashby's agar media were circular, convex, opaque and creamy in color some were dark brown. Whereas, the colonies on Jensen's media were raised, transparent, water droplet type. The colony size varied from ≥2mm to ≤5mm.

Biochemical tests were done to confirm and classify them into different species according to Bergey's Manual of Systematic Bacteriology.

Table 4. 8: Cell morphology of *Azotobacter*

S.N.	Azotobacter isolates	Gram staining	cell shape	colony shape margin	Color	consistency	motility	Catalase
1	**F1a**	Gram – ve	oval	circular, raised	Brown	viscous	-ve	+ve
2	**F1b**	Gram – ve	medium rod	smooth, opaque, raised	dark brown	slimy	+ve	+ve
3	**F1c**	Gram – ve	medium rod	circular, smooth	Creamy	mucoid	+ve	-ve
4	**F1d**	Gram – ve	large rod in cluster	raised, opaque	Brown	viscous	+ve	+ve
5	**F2b**	Gram – ve	medium rod	opaque, confined	Creamy	slimy	+ve	+ve
6	**F2c**	Gram – ve	small rod	circular, raised	Brown	weak slimy	+ve	+ve
7	**F2d**	Gram – ve	medium rod	creamy, convex, glistening	Creamy	mucous	-ve	+ve
8	**F2g**	Gram – ve	oval	brown, raised	Brown	slimy	-ve	+ve
9	**F2h**	Gram – ve	small rod	slimy, raised, translucent	Brown	slimy	+ve	+ve
10	**F2i**	Gram – ve	medium rod	spherical, flat	dull white	dry	+ve	-ve
11	**F2j**	Gram – ve	small rod	large slimy glistening	Transpat ent	viscous	+ve	+ve
12	**F2l**	Gram – ve	small rod	circular, smooth, pulvinate	dull white	weak slimy	+ve	+ve
13	**F3f**	Gram – ve	medium rod	small, circular, glistening	Yellow	viscous	+ve	+ve
14	**F3g**	Gram – ve	medium rod	oval, small	Pink	milky	+ve	+ve
15	**F3h**	Gram - ve	small rod	circular, convex, sticky	Transpar ent	viscous	+ve	-ve
16	**F3i**	Gram - ve	small rod	circular, smooth	Creamy	slimy	+ve	-ve
17	**F3k**	Gram - ve	large rod in cluster	flat, large, creamy	Creamy	slimy	+ve	+ve
18	**F3l**	Gram - ve	large rod	entire, raised, glistening	Brown	slimy	+ve	+ve
19	**F4i**	Gram - ve	oval singly	slimy, circular, convex	pale yellow	dry	+ve	-ve
20	**F4j**	Gram - ve	large rod	large, flat, smooth	Creamy	weak slimy	+ve	+ve
21	**F4k**	Gram - ve	medium rod	circular, flat	dull white	dry	+ve	-ve
22	**F4m**	Gram - ve	cocci	smooth, glistwning, raised	Transpar ent	weak slimy	-ve	+ve
23	**F5a**	Gram - ve	thin long rod	circular, raised, convex	Yellowi sh to white	viscous	+ve	+ve
24	**F5b**	Gram - ve	medium rod	circular, creamy, smooth	Creamy	weak slimy	+ve	+ve
25	**F5d**	Gram - ve	small rod	mucoid, smooth	Brown	mucous	+ve	-ve
26	**F5f**	Gram - ve	long rod	circular, thick, sticky	Creamy	mucous	+ve	+ve
27	**F5h**	Gram - ve	small rod	creamy brown, flat	creamy brown	weak slimy	-ve	+ve

Motility test and colony morphology: Motility test was done in SIM media. Out of 27 isolates, 22 isolates were motile and 5 were non-motile. Different isolates showed diverse characteristics in colony color, shape, margin and consistency. The colonies were transparent, white, creamy, pale, brown to dark brown. Pink and yellow colonies were also present. They were also uneven in consistency because some were viscous, slimy and weak slimy whereas some were mucous, milky and even dry.

Catalase activity test and carbon source utilization: Among 27 isolates, 7 isolates were catalase negative (table 6) hence, were not *Azotobacter species*. By analyzing the result of carbon utilization test, it is clear that among 20 isolates, 10 isolates were matched with the characters of *A. chroococcum*, 6 strains resembled with *A. vinelandii* and 4 strains with *A. beijerinckii*.

Table 4. 9: Classification of *Azotobacter Species*

S.N.	Azotobacter strains	starch hydrolysis	glucose	sucrose	maltose	cyst	result
1	**F1a**	-ve	+ve	+ve	-ve	+ve	*A. beijerinckii*
2	**F1b**	+ve	+ve	+ve	+ve	+ve	*A. chroococcum*
3	**F1d**	+ve	+ve	+ve	+ve	+ve	*A. chroococcum*
4	**F2b**	+ve	+ve	+ve	+ve	+ve	*A. chroococcum*
5	**F2c**	+ve	+ve	+ve	+ve	+ve	*A. chroococcum*
6	**F2d**	-ve	+ve	+ve	-ve	+ve	*A. beijerinckii*
7	**F2g**	-ve	+ve	+ve	-ve	+ve	*A. beijerinckii*
8	**F2h**	+ve	+ve	+ve	+ve	+ve	*A. chroococcum*
9	**F2j**	+ve	+ve	+ve	+ve	+ve	*A. chroococcum*
10	**F2l**	-ve	+ve	+ve	+ve	+ve	*A. vinelandii*
11	**F3f**	-ve	+ve	+ve	+ve	+ve	*A. vinelandii*
12	**F3g**	-ve	+ve	+ve	+ve	+ve	*A. vinelandii*
13	**F3k**	+ve	+ve	+ve	+ve	+ve	*A. chroococcum*
14	**F3l**	+ve	+ve	+ve	+ve	+ve	*A. chroococcum*
15	**F4j**	+ve	+ve	+ve	+ve	+ve	*A. chroococcum*
16	**F4m**	-ve	+ve	+ve	+ve	+ve	*A. beijerinckii*
17	**F5a**	-ve	+ve	+ve	+ve	+ve	*A. vinelandii*
18	**F5b**	-ve	+ve	+ve	+ve	+ve	*A. vinelandii*
19	**F5f**	-ve	+ve	+ve	+ve	+ve	*A. vinelandii*
20	**F5h**	+ve	+ve	+ve	+ve	+ve	*A.chroococcum*

Figure 3 shows 50% of isolated *Azotobacter* were *Azotobacter chroococcum*, 30% were *A. vinelandii* and 20% were *A. beijerinckii* which shows domination of *Azotobacter chroococcum* in Panchakanya forest soil.

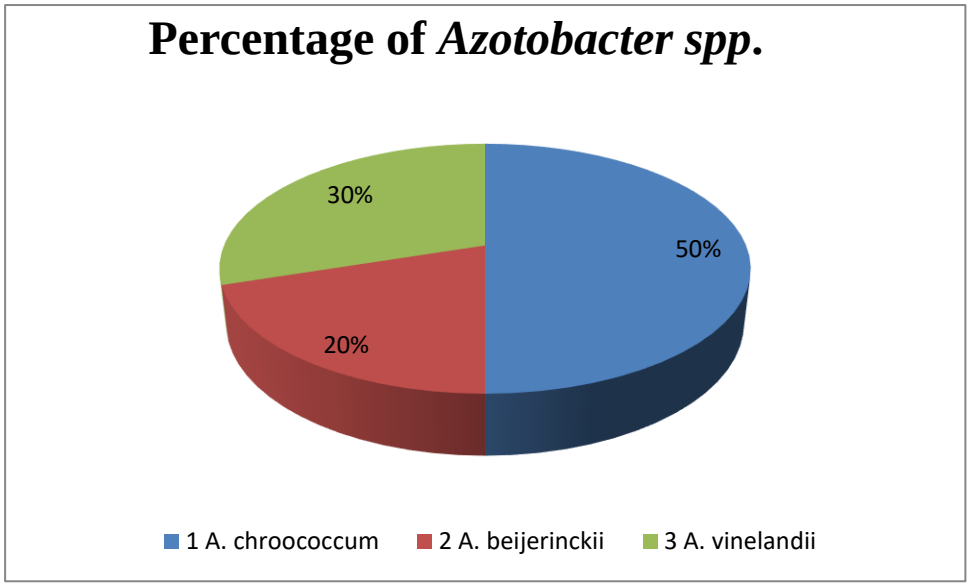


Figure 3: Percentage of different species of *Azotobacter*

4.5 Physico-chemical parameters vs Organisms

The result obtained after the canonical analysis (CA) was plot in figure 3. The canonical analysis suggested that the physico-chemical variables soil texture (sand, silt and clay), organic carbon, pH, bulk density, potassium, nitrogen and electric conductivity are highly associated with organism (Total viable bacterial count) but negatively related to soil microbial biomass carbon. In the contrary, variables of gravel, soil microbial biomass nitrogen and moisture content are not associated with organisms. Similarly, one way analysis of variance (ANOVA) on the canonical analysis suggested that the physico-chemical variables such as, clay, silt, sand, organic carbon, pH, bulk density, potassium, nitrogen and electrical conductivity were found to be influencing factors ($P<0.05$) to shape the population of organisms.

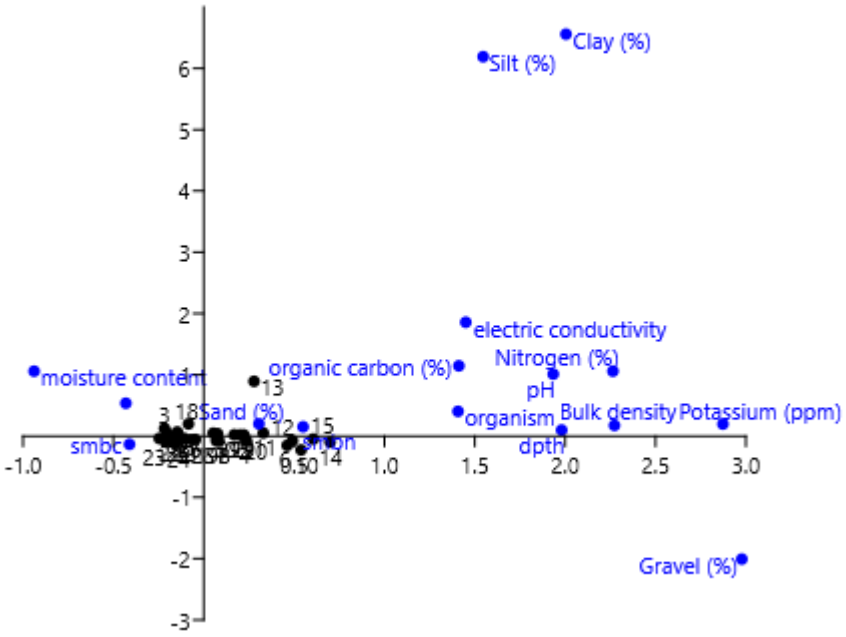
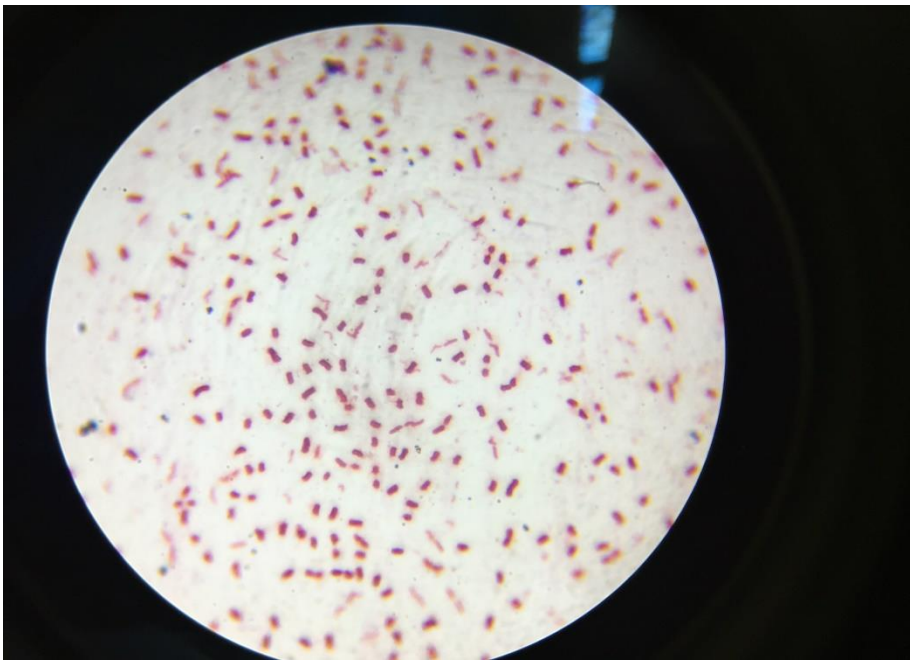


Figure 4: Canonical Analysis (CA) ordination between physico-chemical variables and total plate count

PHOTOGRAPHS

Photograph 1: *Azotobacter* colony on Jensen's media



Photograph 2: Gram staining of *Azotobacter*

Photograph 3:Starch hydrolysis test of *Azotobacter*

Photograph 4: Analysis of soil phosphorus by spectrophotometer

Photograph 5: Analysis of soil nitrogen by kjeldahl

CHAPTER V

DISCUSSION

A forest soil is a natural or only slightly disturbed material that takes centuries to develop under permanent forest cover diss1. Soils change substantially across landscapes, in response to changes in parent materials, to differences in water flow and the responses of plants and the rest of the ecosystem biota (Binkley and Fisher 2019). Soil and forests are intrinsically linked with huge impacts on each other and on the wider environment Forest soil survey serves environmental purposes such as the inventory of carbon (C) stocks and sinks related to climate change (Saby et al. 2008) and sustainable forest management (Burger and Kelting 1999). Accordingly, the objective of this research was to study physical, chemical and microbiological properties of forest soil and to study the association between physico-chemical parameters and number of organisms (TPC).

In this study, soil samples were collected from 10 different locations of Panchakanya forest and its surrounding area. 40 composite samples were collected by random sampling method. 30 samples were analyzed for physico-chemical characteristics of soil and all the samples (40) were studied for microbial analysis. For the physical analysis of soil samples; moisture content, water holding capacity, pH, soil texture, electric conductivity and bulk density were determined. Soil carbon, nitrogen, phosphorus, potassium, soil microbial biomass carbon and soil microbial biomass nitrogen were estimated for the chemical analysis of soil samples. Bacterial load of each sample was determined by performing total viable bacterial count. Another objective of this study was completed by isolating and identifying *Azotobacter spp.* (N_2 fixing bacteria). Three species of *Azotobacter* were identified namely; *A. chroococcum, A. vinelandii, and A. beijerinckii.* Then, to establish the correlation between physico-chemical parameters and number of organisms, canonical analysis was done in Past 3 software.

In this study, moisture content of soil samples ranged from 0.08 to 128.62% with mean value of 24.33% which is higher to the findings of Shishir Paudel

and Jay P Shah in Udayapur district of Eastern Nepal. Momita Chettry, Rijan Ojha and Bhanbindra Niroula also had lower moisture content in Sal Forest of Triyuga municipality and Siwalik Forest of Morang district in comparison to this study. The result of this study is in accordance with the study of Tilak Prasad Gautam and Tej Narayan Mandal carried out in tropical moist forest, Charkoshe Jungle of Sunsari district, Nepal. This wide range of moisture content was due to the fact of different soil type and altitude of location (488m to 635m) from where samples were collected. Sample F2 (0-5 cm) has the lowest moisture content because this sample was collected from the upper layer of soil profile near Bhatabhunge Durbar, where the amount of gravel was maximum. Whereas, sample S1 (10-15 cm) was from farmland and the type of soil was loamy sand.

The range of moisture content of soil samples surrounding the forest area was 2.84% to 128.62% with mean value of 19.45% whereas, forest soil sample had moisture content in the range of 0.08% to 46.93% with mean value of 32.98% which confirms forest soil samples have uniform and higher value of moisture content than samples surrounding the forest area. Presence of organic matter and humus in forest soil could be the reason for higher moisture content and absence of plants in most of the soil samples collected from the area surrounding the forest may be the reason for lower moisture content. Forest soil was found to be the best for plant growth.

Water holding capacity of soil influences crop growth, rotting pattern and ability to supply water to crops during dry period. The data on mean water holding capacity of soils indicated that the soils of forest have distinctly higher percentage of water holding capacity (ranged between 46.5 -78.5 % with mean value of 66.27%) than that of area surrounding the forest (40.5-67.5% with the mean value of 54.73%). In most of the sample's water holding capacity decreased depth wise which is in accordance with the results of Tilak Prasad Gautam and Tej Narayan Mandal and Bhattarai KP and Mandal TN. Irrespective of areas of samples, the value of water holding capacity was from 40.5% to 78.5% with mean value of 60.5%. The study conducted by Shishir Paudel and Jay P Shah (2003) and Chettry M et al (2011) had the water

holding capacity slightly lower than this study. Out of 30 soil samples, only 2 samples have low water holding capacity (<45%), eleven samples were in the categories of medium WHC (45-60%) and rest seventeen samples were in the category of high WHC with more than 60%.

After analyzing the data of water holding capacity, we can conclude that forest soil is more favorable for plant growth than the soil sample studied from area surrounding the forest because forest soil has comparatively higher water holding capacity. The reason could be higher soil organic matter and soil texture. Higher WHC provides favourable living condition for soil organism which in turn improves the quality and fertility of soil (George et al. 1995).

Soil pH of all samples from 0cm to 15cm ranged from 5.17 to 6.6 except sample S5 (pH value 7.6 to 9.2) with mean pH value of 5.92. This range provides the best growing conditions and influences the uptake of nutrients by plants (Suleiman et al. 2017). This report is in accordance with the study of Bhattarai KP and Mandal TN (2020) and Anup et al 2013 done in Sal Forest of Jhapa district and mid hill region of Nepal respectively. Whereas, Chetry M et al (2021) reported highly acidic forest soil in Raja-Rani wetland, Morang. The pH was slightly acidic to neutral to alkaline in the soil samples of area surrounding the forest (5.42-9.2 with mean pH (6.46), whereas, a comparatively lower pH value was recorded in forest soil samples (5.17-5.62 with mean value 5.38). It shows that the soil of Panchakanya forest is slightly acidic in nature. The result indicates that the soil acidity is more in higher altitudes as compared to lower altitudes. In acidic soil, nutrients are absorbed by plants roots very fast and vegetation is not able to consume them quickly and plant dies whereas, in alkaline soil nutrients do not get dissolved easily which limits the absorption of nutrients by plants. This shows forest soil has favourable pH for plant growth.

The acidic soil may be due to the acids released by decomposition of organic residues obtained from forest vegetation. Or it could be due to parent materials high in elements such as silica, intense leaching of basic cations during monsoon season, and the atmospheric nature of aluminum in these soils (Pandey et al. 2018). The lower soil pH in forest land also might due to its

higher slope (Yeshaneh 2015), higher OM content, and less evaporation from the surface in the study area. In acidic soil, availability of P and Ca decreases whereas Fe and Mn increase (George et al. 1995).

Soil bulk density increased with increasing the depth of soil. The bulk density ranged from 0 to 10 cm depth was 0.66 to 1.45 gm/cm^3 with mean value of 1.05 gm/cm^3, which is close to the findings of Gautam TP and Mandal TN (2013) and Chettry M et al (2021) carried out in bhabar belt of Sunsari district, Nepal and Siwalik Forest of Letang, Morang respectively. Whereas, in the research done by Naghdi, Labelle and Solgi (2016) the average bulk density at 0-10 cm depth was 0.74 gm/cm^3. The value of bulk density was greater in soil samples surrounding the forest than the samples from the forests. This may be due to the fact that soil compaction increases the bulk density and soils around the forest are more compact than the undisturbed soil of forest.

The electrical conductivity is related to the total cations and anions in the solution. EC indicates goof nutrient availability for plants, with the low end indicating nutrient poor soil. The electric conductivity in this study ranged from 0.15 mS/cm to 0.52 mS/cm with mean value of 0.24 mS/cm. This value of electric conductivity is lower than 1.25mS/cm to 1.39mS/cm of EC conducted by Noof A El Khamas 2016. Whereas, a research conducted by Shrestha R.K. (2011) showed the value of EC from 0.043 mS/cm to 0.154 mS/cm. Soil electrical conductivity in both locations we in the same range with mean EC value of 0.24 mS/cm in area surrounding the forest soil and mean EC value of 0.25 in forest soil samples.

The texture of most of the soil was loamy sand whereas some samples were in the category of sand and the category of one sample {S5 (0-5)} was silt loam. Sand content increased while silt and clay decreased on increasing the depth. This result is supported by the findings of Gautam TP and Mandal TN 2013 in tropical moist forest of Bhabar belt of Sunsari district of Nepal. Whereas Bhattarai KP and Mandal TN (2020) in Jhapa district of Nepal and Paudel S and Shah JP (2003) in eastern Nepal reported the soil texture as sandy loam. Soil texture corresponds strongly to electric conductivity. Sands have low

conductivity; silts have a medium conductivity and clays have a high conductivity.

The average soil organic carbon was 1.99% which is close to the value of soil organic carbon reported by Bhattarai KP and Mandal TN (2020) in tarai sal forest of Jhapa district. A study conducted by Gautam TP and Mandal TN (2013) in tropical moist forest of Sunsari reported 3.07±0.15% of organic carbon which is higher than the value of organic carbon of this study whereas Chettry M et al (2021) reported lower value of organic carbon in Siwalik Forest, Morang, Eastern Nepal. The soil samples around the forest had maximum OM content of 3.244%, minimum 0.979% with average value of 1.868%. The results were consistent with the findings of Kharal S et al 2018 and Bista and Chauhan et al who reported a higher amount of soil organic carbon in forest land compared to farmlands Dhading, Nuwakot and Chitwan districts of Nepal, respectively. Organic carbon was maximum in the upper layer (0-5 cm) which decreased to minimum in the lower layer (10-15 cm) which may be due to higher accumulation and decomposition of forest litter on the surface.

Tillage practice results in higher decomposition and mineralization of organic carbon. The more a soil is tilled, the more the organic matter is broken down (Glanz 1995). Tillage improves the aeration of the soil and causes a flush of microbial action speeding up the decomposition of OM and also often increases erosion (Kharal et al. 2018). Hence, the forest had the higher soil organic carbon (2.1%) due to high OM accumulation and no tillage disturbance compared to disturbed lands around the forest which had mean OM content of 1.86%. This shows forest soil as better soil than the soil from surrounding area.

The average nitrogen concentration in this study was 0.1016% which is close to the study done by Kafle G. (2019) who reported 0.10% of nitrogen in community forest of Chitwan, Nepal and Krishna Prasad Bhattarai and Tej Narayan Mandal in a study done in Sal Forest of Kechana, Jhapa. Tilak Prasad Gautam and Tej Narayan Mandal reported slightly higher nitrogen concentration in tropical moist forest (charkoshe jhari) in Sunsari district of

Eastern Nepal. The highest soil N (0.27%) was reported from soil sample near the forest (S1 0-5) having minimum N% of 0.07% and mean value of 0.14% of nitrogen and the lowest (0.014%) was observed in the forest sample (F4 5-10 and F4 10-15) having maximum nitrogen % of 0.14% with mean value of 0.06% of nitrogen. Which confirms slightly higher nitrogen percentage in non-forested area in comparison to forested area. This shows soil of non-forested area as comparatively fertile as N_2 is important for plant growth (structure), plant food processing (metabolism) and the creation of chlorophyll. Total nitrogen in the soil decreased depth wise in both forest and land surrounding the forest which is consistent with the findings of Gautam TP and Mandal TN 2013.

The mean value of phosphorus was 2.278mg/kg which is 4.556 kg/hectare. This value is less than the value of phosphorus obtained by KC Anup et al 2013 and Kharal S et al (2018) in a community forest of Syangja, Nepal and forest land of Chitwan district respectively. The highest amount of phosphorus (21.87 mg/kg) was found in soil samples collected from the surrounding area of Panchakanya forest and the lowest (0.662 mg/kg) in forest area. The mean value of available phosphorus surrounding the forest was 3.41 mg/kg and the mean value of available phosphorus in forest soil sample was 1.14 mg/kg. Available phosphorus in the soil sample surrounding the forest (cultivated and non-cultivated area) was significantly higher than in forest. This concludes samples of non-forested area as fertile soil in terms of available phosphorus than forest samples because, adequate amount of available Phosphorus in soil favour rapid plant growth and development, hasten fruiting or maturing and often improve the quality of vegetation (Pierre 1938).

Availability of P in cultivated soil depends on soil pH. Hence very low P in forest land might be due to low pH. The pH in forest land were lower than 5.62, a favorable condition in which P is fixed in the soil as aluminum phosphate ($AlPO_4$) and becomes unavailable (Thomson et al. 2010). The lower P content might also be due to no external sources of P were applied to these natural lands. The high P in area surrounding the forest may be due to greater pH and the residual effect of external chemical fertilizers.

The concentration of potassium in soil varies from 6.30 ppm to 83.40 ppm with the mean value of 30.68 ppm which is higher than Anup KC, Govinda Bhandari, Subigya Prabhat Wagle and Yubraj Banjade who reported 2.54 to 4.23 ppm, but lower than the value of potassium reported by Momita Chetry, Rijan Ojha and Bhabindra Niroula in Siwalik Forest of Morang district. The highest amount of potassium (83.4 ppm) was found in forest soil sample with minimum value of 6.3 ppm and mean value of 28.89 ppm. Meanwhile the average value of potassium in area surrounding the forest was 32.47 ppm with maximum value of 60.5 ppm and minimum value of 11.5 ppm. Bista P 2010 and Kharal S et al 2018 also reported significantly higher potassium level in forest land than cereal-based lowland of Chitwan district of Nepal.

The low level of potassium in lowland area surrounding the forest might be due to higher leaching loss and more K harvest from soils. The available potassium in forest land compared to the land surrounding the forest may be due to its extensive and deep-rooted trees. Trees act as a nutrient pump and extract nutrients from the deep subsoil horizons and recycle it into surface layer through leaf fall (Tewari et al. 2016).

Soil microorganisms are potentially very important to increase the level of soil fertility. Mean microbial biomass carbon was reported as 645.62mg/kg which is close to the findings of Gautam TP and Mandal TN (2013) in tropical moist forest (charkoshe jungle) in Sunsari district of Eastern Nepal and Limbu DK et al 2020 at Tinjure Milke Jaljale area of Taplejung, Tehrathum and Sankhuwasabha of Eastern Himalaya, Nepal. Bhattarai KP and Mandal TN (2020) reported 216.2 ± 11.8 µg/g of SMBC in tarai sal forest of Jhapa district. Highest SMBC (1312.5 mg/kg) was found from forest land with minimum SMBC value of 487.5 mg/kg and mean value of 808.75 mg/kg. Whereas, the lowest SMBC (225 mg/kg) was found in soil sample collected from the area surrounding the forest with mean value of 482.5 mg/kg and maximum value of 1087.5 mg/kg. According to this result SMBC increased along with higher altitude meaning, SMBC is higher in forest land than land surrounding forest. This may be due to higher accumulation of soil organic carbon and total nitrogen in forest because low soil temperature and moisture causes reduced decomposition and slow turnover of organic matter. The reason of high value

of soil microbial biomass carbon in forest may also be due to active and high population of microorganisms in forest ecosystem than the undisturbed area. Differences in the quantity and quality of substrate inputs via varying litter and root types and associated nutrient specificity can be crucial drivers to influence the soil microbial biomass (Feng et al. 2009).

Soil microbial biomass nitrogen in this study was 9.65 mg/kg which is lower than the result of Bhattarai KP and Mandal TN who reported 39 mg/kg of SMBN at Sal Forest of Kechana of Jhapa district, Nepal and Gautam TP and Mandal TN (2013) who reported 59.0±38 µg/g of soil microbial biomass nitrogen in a study conducted in Sal bearing tropical moist forest (Charkoshe Jhari), located in bhabar belt of Sunsari district, Nepal. Limbu DK et al reported the value of SMBN as 23.65 mg/kg. The value of SMBN ranged from 15 to 40 mg/kg in a study conducted by Padalia K et al 2018. Barbhuiya (2006) reported microbial biomass N value 45.29-92.72 mg/kg in undisturbed forest of Meghalaya. Soil microbial biomass was highest (24.032 mg/kg) in forest area with minimum value of 6.712 mg/kg and mean value of 16.108 mg/kg. The lowest value of SMBN (2.53 mg/kg) was in the sample from area surrounding the forest with maximum value of SMBN of 17.13 mg/kg and mean value of 9.65 mg/kg. This observation shows higher value of soil microbial biomass nitrogen in forest land. This may be due to variation in accumulated plant debris and fine roots in the forest with multiple tree species, which favour the intensification of microbes and hence more C and N are accumulated in the microbial biomass (Kara and Bolat 2008).

The total viable bacterial count in this study was 1.5×10^{6} cfu/ml to 7.9×10^{8} cfu/ml. whereas 0.6×10^{10} cfu/ml to $4.3\text{x}10^{10}$ cfu/ml was reported by Olsen RA and Bakken LR (Olsen and Bakken 1987) and $2.8\text{x}10^{4}$ cfu/ml to $4.0\text{x}10^{4}$ cfu/ml was reported by Noof A. 2016. In this study out of 20 isolates of *Azotobacter* 10 were *A. chroococcum*, 6 were *A. vinelandii* and 4 were *A. beijerinckii* which means 50% isolates are *A. chroococcum*, 30% are *A. vinelandii* and 20% are *A. beijerinckii*. In a study conducted by Upadhyay S et al (2015) 72.7% of isolates were *A. chroococcum*, 13.6% isolates were *A. vinelandii* and 13.6% were *A. beijerinckii*.

CHAPTER VI

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Results of the study show evidence of poor soil health in land surrounding the Panchakanya forest compared to the Panchakanya forest area in Sunsari district of Nepal. This is due to lack of plants and vegetation.

Nitrogen fixing bacteria (*Azotobacter spp*.) are found in forest soil sample but samples collected from the surroundings of forest were devoid of *Azotobacter spp.* which means *Azotobacter* species are normally found in the rhizosphere of plants. The bacteria showed Gram negative, catalase positive, cyst forming nature. On carbon source utilization behavior, *Azotobacter* showed its species variation as out of 20 strains, 10 were confirmed as *A. chroococcum*, 6 strains matched with *A. vinelandii* and 4 strians confirmed the characters of *A. beijerinckii*. From this result we can also conclude that in Panchakanya forest soil, *A. chroococcum* are dominant than other species of *Azotobacter*.

The statistical analysis between the physico-chemical variables and population of microorganisms suggested that the soil texture, pH, bulk density, electric conductivity, potassium, nitrogen and organic carbon are highly associated with population of microorganisms but negatively related to soil microbial biomass carbon. In the contrary, variables of gravel, moisture content, soil microbial biomass nitrogen are not associated with organisms. Similarly, ANOVA on the canonical analysis suggested that the physico-chemical variables like; clay, silt, sand, pH, bulk density, electrical conductivity, potassium, nitrogen and organic carbon were found to be influencing factors to shape the population of organisms.

6.2 Recommendation

1. In this study, physico-chemical and microbiological characters of forest soil of Panchakanya of only one season were studied. To have a better understanding of different properties of soil, analysis and study of soil samples of all season is important.

2. For better knowledge of soil profile, analysis of soil samples up to 35-40 cm depth is adequate.

3. Molecular identification of *Azotobacter species* could be more accurate.

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APPENDIX

APPENDIX-I

Materials and Equipments:

Equipments used

Autoclave
Weighing machine
Hot air oven
Incubator
Microscope
Refrigerator
Dessicator
Digital thermometer
pH meter
Spectrophotometer
Flame photometer

Glasswares

Test tubes
Beakers
Pipettes
Petri plates
Conical flask
Glass rod and glass tubes
Reagent bottle
Slides
Measuring cylinder

Microbiological and Biochemical media

Ashby's agar
Jensen's agar
Nutrient agar
Plate count agar
Starch agar
Glucose agar
Sucrose agar
Maltose agar
Urease agar

Miscellaneous

Whatmann filter paper
Asbestos gauze
Aluminium foil
Inoculating loop/ needles
Cotton plugs
Bunsen burner
Zipped polythene bag
Ice box
Borer
Digital and medical thermometer
Labeling tape
Marker
Test tube holder
Detergent

APPENDIX-II

Composition and preparation of different culture media

1. Ashby's media

Ingredients	Amount (gram/litre)
Mannitol	20
Dipotassium phosphate	0.2
Maganesium sulfate	0.2
Sodium chloride	0.2
Agar	15
pH	7.4±0.2
Water	1000ml

The mixture of agar and water was boiled to dissolve completely then autoclaved at 15 lbs pressure (121ºC) for 15 minutes. The final ingredient solution was cooled at room temperature and then added to sterile petriplates at around 40ºC-45ºC.

2. Jensen's media

Ingredient	Amount (gram/litre)
Sucrose	20
Dipotassium phosphate	1
Magnesium sulfate	0.5
Sodium chloride	0.5
Ferrous sulfate	0.1
Sodium molybdate	0.005
Calcium carbonate	2
Agar	15

Required amount of Jensen's media was weighed and mixed with water in conical flask. The flask was boiled to dissolve the ingredients and then autoclaved at 15lbs for 15 minutes.

3. Nutrient Agar

Ingredient	Amount (gram/litre)
Peptone	5
Sodium chloride	5
Beef extract	1.5
Yeast extract	1.5
Agar	15
Final pH	7.2

For the preparation of nutrient agar, 37gram agar was suspended in 1000 ml of distilled water, boiled carefully and then autoclaved at 121ºC for 15 minutes. Nutrient agar plates were prepared by cooling the media to 40-45ºC.

4. Starch Agar

Ingredients	Amount (Grams/Litre)
Beef extract	3
Soluble starch	10
Agar	12
pH	7.3±0.2

Suspend 25gm of powder in 1 L of purified water and mix thoroughly. Heat and boil for 1 min, autoclave at 121ºC for 15 min.

5. Peptone broth

Ingredients	Amount (Gram/Litre)
Peptone	10
Sodium chloride	5
Final pH (at 25ºC)	7.2±0.2

APPENDIX-III

Methodology of biochemical test for the identification of Azotobacter

A. Motility Test

This test is done to determine if an organism is motile or not. Bacteria are motile by means of flagella. The motility media used for motility test are semisolid, making motility interpretation macroscopic. The media used in this study was SIM.

Procedure: the test organism was stabbed in the SIM medium and incubated at 37ºC for 24- 48 hours. Motile organisms migrate from the stab line and diffuse into the medium causing turbidity. Whereas non-motile bacteria show the growth along stab line only.

B. Catalase Test

This test is performed to detect the enzyme catalase. During aerobic respiration int the presence of oxygen, microorganisms produce hydrogen peroxide (H_2O_2) which is lethal to the cell. The enzyme catalase present in some microorganisms breaks down hydrogen peroxide to water and oxygen which helps them in survival.

Procedure: 3% H_2O_2 was taken clean and dry test tube (3 ml). a small amount of culture from nutrient agar plate was added and mixed with the help of glass rod. Positive test is indicated by the formation of bubbles of oxygen gas.

C. Starch Hydrolysis Test

The ability to degrade starch is used as a criterion for the determination of amylase production by a microbe. In this study starch hydrolysis test was done to identify *A. chroococcum*. Starch in the presence of iodine produces a dark blue coloration of the medium and yellow zone around a colony in an otherwise blue medium indicates amylolytic activity.

Procedure: A test bacterium was taken by sterile loop and single streak inoculation was done in starch agar plate. After 48 hours of incubation at

37ºC, iodine solution was added with a dropper and left for 30 seconds. Positive test showed clear spaces/zone around bacterial growth.

D. Fermentation of Carbohydrates

Carbohydrates are organic molecules that contain carbon, hydrogen and oxygen in the ratio $(CH_2O)_n$. Fermentative degradation of various carbohydrates such as glucose, sucrose, maltose, cellulose by microbes under anaerobic condition is carried out in a fermentation tube. The fermentation broth contains ingredients of nutrient broth, a pH indicator (phenol red) which is red at neutral pH (7) and turns yellow at or below a pH of 6.8 due to the production of an organic acid.

Procedure: the organism to be tested was added in glucose, sucrose and maltose fermentation broth and incubated at 37ºC for 48 hours. After incubation the test tubes were observed. Yellow colored tubes show positive fermentation test whereas red colored shows negative.

E. Cyst Staining

A cyst is a resting or dormant stage of a microorganism, usually a bacterium or a protest, that helps the organism to tide over an unfavorable environmental condition. Giemsa staining is performed to observe cysts under microscope.

Procedure: Heat fixed bacterial smears were placed in the sulphation reagent for 10 minutes then removed and washed in running tap water for 5 minutes. The slides were then placed; smear side down in a small of the Giemsa stain, left for 30minutes. After further washing to remove excess stain, the slides were air dried and observed under oil immersion for cyst.

APPENDIX-IV

Information on sample location

Samples	lattitudes	longitudes	elevation (m)	soil temperature (°C)	air temperature (°C)
soil (S1)	26°49.807'	87°17.934'	488	28	28.5
Soil(S2)	26°49.763'	87°17.546'	565	26.5	27
Soil (S3)	26°49.773'	87°17.430'	489	29.9	25
Soil (S4)	26°50.024'	87°17.649'	513	25.2	28
Soil (S5)	26°50.087'	87°18.067'	592	21.3	21.5
Forest soil (F1)	26°47.743'	87°17.713'	502	26.3	27.9
Forest soil (F2)	26°50.007'	87°18.057'	632	26.6	27.6
Forest soil (F3)	26°50.078	87°17.888'	635	27.4	29.9
Forest soil (F4)	26°49.933'	87°17.822	595	26.5	29.8
Forest soil (F5)	26°49.821'	87°17.646'	581	27.1	29.5

APPENDIX-V

Chemicals and reagent

Conc. sulfuric acid

Sodium hydroxide (30%)

Boric acid (2%)

Hydrochloric acid

1N potassium dichromate

Ferrous sulphate

Ferroin indicator

Ammonium acetate / acetic acid solution

Potassium chloride

Hydrogen peroxide (30%)

Conc. hydrofluoric acid

Potassium dihydrogen orthophosphate

Deionized water

Soda lime

Chloroform

0.5 M potassium sulfate

70 mg mercury (II) oxide

Orthophosphoric acid

0.4 M ferrous ammonium sulfate

1,10 phenanthrone ferrous sulfate complex

Iodine solution