PHYSICOCHEMICAL AND MICROBIOLOGICAL ANALYSIS OF WATER SAMPLES FROM *LAXMI POKHARI* -THE PSYCHROPHILIC LAKE OF SIKKIM

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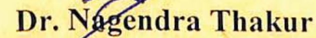
Certificate

This is to certify that the thesis entitled "**Physicochemical and Microbiological Analysis of Water Samples from *Laxmi Pokhari*- The Psychrophilic Lake of Sikkim**" submitted to **Sikkim University** for the award of **Master of Philosophy (M. Phil.) Degree in Microbiology** embodies the results of *bona fide* research work carried out by **Mr. Mingma Thundu Sherpa** bearing **Roll No. 12PDMB04** and **Registration No. 12SU6398,** under my guidance and supervision. No part of the thesis has been submitted for any other degree, diploma, associate-ship and fellowship.

All the assistance and help received during the course of the investigation have been acknowledged by him.



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ABBREVIATIONS

$CaCO_3$ =	Calcium Carbonate
Cl^- =	Chloride
D.O. =	Dissolved Oxygen
DNA =	Deoxy ribonucleic Acid
E =	East
GSI =	Geological Survey of India
HCl =	Hydrochloric Acid
L =	Liter
MHA =	Mueller Hinton Agar
m =	meter
mg =	Milligram
ml =	Milliliter
Mt. =	Mountain
MPN =	Most Probable number
N =	North
No. =	Number
nm =	Nanometer
NaCl =	Sodium Chloride
NaOH =	Sodium Hydroxide
Na_2SO_3 =	Sodium Sulfite
$NaNO_2$ =	Sodium Nitrite
O.D. =	Optical Density
P alkalinity =	Partial alkalinity
PCR =	Polymerase Chain Reaction
pH =	power of hydronium ions
ppm =	Parts per million
s =	Seconds
SiO_2 =	Silica Oxide
T.D.S. =	Total Dissolved Solids

Summary

SUMMARY

Pulchritudinous landscape blossomed by the wild, lush, vibrant doily of chromatic *Dendrobium*[1] orchids, picturesque *Gurans*[2] and variegated valley, spreads redolence bouquet of aromatic air in the Land of Snow Lion – Sikkim. Under the celestial custodian of Mt. Kanchendzonga[3], this blessed land of Holy Lamas[4], is an uncultivated coliseum of antediluvian cult. Sikkim hosts many natural virgin lakes. The Nepali word for lake is *Pokhari*[5]. For the first time, a detail geographical and Microbiological analysis of the *Laxmi* lake of Sikkim was carried out. The Snow-fed milky lake, *Laxmi* pokhari located at an altitude of 4145m, 27°29'58.4" north, 088°09'48.2" east of west Sikkim were the site of sampling and survey. *Pokhari* is regarded as holy one both in Buddhist and Hindu prospects and her water is taken away as holy water by the *Pokhari* goers. Water sample were analyzed for its physicochemical properties and micro-flora of the *Laxmi Pokhari.*

The pH measured was 6.7, Temperature was below zero degrees as it was completely frozen, TDS/Salinity was 1.3ppm, Conductivity was 28.5μS/cm and other chemical analysis of the *Laxmi Pokhari* water was done which showed 0.5 ppm fluoride; 100 ppm nitrite; 30 ppm chloride; 500 ppm sulphite and few other chemical compounds. The geographical parameter was also analyzed.

Microbiological assay were done and thirty one bacteria were isolated from *Laxmi Pokhari* and was characterized through various biochemical test and physical growth conditions were checked for optimization and tolerance.

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[1] ***Dendrobium*** = The State Flower of Sikkim, an orchid *Dendrobium nobile.* Fam. Orchidaecae.

[2] ***Gurans*** = The State Tree of Sikkim, *Rhododendron sp.*; Fam. Ericacae.

[3] **Mt. Kanchendzonga** = World's 3rd highest mountain, falls in Himalayan range; Height = 8586m.

[4] **Lama** = A Tibetan spiritual head of a monastery or teacher or priest.

[5] **Pokhari** = The Lakes

Introduction

INTRODUCTION

Water is the panacea of the earth, without it life in this world is impossible. They have the direct sway on animals and human beings since an ancient period. The Himalayas are regarded as the water tower of the world. The major forms of the Himalayan water reservoir are glaciers, lakes and springs. Both Hindu and Buddhist populace regard *Pokhari* (lake) as the god and goddess. People even used to took away her sanctified water and drink it as a blessed potion for its alleged curative properties. In early period, diseases were then categories or rather claimed to be a symbol of committed sin or gluttony. The only ways to get rid their aliments was to search and bath in the holy river, lakes or in natural hot water bodies. Thus in order to relief from their diseases or sins, through ages people have been touring different places to drink or bathing in rivers like the Ganges of India, River Nile of African Continent, River Yangtze in China (Goodrich, J.N. and Uysal, M. 1994). The lakes of Sikkim have been regarded as the blessed water by guru Padmasambhava and have been in use for religious purposes since 8th century AD.

The word lake comes from Middle English *Lake* ("lake, pond and waterway"), from old English *lacu* ("Pond, pool, stream"), from the Proto-Indo-European root *Leg* (*"to leak, drain"*). The word *Pokhari* came from Nepali dialect which means, "pool or small Lake". A lake is a body of relatively still liquid of considerable size, localized in a basin, that is surrounded by land apart from a river or other outlet that serves to feed or drain the lake (Hairston, N. G, 2002). Generally there are two types of lakes, artificial and Natural. Natural lakes are generally found in mountainous areas, rift zones and areas with ongoing glaciations. Other lakes are found in endorheic basin or along the courses of mature rivers. In some parts of the world there are many lakes of chaotic drainage patterns left over from the last Ice Age. All lakes are temporary over geologic time scales, as they will slowly fill in with sediments or spill out of the

basin containing them. Lakes of all the types share many ecological and biogeochemical processes and their study falls within the discipline of 'Limnology'. (Hairston, N. G, 2002). Lakes are excellent habitats for the study of ecosystem dynamics like interactions among biological, chemical and physical processes which are frequently either quantitatively or qualitatively distinct from those on land or in air. Because the boundaries between water and land are distinct, there is tight coupling among many ecosystem components. There are a number of natural processes that can form lakes. A recent tectonic uplift of a mountain range can create bowl-shaped depressions that accumulate water and forms lakes. The advance and retreat of Himalayan glaciers can scrape depressions in the surface where water accumulates and forms lakes. Lakes can also form by means of landslide or by glacier blockages.

Minerals like carbonate, salt and other trace minerals salts are effective against high blood pressure, gastroenteritis and relief to certain cardiovascular aliments but at the same time some elements present in higher concentrations above their threshold values can be harmful e.g. chloride, fluoride, sulphate and nitrate may cause diseases when present in higher concentrations in accordance with APHA and WHO standard. Thus the presence of minerals salt deposit in some high altitude lake water is important to study as people are using for various purposes such as drinking and bathing etc. The various properties of this kind of *Pokhari* are also dependent upon the micro-biota present in it and thus it is interesting to study the micro-flora of *Pokhari.* There are the microorganisms which can thrive under extreme cold temperature. These microorganisms are called *Psychrophiles* which have the ability to reproduce at 0°C.

The ideal growth temperature of the psychrophiles may vary from 0°C to 20°C (Schmidt-Nielsen, 1902 & Forster, 1887). For a long period psychrophiles were considered as exotic organisms, however in the last few years the number of psychrophilic isolates increased considerably. Many bacteria and Achaea have been isolated from cold habitats such as *Actinobacteria, Firmicutes, proteobacteria, Betaproteobacteria, Bacteroidetes, Euryarchaeota,*

Psychromonas, Marinomonas and Shewanella (Dang et al., 2009; Lauro et al., 2007; Nogi, 2008). It is important to study psychrophiles of *Pokhari* as their chemical constituents and various metabolites are stable at varying low temperatures. The cryo-enzymes of these microbes got a special interest for the scientists from all over the world since these enzymes are usually stable at lower temperature, Resist to chemical reagent and extreme pH in comparison to their mesophilic homologues. These cryo-enzymes have vast applications in industries. Also, the water of *Pokhari* might be contaminated with pathogenic microorganisms which can lead the spread of diseases. Thus it is necessary to detect and isolate the pathogenic microorganisms if present in *Pokhari.* Sikkim which harbour several Natural *Pokhari* located at the high alpine regions provides an opportunity to study them and developed several applications in near future.

Sikkim is considered as "The Blessed Land" and is guarded by an ancestral deity-Mount Kanchendzonga. This land locked state of India has many deep unfathomed beliefs, traditional knowledge and many virgin areas where it has numerous pristine landscapes covered with psychedelic orchids *Dendrobium* and *Cymbidium.* The aromatic and variegated valley when blossoms with *Gurans* creates mystiqueness of divine aura and makes belief the existence of Mother Earth – the Nature. Geographically located between 27°04'46" and 28°07'48" North latitude and 88°00'58" and 88°55'25" East longitude on the Eastern Himalayas, Sikkim is renowned for its tourism potential, Bio-organic farming and agricultural practices and also for its important cross border strategic talks.

Sikkim is a mixed bowl of culture where many ethic tribes have evolved their cultural identity. *Lepchas, Bhutias* and the *Sherpas* are the most predominant inhabitant tribes of Sikkim. Apart from them, there are many tribes of ancient kiratas like *Limboos, Khombu-Rai's* and *Yakhas;* ancient tribes of Khasas like *Bhahums, Chettris, Kamis, Damai's, Sarkis*, Newars and other mixed communities of Biharis, Bengalis, Marwaris and also

from other cultures. There are also the analogous ethnic tribes of kiratas like *Sunwars, Magars, Gurungs, Bhujels, Thamis* and analogous ethnic tribes of Khasas like *Thakuris , Jogi/Sanyasis, Majhis etc* (Subba, 2009; Sherpa, 2012; Das et al. 2012). Sikkim houses many natural lakes within its geography. It is a major tourist destination state of India where the nature is in its juvenile form and a refresh season greets its visitors. It is believed that the water from these lakes, used as a social medicine, religious customs and practices.

There are numerous lakes in Sikkim; *Lakshmi Pokhari, Dudh Pokari, Samiti Pokhari, Menmecho lake; Karthok lake, Kechoparli and Majur Phokhari* west Sikkim, *Gurudongmar Pokhari, Tso Lhamu lake* of and *Green lake* of north Sikkim, *Changu lake, Menmecho lake Kupup lake* and *Lam Pokhari* of east Sikkim. These lakes are virgin in context of research is concern; with the insufficient amount of research has lead to very few literatures on the lakes of Sikkim.

In the present study, the *Laxmi Pokhari* (lake) has been discussed with special emphasis on the physiological analysis of the *Pokhari* and the microbial study of it. *Laxmi Pokhari* is located in the west Sikkim, Sikkim, India at an altitude of 4145m, (27°29'58.4" north, 088°09'48.2" east).

Rationale & Scope of Study

RATIONALE AND SCOPE OF STUDY

Sikkim is the homeland of many high altitude Lakes (*Pokhari*) where most part of Year, Lake is covered with snow and the temperature goes to 0°C and even goes below 0°C. Pokhari of Sikkim has huge religious associated faith and belief system. There is absolutely little and very informal amount of literature accessible on all the Lakes of Sikkim.

The major thrust areas of the research on *Pokhari* are detection of the chemical compounds dissolved in the water, detection of pathogenic microbes. The intermingling web of culture, society, religion, faith and medicine is the main significant of this region and our research.

Chemical compounds like sulphate, fluoride content etc. and higher concentrations of heavy metals or halogens can cause several diseases or syndromes, which are detrimental to health. As, these lakes are frequently visited by many tourist both foreign as well as nationals, the detection of chemicals compounds are necessary.

Pathogenic microorganisms might be present at the lakes as they are contaminated by several migratory birds with their micro-flora. As many diseased birds may take bath, there might be a possibility of pathogenic microbes within the system. Thus it is necessary to detect and isolate such pathogenic microorganisms if present in *Pokhari*.

Understanding the evolution has been a distant dream for mankind. Research on this extremophiles will bring Sikkim into limelight with the discovery of novel microorganisms in the world. This is the biggest potential of Sikkim which has yet to be done.

Aims
&
Objectives

AIMS AND OBJECTIVES

The lakes of Sikkim are natural lakes and are regarded as "Source of Life" in Himalayas. These are ancient and have a lot of religious value since ages. There are many natural lakes in Sikkim and very few are documented. The major aim of the present study is to map the locations of *Laxmi Pokhari* (lakes), impact of lakes on the visitors and physicochemical and Microbiological analysis of water of *Laxmi Pokhari* which is located in Dzongri, west Sikkim (India). The major objectives for the present studies are:

- To document or map the location of *Laxmi Pokhari,* cold lake of Sikkim.
- To investigate the physicochemical analysis of the lakes

a) Physical characteristics like pH, conductivity, total dissolved oxygen etc.

b) Concentration of various kinds of minerals and compounds.

- To study the micro flora of *Laxmi Pokhari*

a) Isolation of psychrophilic bacteria

b) Characterization of isolates

c) Detection of pathogenic bacteria

Literature Review

LITERATURE REVIEW

An extremophile (from Latin words *extremus* meaning "extreme" and *philia* meaning "love") is an microorganism that can thrives in an extreme environment and the enzymes they produce are called extremozymes. The term extremophile was first used by MacElroy in 1974. "Extremes Environment" includes psychically or geochemically extreme conditions that are detrimental for one organism may be essential for the survival for another organism on Earth (Rothschild, L. J & Mancinelli, R. L, 2001). Extremophiles thrive under conditions that would kill most other creatures and many cannot survive in the normal anthropogenic global environments. Extreme environment includes those with either high (55 to 121 °C) or low (-2 to 20°C) temperatures, high salinity (2-5 NaCl) and either high alkalinity (pH>8) or high acidity (pH <4) (Madigan, M.T & Marrs, B.L, 1997; Rothschild, L. J & Mancinelli. R. L, 2001). It could be argued that extremophiles should include organisms thriving in biological extremes (nutritional extremes, extremes of population density, parasite, prey and so an on) condition. In contrast, organisms that live in moderate environments may be termed mesophiles/neutrophiles (Rampelotto, P. H, 2010; Rothschild, L. J & Mancinelli, R. L, 2001).

Various extremophiles can tolerate other extreme conditions including high pressure, high levels of radiation or toxic compounds or conditions that we consider unusual, such as living in rock deep below the surface of the earth or living in extremely dry areas with low water and nutrient supply (Mortia, 1975; Baross and Morita, 1978). In addition, extremophiles may be found in environments with a combination of extreme conditions such as high temperature and high acidity, high temperature and high alkalinity or high pressure and low temperature. The limits of temperature, pressure, pH, salinity and water activity at which life can thrive have not yet been precisely defined. Most of the extremophiles that have been identified to date belongs to the domain of the Achaea. However, many extremophiles from the eubacterial and

eukaryotic kingdoms have been identified recently and characterized (Rothschild and Mancinelli, 2001; Madigan and Marrs, 1997; Mortia, 1975).

PSYCHROPHILES

Psychrophiles or cryophiles are extremophilic organisms that are capable of growth and reproduction in cold temperatures. The old definition of psychrophiles applied to those organisms that produced a visible colony in 1 week at 0°C (Richard *et. al*, 2001). Specifically, their basic temperature are 20°C for maximum growth, 15°C or lower for optimal growth, and 0°C or lower for minimum growth (Morita, 1975). Microorganisms capable of growing at 5°C or lower are psychrotrophs, regardless of the optimum temperature for growth. The psychrotrophs are cold-tolerant bacteria, but their maximal growth temperature ranges above 20°C and in many cases their optimal growth temperature is also above 20°C. A better term for these organisms that withstand cold temperatures is psychrotolerant (Richard *et. al,* 2001).

Bacteria capable of growing at 0°C were first reported by Foster in 1887 and 1892. The source materials for these bacteria were from fish, natural water, foods, waste, rubbish, soil surface and intestines of fish. The term "Psychrophile" was first used in 1902 by Schmidt-Nielsen.

THE ENVIRONMENT AND ITS MICROFLORA

Eighty percent of earth's biosphere is permanently cold. Permafrost covers 20% of the earth's surface: 80% of Alaska, 50% of Canada, 20% of China and 50% of Russia are covered by permafrost (Baross and Morita, 1978). The Polar Regions comprise about 14% of the earth's surface. Approximately 71% of the earth's surface is ocean and more than 90% (by volume) of the oceans are colder. Other cold environments include caves, the tops of mountains, certain rivers and streams, the upper atmosphere, snow and ice and the water below the

thermocline of freshwater lakes; each has its own microbial flora (Baross and Morita, 1978).

MICROBIAL DIVERSITY IN VARIOUS COLD ENVIRONMENTS

Air samples collected near the earth's surface up into the stratosphere exceeding 27,000m have shown the presence of viable bacteria, viable fungi, pollen and other microscopic particles. A high incidence of viable bacteria has been reported within the troposphere at approximately 10,000m, where the temperature may be lower than -40°C (Griffin, 2008; Pearce *et al.*, 2009; Wainwright et al., 2004). The highest incidence of bacteria appears to be at altitudes slightly higher than 10,000m, where the temperature is lower than 10°C (Priscu & Christner, 2004; Priscu *et al.*, 2007). Although this temperature is well within the cardinal temperature range of psychrophile and psychrotrops, there are no reports of psychrophiles in the upper atmosphere (Priscu & Christner, 2004).

There are many glaciated and subterranean caves in which the permanent temperature is above 10°C to below freezing (Priscu *et al.*, 2007). In addition to the low temperature, there is an absence of light, low levels of organic material and relatively high moisture. Generally, there is an absence of psychrophiles in these caves, but psychrotrophs are found.

About 98% of the surface of Antarctica is ice, leaving 2% of the continent ice free. The lowest temperatures on Earth occur on this continent and it has the lowest precipitation and relative humidity levels, making it the driest areas on Earth. As a result, dry valleys and ice free areas occur in addition to many other types of environment (e. g., temporarily and permanently frozen lakes, mosses and peat bogs), with all sharing low temperature. In the dry valley of Antarctica, numerous psychrotrophs and mesophiles have been isolated.

The discovery of microorganisms in permafrost was initiated in the H1930s in the Trans-Baikal and North Ural regions, Central Yakutia and Arctic island

(Gilichinsky, 1995). Permafrost cores yielded numerous microbes. Microbes have been reported in all the Arctic and Antarctic permafrost environments except in the lower strata of permafrost ice of Lake Vostok in Antarctica.

About 71% of the Earth's surface is covered by ocean. The area below the thermocline (over 3000m deep) is approximately 5°C or colder. Thus, the deep sea is permanently cold. Gradually, as the higher latitudes are approaches, the depth of the thermocline decreases until it is at the surface of the ocean (Lauro & Bartlett, 2008; Nogi, 2008). In the deepest portions of the oceans, barophiles can be isolated that are also psychrotrophic or psychrophilic. At the edge of the ice where ice is melting or being formed, there is a sea ice environment with an associated microbial community.

Where the seawater is being frozen, a sea ice microbial community resides. The freezing process helps concentrate the dissolved nutrients and when the ice melts the concentrated nutrients in the ice are release so that sufficient energy is present to permit the growth of this microbial community. Within this sea ice community a variety of bacteria are found displaying numerous morphological types. Seventy percent of the bacteria in the sea ice community are free living.

BIODIVERSITY OF PSYCHROPHILES

The lowest temperature limit for life seems to be around -20 °C, which is the value reported for bacteria living in permafrost soil and in sea ice. Microbial activity at such temperatures is restricted to small amounts of unfrozen water inside the permafrost soil or ice and to brine channels. The first known species of psychrophiles descried taxonomically are Vibrio (Moritella gen. nov.) marinus MP-1 and Vibrio (Colwellia gen. nov.) Psychroerythrus, both isolated in 1964 (Russell, 1998). The biodiversity among psychrophiles in the various cold environments has yet to be studied extensively. Nevertheless, the various species within the genera *Achromobacteria, Alcaligenes, Altermonas,*

Aquaspirillum, Arthrobacter, Bacillus, Bacteroides, Brevibacterium, Clostridium, Colwellia, Cytophaga, Flavobacterium, Gelidibacter, Methanococcoides, Methanogenium, Methanosarcina, Microbacterium, Micrococcus, Moritella, Octadecabacter, Phormidium, Photobacterium, Polaribacter, Polaromonas, Pseudomonas, Psychroserpens, Shewanella, and *Vibrio* have been found to be psychrophilic.

The main importance of psychrophiles is their proteins, enzymes, metabolites and their adaptive mechanism. As they can adapt to the most stressful conditions and grow in tandem, their metabolites are cryo-stable. The cold enzymes along with the producing bacteria cover a broad spectrum of biotechnological applications such as medical and pharmaceutical, fine chemical synthesis, food industry and domestic application. They include additives in detergents, additives in food industries (fermentation, cheese manufacture, bakery and meat tenderization), environmental bioremediation (digester, composting, oil degradation or xenobiotic biology application). A number of relatively straight forward reasons for applications of cold active enzymes in biotechnology have been mentioned by various authors (Russell, 1998; Margesin, 1999; Gerday *et al.*, 2000; Caviccioli *et al.*, 2002).

Table1. Microorganism in relation to temperature requirements for growth

Temperature for growth (degrees C)

Group	Minimum	Optimum	Maximum
1.Psychrophiles	Below 0°C	10-15°C	Below 20°C
2.Psychrotroph	15-30°C	15-30°C	Above 25°C
3.Mesophile	30-40°C	30-40°C	Below 45°C
4.Thermophile	45°C	50-85°C	Above 100°C

T.D. Brock, 2004.

Thus, the application in biotechnology is boon for mankind. Psychrophiles are of particular interest to astrobiology, the field dedicated to the formulation of theory about the possibility of celestial life and the geo-microbiology, the study of microbes active in geochemical processes.

BIOTECHNOLOGICAL IMPORTANCE OF PSYCHROPHILES

The detection and isolation of Psychrophilic bacteria has increased for last two decades. This may be due to progress in molecular biology, such as amplification of the 16S rRNA gene, which allows detection of DNA sequences of many different organisms at a time (Reysenbach & Shock, 2002). The hunt has also been fuelled in the past several years by industry's realization that the "Survival Kits" possessed by extremophiles can potentially serve in an array of application. The main importance of psychrophiles is their proteins, enzymes, metabolism and their adaptive mechanism. The applicability of cryo-enzyme as biocatalysts is gaining wide industrial and biotechnological interest. Thus, the application of psychrophiles in biotechnology and Industry is a great boon for mankind

PSYCHROPHILIC ENZYMES AND ITS APPLICATION

Psychgrophiles became an important resource for bio-prospecting because of their unique cold adoptions, which help them to successfully live in such frigid living conditions. The Psychrophiles survive successful in such extreme living conditions is by optimizing various basic cell processes like enzyme function, nutrient transport and cell membrane function (Kahlke & Thorvaldsen, 2012, Chintalapati *et al.* 2004). The most important of these adoptions, which has immense potentials to be exploited, are the production of poly unsaturated fatty acid (PUFA) and cold active enzymes. The membranes and proteins in these microbes have a special property of increased structural flexibility that enhance

the catalytic function and the presence of unsaturated fatty acid help in easy nutrient cell transportation, due to the better fluidity. When temperature drops, Psychrophiles produce cold shock protein (CSP) or antifreeze proteins that enhance the activity of enzymes by improving enzyme kinetics and stabilizing microtubules. Psychrophilic enzymes have the advantage of having a low temperature optimum for activity with enhance specific activity at low temperatures and rather high thermo-ability.

The useful application of cold active enzymes are wide spread to a large number of industries like textile industry, food and dairy industry, brewing and wine industry, laundry etc. Cold active hydrolytic enzymes like lipases, proteases, cellulases and amylases can be used as an active agent detergents applied for cold washing. This reduced energy consumption and prevents wear and tear of textile fibers. Industrial dehairing of skins and hides also uses psychrophilic proteases or Keratinease at low temperatures saving energy and reducing undesirable effect of toxic chemicals. Other potential applications of Psychrophilic enzymes apart from these are in processes such as the hydrolysis of lactose in milk using galactosidase, stone washing and bio-polishing of textile products using cellulases, extraction and clearing fruit juices using Pectinases, meat tenderization or taste improvement of refrigerated meat using proteases, betterment of bakery products using glycosidase (Amylases, Proteases and Xylanases), softening of wool or cleaning of contact lenses using proteases.

Brewing and wine industries use other cold active enzymes as a better alternative to mesophilic enzymes. This can also work in cheese manufacturing and as animal feed supplements etc. Both Psychrophilic microorganism and their enzymes such as oxidase, peroxidase and catalase have been proposed as alternate options for the bioremediation of waste water and solids that are polluted by hydrocarbon (dehydrogenases) or in biotransformation (aminotransferases, mathylases and alanine racemase) (Nicholis *et al.* 1999)

Psychrophiles are a good source of polyunsaturated fatty acid (PUFA) which can be extensively used in pharmaceutical industry for developing new therapeutic

agents, because of the antibiotic properties of some microbes (Nichols *et al.* 1999, Singha, 2012). The use of cold active enzymes in food industry reduces the risk of contamination and the flavor is not destroyed at high temperature and it conserves the nutritional quality of food. Diverse starch modifying enzymes Xylanases and proteases can be used to bring down the fermentation time of dough and improves its properties (Struvay, 2012). In addition to these cold adapted lipases are preferred as flavor modifying enzymes in the mass production of fermented food, cheese manufacture, beer treatment and for biotransformation reactions in chemical processes.

Cold active beta-galactosidases are helpful in removing lactose from refrigerated milk at low temperature so that it can be consumed by people who are allergic to lactose (Fernandes et al. 2002). Proteases from Psychrophiles are also used widely in the food industry for the treatment of beer in bakeries and in the fast maturing of cheese. Patents involving enzymes such as proteases from cold-adapted bacteria have already been filed: Baeck and Quamrul, ZA9610820; Mikia and Katsuhisa, WO9743406; Quamrul and Eiichi, US6200793; Quamrul and Eiichi, WO9730172; and Eiichi, ZA9601237 (Marx et al., 2006).

Industrially Important Psychrophilic enzymes

Table 2: Selected potential biotechnological applications of cold-adaptive enzymes

Applications	Enzymes	References
Food and Feed industry Animal feed for the improvement of digestibility & assimilation	Lipase, Protease, glucanease and xylanase	Collins et al. (2005); Hatti Kaul et al. (2005); Huang et al. (2009); Ueda et al. (2010).
Detergents & cleaning industry Additive to detergents for washing at room temperature	Lipase &Protease	Tutino et al. (2009); Wang et al. (2010).
Environmental Biotechnology Bioremediation, degradation & removal of xenobiotics	Lipase, Protease & xylanase	Joseph et al. (2008); Wang et al. (2010).
Tanning & Hide industry Bioleaching in paper & pulp industry	Collagenase	Zaho et al. (2005)
Anti bacterial agent Antibacterial & antioxidant	Lysozymes	Aurilia et al. (2008)
Textile industry Stone washing Desizing denim jeans	Cellulase Amylase	Ueda at al. (2010) Gerday et al. (2010)

CELLULASE

Cellulose is the extensively used most organic polymer which is an exhaustible source of raw material for different products (Klemm 2002). It was initially used for the bioconversion of biomass which gave way research in the industrial application of enzyme in various fields such as food, feed, textiles, and detergents and in the paper industry. Cellulases have become the third largest group of enzyme used in industry. Since a decade they are used in the bio stoning of denim garments replacing the use of palmic stones which were traditionally employed in the industry (Belghith *et al.*, 2001). Cellulases have also been used in softening (Galante *et al.*, 2003) and in providing variation in

the color density of fibers. Cellulase in particularly ECIII and CBHI, are commonly used in detergents for cleaning textiles. In food industry, cellulases are used in extraction and clarification of fruits and vegetables juices, production of fruit nectars and purees, and in the extraction of Olive oil. Cellulases are used in carotenoid extretion in the production of food colouring agents (Bedford *et al.,* 2003) and are also used to improve the nutritive quality of forages (Aktar, 1994). In the pulp and paper industry cellulose and hemicellulases have been employed for mechanical pulping for modification of the coarse mechanical pulp and hand sheet strength properties (Franks *et al.*, 1996).

AMYLASES

Amylases are starch degrading enzyme that catalyses the breakdown of starch into sugar. They are widely distributed in plant, animal and bacteria kingdom. The starch requires a combination of enzymes for its complete hydrolysis. These enzymes include Alfa-amylases, Glucoamylases or Beta amylases (Poonam, N. & Dalel, S. 1995). Alfa-amylase is an endozyme which leads to the formation of linear and branch oligosaccharide, while the rest are exoenzyme and attacks the substrate from the non-reducing end producing oligosaccharide/monosaccharide. Amylase is used in bread making and to break down complex sugar, such as starch into simple sugars. Alpha and beta amylases are important in brewing beer and liquor made from sugar derivative from starch. Liquefactions are the first important steps in starch processing.

PROTEASES

Protease is the enzyme that converts the protein into amino acid and peptides. They are classified according to the nature of their catalytic activity. Today the amount of proteolytic enzymes produced throughout the world on a commercial scale is large as compare to the other biotechnologically modified enzymes (Mozersky *et al.*, 2002; Fan et al., 2001; Cowan, D. 1996). In the food, leather, pharmaceutical and textile industry, these are the major used enzymes. Serine alkaline protease is used as addition to detergents for laundering. Proteinases

showing high keratinolytic and elastolytic activities and are used for soaking in the leather industry. The proteases that can catalyze reactions under extreme condition i.e. high temperature and extreme pH will be valuable for industrial application.

LIPASES

Lipases are regarded as the most versatile enzymes of the industries which bring about a range of bioconversion reaction, which includes hydrolysis, inter esterification, esterification, acidolysis. The esters produced play a vital role in the food industry as flavor and aroma constituents. Whereas long chain methyl and ethyl esters of carboxylic acid moieties provide valuable oleo chemical species that may function as fuel for diesel engines, ester of long chain carboxylic acid and alcohol moieties have application as lubricants and additives in cosmetic formulation. Other applications includes are the removal of the pitch from pulp produced in the paper industry, for the hydrolysis of milk fat in the diary industry, removal of non-cellulosic impurities from raw cotton before processing into dyes and finished product, drug formulations in the pharmaceuticals industry and in the removal of subcutaneous fat in the lather industry. Lipases occur throughout the earth's flora and fauna. Extensively and abundantly, they are produced by various microbial communities like bacteria; fungi and yeast.

Besides this there are a number of advantages in using extremophiles in industrial applications, particularly in the production of bio-fuels. The continuous diminishing of fossil fuel reserves and the current global energy situation has revealed an exigent need for the development of alternative fuel sources. Microbial catalysis, in the conversion from biomass to bio-fuel has gained much impetus as biotechnology has evolved to its current status (Barnard *et al.*, 2010). Extremophiles are robust organisms producing stable enzymes, and are often able to tolerate changes in environmental conditions, such as pH and temperature. The potential application of such organisms and their enzymes in biotechnology is enormous, and a particular application is in bio-fuel

production.Psychrophiles readily ferment pentose and/or hexose sugars from biomass and, in some cases, even structurally complex carbohydrates, a quality which is particularly important for production of second-generation bio-fuels Sommer et al., 2004; Rogers et al., 1982). Furthermore, psychrophilic industrial fermentations are less prone to microbial contamination.

Materials

MATERIALS

4.1. Materials used for measuring and mapping of the geographical features of the sample sites.

1. Global Positioning System - GPSMAP78S, Garmin©2010, Olathe, Kansas, United States of America (USA).
2. Google Earth – Software; Google ©, USA.
3. Arc GIS – Software; Google ©, USA.
4. GSI (Geological Survey of India) Website: www.portal.gsi.gov.in

4.2. Materials used for sample collection.

Steel Container (Flask), Capacity 0.75 liter, Mega Slim®, Nashville, United States of America (USA).

4.3. Materials used for the determination of the Physical Analysis of the water.

ORION 5 STAR Thermo Scientific, India.

4.4. Materials used for the determination of the Chemical Analysis of the water.

1. Total Hardness Test Kit (WT001C AQUA *CHECK*™) HiMedia, Mumbai, India.
2. Alkalinity Test Kit (WT003A AQUA *CHECK*™ HiMedia, Mumbai, India.
3. Chloride Test Kit (WT004A AQUA *CHECK*™)

HiMedia, Mumbai, India.

4. Sulphite Test Kit (WT005A AQUA *CHECK*™)

HiMedia, Mumbai, India.

5. Residual (Free) Chlorine Kit (WT006 AQUA *CHECK*™)

HiMedia, Mumbai, India.

6. Nitrite Test Kit (WT007A AQUA *CHECK*™)

HiMedia, Mumbai, India.

7. Orthophosphate Test Kit (WT008A AQUA *CHECK*™)

HiMedia, Mumbai, India.

8. Iron Test Kit (WT010 AQUA *CHECK*™)

HiMedia, Mumbai, India.

9. Fluoride Test Kit (WT012 AQUA *CHECK*™)

HiMedia, Mumbai, India.

4.5. Materials for the Isolation and Cultivation of the microorganisms.

4.5.1. Microbiological Media.

Luria Bertani Agar

Carbohydrate Fermentation Broth

Urea Agar

Gelatin Agar

Other bacteriological media components

Agar Agar Type-1	HiMedia, Mumbai, India.
Bacteriological Peptone	HiMedia, Mumbai, India.
Trypticase	CDH (P) LTD., West Bengal, India
Yeast Extract	HiMedia, Mumbai, India.
Beef Extract	HiMedia, Mumbai, India.
Starch	HiMedia, Mumbai, India.
Trypticase Soya Agar	HiMedia, Mumbai, India.
Mueller Hinton Agar	HiMedia, Mumbai, India.
Gelatin	HiMedia, Mumbai, India.
Double Toned Milk	Amul, Gujarat, India.

4.5.2. Chemicals.

Ethyl Alcohol	Bengal Chemicals, West Bengal, India.
Magnesium Chloride	Merck, Mumbai, India.
Mercuric Chloride	Merck, Mumbai, India.
Sodium Chloride	Merck, Mumbai, India.
Potassium Di hydrogen Phosphate	HiMedia, Mumbai, India.
Phenol Red	Merck, Mumbai, India.
Urea	Merck, Mumbai, India.

Hydrogen Peroxide	Merck, Mumbai, India.

4.6. Materials for the Staining of the microorganisms.

Crystal violet	HiMedia, Mumbai, India.
Copper Sulfate	Merck, Mumbai, India.
Gram's Iodine Solution	STANBIO, Kolkata, India.
Safranine	HiMedia, Mumbai, India.

4.7. Materials for the Biochemical Analysis of the microorganisms.

Carbohydrate Fermentation Test.

D (-) Arabinose	HiMedia, Mumbai, India.
D (+) Dextrose	HiMedia, Mumbai, India.
D (+) Galactose	HiMedia, Mumbai, India.
D (+) Lactose	HiMedia, Mumbai, India.
D (+) Mannose	HiMedia, Mumbai, India.
D (+) Maltose	HiMedia, Mumbai, India.
D (+) Rhamnose	HiMedia, Mumbai, India.
D (+) Sucrose	HiMedia, Mumbai, India.
D (+) Xylose	HiMedia, Mumbai, India.
D (+) Cellobiose	HiMedia, Mumbai, India.
D (+) Melibiose	HiMedia, Mumbai, India.

D (-) Mannitol	HiMedia, Mumbai, India.
D (-) Inositol	HiMedia, Mumbai, India.
D (-) Fructose	HiMedia, Mumbai, India.

4.8. Antibiotics used for Antibiotic Assay.

Amoxicillin	HiMedia, Mumbai, India.
Ampicilin	HiMedia, Mumbai, India.
Chloramphenicol	HiMedia, Mumbai, India.
Erythromycin	HiMedia, Mumbai, India.
Tetracycline	HiMedia, Mumbai, India.

4.8. Laboratory Equipments and Instruments.

Cooler Incubator	REMI, Kolkata, India.
Spectrophotometer (uv-vis range)	Biophotometer Plus Model AG6132
Laminar Air Flow System	Klenzaids Pvt. LTD.; Kolkata.
Weighing Balance	METTLER TOLEDO, Switzerland.
Autoclave	Instrumentation India, Kolkata, India.
Incubator cum Shaker	REMI, Kolkata, India.
Freezer -80°C	REMI, Kolkata, India.
Freezer -20°C	REMI, Kolkata, India.
Freezer 4°C	Samsung, India.

Compound Microscope	Olympus, Model - 808209.
Phase Contrast Microscope	Olympus, Model – CKX41
Water Purification System	Millipore
Hot Air Oven	N.A.Instruments & Equipments, Kolkata, India
Microwave	Samsung, India.

4.9. Glass wares

Petri plates	Borosil, India.
Test Tubes	Borosil, India.
Spreader	Borosil, India.
Pipettes	Borosil, India.
Conical Flask	Borosil, India.
Reagent Bottle	Borosil, India.
Glass slides	HiMedia, Mumbai, India.
Cover Slip	Blue Star, India.
Dropper	Borosil, India.

4.10. Other Materials

Inoculum Loop	HiMedia, Mumbai, India.
Spirit Lamp	Borosil, India.
Absorbent Cotton	Bengal Surgical, West Bengal, India.

Non-Absorbent Cotton	Bengal Surgical, West Bengal, India.
Test Tube Rack	Tarson, India.
Cryo Vials	Tarson, India.
Micropipette	Gilson, France.
Micro tips	Tarson, India.
Cryo Vial Stand	Tarson, India.
Paraffin Tape	Bengal Surgical, West Bengal, India.
Blotting Paper	Bengal Surgical, West Bengal, India.

Methods

METHODS

5.0. Methods

5.1 Sampling

Water samples were collected in sterile containers for their microbiological and physicochemical analysis from *Laximi Phokari.* These samples were collected on the basis of their extensive usage as a traditional socio medicine to the community, popularity in terms of tourism and its basis of being a pilgrimage center.

5.2. Mapping of the sampling site.

By the help of GPS78SMAP device (Global Positioning System), the geographical coordinates were found out. To describe any geographical location one has to measure the latitude and longitude of the area. Thus, on locating the coordinates, the exact area gets mapped. Next, the elevation of the land from the sea level is calculated to position the area of the land. Ascent of the land depicts the height of the site at which it is present. By the help of compass, the exact direction of the site studied can be easily quantified. After measuring all the co-ordinates, elevation, latitude, longitude, the elevation of the site, the ascent of the site and the atmospheric temperature of the site was measured and they were mapped in Google Earth and Arc GSI Software.

5.3. Description of the sampling site.

From the coordinates obtained by the GPSMAP78S, the data was inserted into the Geological Survey of India (GSI), website www.portal.gsi.gov.in. From there, the District Resource Map was obtained for the site specific district of Sikkim. With the help of the reference scale and legends, the geology and the earth science of the site was obtained and analyzed. The site i.e. the ponds where the bath is located was measured and a sketched diagram was designed. The

basic length parameters was measured and calculated by the reference scale. All the physical parameters were observed and discussed, to describe the sampling site.

5.4. Methods used for sample collection.

The water samples for the physicochemical analysis and microbiological analysis, was aseptically collected in a sterile containers. These containers were then sealed tightly and were brought to the laboratory for the tests. At the laboratory the water was kept in cooler Incubator at 4°C to maintain the microbes viability and reduce their metabolic activity.

5.5. Physicochemical Analysis of the water samples.

5.5.1. Methods used for the analysis and determination of the physical characteristics of the water.

By the help of Orion 5 Star pH benchtop automatic instrument, the four physical parameters were measured – pH, Temperature of the Hot spring at the source, D.O. and Conductivity was monitored and measured.

Determination of Total Dissolved Solids (T.D.S.) of water

Procedure:

1. The weight of the evaporating dish was measured.
2. The sample was nicely vortexed and uniformly mixed.
3. 500 ml of the water sample to be tested was filtered through the Whitman filter.
4. The filtrate was transferred into the evaporating dish.
5. The evaporating dish was set on a hot water bath of 100°C and the contents were allowed to evaporate.

6. After 1 hour, the weight of the dish along with contents left after cooling in a dessicator was measured.
7. All the observations and results were noted down and the T.D.S. was calculated by the provided formula.

Formula:

$$T.D.S.\ (mg/lt.) = ((B-A) / V) \times 10^6$$

Where,

A= Initial weight of the dish (g)

B= Final weight of the dish (g)

V= Volume of the water sample taken (ml)

5.5.2. Methods used for the analysis and determination of the chemical characteristics of the water.

I. Total Hardness Test Kit WT001D AQUA *CHECK*™ water analysis system (HiMedia Laboratories Pvt. Ltd., Mumbai, India.)

Detection Range :

2 – 40 mg/L (ppm) as $CaCO_3$

5 – 100 mg/L (ppm) as $CaCO_3$

Procedure:

For the Total Hardness range ≤ 40 mg/L (ppm)

1. The aqua test jar was filled with the water sample up to the 10 ml mark.
2. 1 spoonful of the powder Reagent 1D-1 was added with the tiny spoon provided.
3. It was nicely mixed, so as to dissolve the powder completely.

4. 4-5 drops of the Reagent 1D-2 was added and thoroughly mixed.
   The change in the color of the solution was observed.
5. The color of the solution should be either Blue or Red. If the solution turns Red in color then the Reagent 1D-3 was added drop wise. The number of the drops added was counted while mixing, until the color changed from Red to Blue.
6. The observations were noted down.
7. The Total Hardness level in the water sample was calculated.

For the Total Hardness range > 40 mg/L (ppm)

1. The aqua test jar was filled with the water sample up to the 10 ml mark.
2. 1 spoonful of the powder Reagent 1D-1 was added with the tiny spoon provided.
3. It was nicely mixed, so as to dissolve the powder completely.
4. 4-5 drops of the Reagent 1D-2 was added and thoroughly mixed.
5. The change in the color of the solution was observed.
6. The color of the solution should be either Blue or Red. If the solution turns Red in color then the Reagent 1D-4 was added drop wise. The number of the drops added was counted while mixing, until the color changed from Red to Blue.
7. The observations were noted down.
8. The Total Hardness level in the water sample was calculated.

<u>Calculation:</u>

The Hardness level in the water test sample is to be calculated as per the formula.

Total Hardness as ppm $CaCO_3$ =

2 x (Number of Drops of Reagent 1D-3)

OR

5 X (Number of Drops of Reagent 1D-4)

II. Alkalinty Test Kit WT003A AQUA *CHECK*™ water analysis system (HiMedia Laboratories Pvt. Ltd., Mumbai, India.)

Detection Range :

10 – 200 mg/L (ppm) as $CaCO_3$

100 – 2000 mg/L (ppm) as $CaCO_3$

Procedure:

For the Alkalinity range ≤ 200 mg/L (ppm)

1. The aqua test jar was filled with the water sample up to the 10 ml mark.
2. 2 drops of the Reagent 03A-1 was added and mixed well.
3. If pink color appears it would indicate presence of the P alkalinity.
4. The reagent 03A-2 was added drop wise by counting the number of drops while mixing, until the pink color disappears (N drops).
5. To the above solution ½ spoonful of the Reagent 03A-3 was added. The color of the solution will turn into Green.
6. Reagent 03A-2 was added to it by counting the number of the drops while mixing, until the color of the solution changes from Green to Reddish Violet (N_1 drops).
7. The change in the color of the solution was observed.
8. The Alkalinity level in the water sample was calculated.

For the Alkalinity range > 200 mg/L (ppm)

1. The aqua test jar was filled with the water sample up to the 10 ml mark.
2. 2 drops of the Reagent 03A-1 was added and mixed well.
3. If pink color appears it would indicate presence of the P alkalinity.
4. The reagent 03A-4 was added drop wise by counting the number of drops while mixing, until the pink color disappears (N drops).
5. To the above solution ½ spoonful of the Reagent 03A-3 was added. The color of the solution will turn into Green.

6. Reagent 03A-4 was added to it by counting the number of the drops while mixing, until the color of the solution changes from Green to Reddish Violet (N_1 drops).
7. The change in the color of the solution was observed and was noted down.
8. The Alkalinity level in the water sample was calculated.

Calculation:

The P Alkalinity level in the water test sample is to be calculated as per the given formula.

P Alkalinity ppm as $CaCO_3$

= 10 X (N drops of Reagent 03A-2)

OR

= 100 X (N drops of Reagent 03A-4)

The Total Alkalinity in the water test sample is to be calculated as per the given formula.

Total Alkalinity ppm as $CaCO_3$

= 10 X ($N + N_1$) drops of Reagent 03A-2

OR

= 10 X ($N + N_1$) drops of Reagent 03A-

III. Chloride Test Kit WT004A AQUA *CHECK*™ water analysis system (HiMedia Laboratories Pvt. Ltd., Mumbai, India.)

Detection Range :

10 – 200 mg/L as Chloride

50 – 1000 mg/L as Chloride

Procedure:

For the Chloride range ≤ 200 mg/L (ppm)

1. The aqua test jar was filled with the water sample up to the 10 ml mark.
2. 1 tiny spoonful of Reagent 04A-1 and 2 drops of the Reagent 04A-2 were added to it.
3. The solution was nicely mixed.
4. Reagent 04A-3 was added drop by drop and the number of drops was counted while mixing, until the color changed to Bluish Violet.
5. The change in the color of the solution was observed and was noted down.
6. The Chloride level in the water sample was calculated.

For the Chloride range > 200 mg/L (ppm)

1. The aqua test jar was filled with the water sample up to the 2 ml mark.
2. 1 tiny spoonful of Reagent 04A-1 and 2 drops of the Reagent 04A-2 were added to it.
3. The solution was nicely mixed.
4. Reagent 04A-3 was added drop by drop and the number of drops was counted while mixing, until the color changed to Bluish Violet.
5. The change in the color of the solution was observed and was noted down.
6. The Chloride level in the water sample was calculated.

Calculation:

The Chloride level in the water sample was calculated as per the given formula.

Chloride in mg/L (ppm) as Cl^- =

10 X (Number of drops of 04A-3)

OR

50 X (Number of drops of 04A-3) for 2 ml sample

IV. Sulphite Test Kit WT005A AQUA *CHECK*™ water analysis system (HiMedia Laboratories Pvt. Ltd., Mumbai, India.)

Detection Range:

5 – 100 mg/L as Na_2SO_3

Procedure:

1. The aqua teat jar was filled with the water sample up to the 10 ml mark.
2. 2 drops of the Reagent 05A-1 was added and pink color appeared.
3. If Pink color appears then the Reagent 05A-2 was added and if the color does not appear then the Reagent 05A-2 was not added and the following steps were carried out.
4. 1 spoonful of the Reagent 05A-3 was added to the solution with the spoon provided.
5. The solution was nicely mixed to dissolve the powder completely.
6. The Reagent 05A-4 was added drop by drop and the number of drops added were counted while mixing, until the color changed from colorless to Violet blue.
7. The observations were noted down.
8. The Sulfite level in the water sample was calculated.

Calculation:

Sulfite as Na_2SO_3 mg/L (ppm) = 5 X (Number of drops of Reagent 05A-4)

V. Residual (Free) Chlorine Test Kit WT006 AQUA *CHECK*™ water analysis system (HiMedia Laboratories Pvt. Ltd., Mumbai, India.)

Detection Range :

0.1 – 3.0 mg/L as Chlorine

Procedure:

1. The aqua test jar was filled with the water sample up to the 10 ml mark.
2. 4-5 drops of the Reagent 06A was added and mixed well.
3. 2-3 drops of the Reagent 06B was added and mixed well.
4. The mixture was allowed to stand for few minutes until the blue color develops completely.
5. The observations were noted down.
6. If the solution color changes to Blue then drop by drop the Reagent 06C was added by counting the number of drops while mixing, until the Blue color disappears.
7. The Residual (Free) Chlorine level in the water sample was calculated.

Calculation:

The Residual (Free) Chlorine level in the water sample was calculated as per the given formula.

Residual (Free) Chlorine as ppm = 0.1 X (Number of drops of Reagent 06C)

VI. Nitrite Test Kit WT007A AQUA *CHECK*™ water analysis system (HiMedia Laboratories Pvt. Ltd., Mumbai, India.)

Detection Range :

5 – 100 mg/L as $NaNO_2$

50 – 1000 mg/L as $NaNO_2$

Procedure:

For the Nitrite range ≤ 100 mg/L (ppm)

1. The aqua test jar was filled with the water sample up to the 10 ml mark.
2. 2 drops of the Reagent 07A-1 was added and mixed well.
3. The Reagent 07A-2 was then added drop wise by counting the number of drops added while mixing till pale Bluish Green color appeared.

4. The observations were noted down.
5. The Nitrite level in the water sample was calculated.

For the Nitrite range > 100 mg/L (ppm)

1. The aqua test jar was filled with the water sample up to the 1 ml mark. It was diluted to 10 ml by adding 9 ml of distilled water to it.
2. 2 drops of the Reagent 07A-1 was added and mixed well.
3. The Reagent 07A-2 was then added drop wise by counting the number of drops added while mixing till pale Bluish Green color appeared.
4. The observations were noted down.
5. The Nitrite level in the water sample was calculated.

Calculation:

The Nitrite level in the water sample was calculated as per the given formula.

Nitrite as ppm $NaNO_2$

= 5 X (Number of drops of Reagent 07A-2)

OR

= 50 X (Number of drops of Reagent 07A-2 for the diluted water sample)

VII. Orthophosphate Test Kit WT008A AQUA *CHECK*™ water analysis system (HiMedia Laboratories Pvt. Ltd., Mumbai, India.)

Detection Range :

0 – 40 mg/L (ppm) as PO_4

Procedure:

1. **Sample Preparation**: The pH of the water should be preferably neutral. The water sample should be neutralized if it is of highly acidic or alkaline in nature. The aqua test jar was filled with the water sample up to the 2.5 ml mark.
2. Equal volume of the Reagent 08A-1 was added.
3. Stopper was placed on the test jar and the contents were mixed thoroughly. It was allowed to stand for 5 minutes.
4. The color developed was matched with that of the standard color chart provided.
5. The observations were noted down and the Orthophosphate level in the water sample was calculated.

Calculation:

Orthophosphate as mg/L (ppm) = Value obtained from matching the color with respect to the standard color chart.

1. The test jar was closed with the stopper and the contents were mixed for 1-2 minutes.
2. 1 spoonful of the Reagent 09C was added.
3. The solution was allowed to stand for 5 minutes.
4. The color developed was compared with respect to that of the standard color chart provided.
5. The observations were noted down. the Reactive Silica level in the water sample was calculated.

VIII. Fluoride Test Kit WT012 AQUA *CHECK*™ water analysis system (HiMedia Laboratories Pvt. Ltd., Mumbai, India.)

Detection Range :

0.5 – 2 mg/L (ppm) as Fluoride

Procedure:

1. The aqua test jar was filled with the water sample up to the 10 ml mark.
2. 3 drops of the Reagent 012A was added to it and the contents were mixed nicely.
3. 8 drops of the Reagent 012B was added and the contents were mixed and it was allowed to stand for 4-5 minutes.
4. The observations were noted down.
5. The Fluoride level in the water sample was calculated by comparing the developed color with respect to the standard color chart provided.

Calculation:

Fluoride as mg/L (ppm) = value obtained by the standard color comparison.

IX. Nitrate Test Kit WT013 AQUA *CHECK*™ water analysis system (HiMedia Laboratories Pvt. Ltd., Mumbai, India.)

Detection Range :

0 – 100 mg/L as Nitrate

Procedure:

1. The aqua test jar was filled with the water sample up to 1 ml mark.
2. 1 spoonful of the Reagent 013A was added and thoroughly mixed.
3. 5 drops of the Reagent 013B was added to it.
4. 1 spoonful of the Reagent 013C was added.
5. The contents were nicely mixed and were allowed to stand for 5 minutes until a color gets developed.
6. The volume of the solution was made up to the 10 ml mark with the distilled water.
7. The observations were noted down.
8. The Nitrate level in the water sample was calculated as per the color obtained and compared with respect to the standard color chart.

Calculation:

Nitrate as mg/L (ppm) = value obtained by the standard color comparison.

5.6. Microbiological Analysis of the water samples.

5.6.1. Enumeration of the microbial load by total plate count.

The viable bacteria or microorganisms capable of utilizing nutrients from the agar based plate, when develops a colony, that is enumerated and counted and depending on the dilution factor, the approximate microbial load of the sample is determined. Thus a faint idea about the microbial load in the sample can be understood. Serial dilution of the sample is done and pour plated on Plate Count Agar (PCA) plates. After sufficient incubation, the number of colonies developed is calculated and through the formula, Total Plate count or viable count of the sample is determined.

$$\text{Total Number of cells/ml} = \frac{\text{(Number of colonies (average of three}}{\text{Amount Plated} \times \text{Dilution}}$$

5.6.2. Isolation of the bacterial samples from the *Laxmi Pokhari.*

Luria bertenin Agar (LB) were used for the isolation of the Psychrophilic microorganism from the high altitude lake (*Laxmi pokhari*). One milliliter sample from of each water sample was added to LB Agar (10 ml) and incubated in Environmental Chamber at 15°C for 7 days. Growth was followed by measuring the increase in turbidity at 600 nm. Then, the culture was streaked onto a Luria bertenin Agar plate. Isolation of pure culture was done by using spread plate method and streak plate method (Rath and Subramanyam, 1998).

5.6.3. Characterization of the bacterial samples from the *Laxmi Pokhari.*

5.6.3.1. Culture characteristics and morphology.

General Morphology of the microorganisms as observed under the compound microscope.

Shape: Spheres / Short rods / Long rods / Filaments / Commas / Spirals

Arrangement: Single / Pair / Chains / Clusters

Capsules: Present or Absent

Gram Stain: Positive or Negative

Motility: Motile or Non Motile

Cultural Characteristics of the colonies on Agar plate.

Colony color: Golden; Yellow; White; Glistening; Other pigmentation.

Form: Circular; Irregular; Rhizoid

Margins: Entire; Lobate; Undulate; Serrate; Filamentous

Elevation: Flat; Raised; Convex; Umbonate

Density: Opaque; Translucent; Transparent

Growth on Broth Media

Surface Growth: Ring; Pellicle; None

Clouding: Slight; Heavy; None

Sediment: Abundant; Scanty; Granular; Flaky; Flocculent; None

5.6.3.2. Staining

Gram Staining was carried out for each of the isolates. Crystal Violet (primary stain) was flooded to the heat fixed, air dried smear and was kept for 30 s. Then it was washed with distilled water and Gram's Iodine solution was flooded to the stained slide and kept for 60 s. the Iodine was washed off with 95% Ethanol

followed by distill water. Later on, counter stain safranin was added to the smear and kept for 30 s and was washed off by distilled water and the slide was air dried. The observations were noted down (Aneja, *Experiments in Microbiology, Plant Pathology and Biotechnology*, 4th Edition).

5.6.3.4. Tolerance to various degrees of Temperature.

The isolates were inoculated in different LB Broths respectively and were incubated at 0°C, 5°, 10°C, 15°C and 20°C in the Environmental Chamber. They were kept for 48 hours incubation. After incubation the O.D. of the broth cultures were measured at 600 nm and 660 nm. All the data's were noted down and the temperature tolerance capability of the bacteria was interpreted with comparing the respective O.D. values against that of O.D. at 60°C. Thus O.D. value of bacterial culture grown at 15°C was our Control. Blank was prepared by taking the uninoculated media broth.

5.6.3.5. Tolerance to various pH.

The isolates were inoculated in different LB Broths respectively and were incubated at various pH ranges. The pH was adjusted by adding 0.1 N HCl for making the condition acidic and 0.1 N NaOH to make the condition alkaline. The cultures were inoculated at the pH of 3.0, 5.0, 7.0, 9.0, 1.0 and 12.0 and 15°C in the Environmental Chamber. They were kept for 48 hours incubation. After incubation the O.D. of the broth cultures were measured at 600 nm and 660 nm. All the data's were noted down and on comparing the various O.D. values of various pH conditions against the O.D. value of bacterial cultures grown at pH 8.0, the pH tolerance capability of the bacteria were interpreted. Thus O.D. value of bacterial culture grown at pH 8.0 was our Control. Blank was prepared by taking the uninoculated media broth.

5.6.3.6. Tolerance to various concentrations of NaCl.

The isolates were inoculated in different LB Broths respectively and were incubated at various concentrations of NaCl. The cultures were inoculated at the NaCl concentrations 0%, 1%, 2.5%, 5%, 10% and 20% in the Environmental Chamber. They were kept for 48 hours incubation. After incubation the O.D. of the broth cultures were measured at 600 nm and 660 nm. All the data's were noted down and on comparing the various O.D. values of various concentrations of NaCl conditions against the O.D. value of bacterial cultures grown at concentrations of 2% NaCl, the tolerance capability of the bacteria to various concentrations of NaCl were interpreted. Thus O.D. value of bacterial culture grown at the concentrations of 2% NaCl was our Control. Blank was prepared by taking the uninoculated media broth.

5.6.3.7. Antibiotic Sensitivity of Isolates.

The isolates were tested for their response to various antibiotics by Disc Diffusion Method. Hence, a series of Antibiotic Sensitivity was carried out for the isolates. Kirby Bauer Antibiotic Sensitivity method was followed. 0.1 ml of isolate was streaked by sterile cotton swabs in different respectively labeled Mueller Hinton Agar (MHA) plates. Then after 15 minutes of standing, one sterile antibiotic disc was placed at the center of the MHA plate. For each Antibiotic, triplicates were done. For a single isolate, its Antibiotic Sensitivity was measured against 5 various antibiotics. Thus all the isolates were tested against the following antibiotics; Amoxicillin, Ampicilin, Chloramphenicol, Erythromycin and Tetracycline. All the Antibiotics were tested against E. coli culture and the zone of diameter of inhibition was taken as a control and sterile discs dipped in sterile water was the control. The plates were incubated at 15°C for 48 hours. After incubation, the plates were observed for their zone of inhibition and their diameter was measured and recorded. By comparing the diameter size of the zone of inhibition, with the standard chart provided for the

Assay, the isolates were interpreted as Resistant (R) or Intermediate (I) or Susceptible (S) to the respective antibiotics.

5.6.4. Identification of the bacterial samples.

5.6.4.1. Carbohydrate Fermentation Test.

Fermentative degradation of various carbohydrates by microbes under anaerobic conditions is carried out in a fermentation tube. A Fermentation tube is a culture tube that contains a Durham tube (a small tube placed in an inverted position in the culture tube) for the detection of gas production as an end product of metabolism. The Fermentation broth contains the ingredients of nutrient broth, a specific carbohydrate and a pH indicator (Phenol red), which is red at neutral pH (7.0) and turns yellow at or below a pH of 6.8 due to the production of an organic acid (Aneja, *Experiments in Microbiology, Plant Pathology and Biotechnology*, 4th Edition).

All the individual isolates were inoculated in Carbohydrate Fermentation Broth and was kept at 15°C for 48 hours. After incubation, the broths were observed for any growth and color change.

5.6.4.2. Catalase Activity

During aerobic respiration in the presence of oxygen, microbes produce Hydrogen Peroxide (H_2O_2) which is lethal to the cell. The enzyme catalase breaks down Hydrogen peroxide to water and oxygen. Release of free oxygen gas in the form of bubbles is a positive catalase test. To all the individual isolates, 3% H_2O_2 was added. The observations were noted down.

5.6.4.3. Protease Activity.

The ability of microbes to degrade protein refers to their proteolytic activity. 0.1 ml of fresh isolates culture broth was added to LB plate supplemented with 10%

double toned milk. It was incubated at 15°C for 48 hours and the observations were noted down. Formation of colonies will show that the microbes have protease activity.

5.6.4.4. Starch Hydrolysis Test

Starch is a complex carbohydrate (polysaccharide) composed of two constituents – amylase, a straight chain polymer of 200-300 glucose units and amylopectin, a larger branched polymer with phosphate groups. Starch consists of eight or more monosaccharide units and disaccharide units. These monosaccharides and disaccharides, enter into the cytoplasm of the bacterial cell through the semi permeable membrane and thereby used by the endoenzymes. Amylase is an exoenzyme that hydrolysis or cleaves starch into its composite units of monosaccharide and disaccharide sugars. The ability to degrade starch is used as a criterion for the determination of amylase production by the microbes. Iodine solution is used as an indicator. Starch in the presence of iodine solution produces a dark blue coloration of the medium and the formation of a yellow zone around the colony indicates the amylolytic activity of the microbes (Aneja, *Experiments in Microbiology, Plant Pathology and Biotechnology*, 4th Edition).

All the individual isolates were spread plated in Starch Agar Media and was kept at 15°C for 48 hours. After incubation, the 2% Iodine solution was flooded to the plates and were observed for any color change.

5.6.4.5 Detection of *coli – aerogenes* contamination in water.

All the isolates were streaked into Violet Red Bile Agar plates (A specialized differential media used for the selective isolation, detection and enumeration of *coli – aerogenes* bacteria in water, milk and other dairy food products.) Presence of growth indicates the fecal contamination and further MPN Test must be carried out to confirm the presence of *coli – aerogenes* bacteria in water. The plates were incubated at 15°C for 48 hours. After incubation, the plates were observed for any growth.

Results

RESULTS

1. Mapping of the sampling site.

It is very important to know the physical location of *laxmi Pokhari* as it is visit by many people. One of the way to know the physical location of any place is by Global Positioning System (GPS) mapping. The Coordinates of the sites where *Laxmi Pokhari* and their water sources were located, lies between **27°29'58.4" N** and **27°29' 59.1" N latitudes and 88°09'48.2" E** and **88°09'48.1" E longitudes**. The elevation range of the locations ranged from 4154 m to 4190 m above the sea level as measured by the GPS MAP78S (Table No.1).

Table No. 1: GPS Data

SNo.	Location	Coordinates	Elevation	Atmospheric Temperature
1.	Zero Point	27°29.584' N & 88°09.482' E	4154 m	12°C
2.	Sampling Point 1	27°29.591' N & 88°09.481' E	4155 m	10°C
3.	Sampling Pont 2	27°30.004' N& 88°09.478' E	4160m	10°C
4.	Sampling Point 4	27°30.012' N & 88°09.449' E	4190m	11°C
5.	Sampling Point 5	27°29.593' N 88°09.439' E	4183 m	11°C
6.	Sampling Point 6	27°29.574' N & 88°09.45' E	4186m	13°C
7.	Sampling Point 7	27°29.572' N & 88°09.469' E	4168m	10°C

GPS Data: The data was measured by GPS MAP 78S. *The atmospheric temperature value mentioned here is of as measured at 22th December, 2013,12:37 p.m.

2. Physicochemical Analysis of the water samples.

Physicochemical analysis consists of two parts:-

Part A – Physical Analysis: These are many parameters which are used to describe the physical state of the water e.g., Temperature, pH, Temperature etc. In the present study parameters like Temperature, pH, Conductivity and T.D.S. of water was measured on site by the help of portable Orion benchtop.

It was observed that the *Pokhari* had the highest temperature around 10°C and minimum temperature of 8°C as measured on December, 2013. With respect to pH, it was observed that they ranged from 6.7 – 7.0 whereas for conductivity, the highest conductivity was 28.5µS/cm. The water of Laxmi *Pokhari* showed the Salinity of 20 ppm (Table No. 2).

Table No. 2: Physical Analysis of water

S. No.	Name of the physical test	Millipore Water (Control)	Water Sample
1.	Temperature	25°C	10
2.	pH	6.9	6.7
3.	Salinity (ppm)	Nil	1.3
4.	Conductivity (µS/cm)	Nil	28.5
5.	T.D.S. (mg/lt.)	Nil	0 ppm

Physical Analysis of water: Water sample were collected from *Laxmi Pokhari*. The data was measured by Orion Digital 5 star pH benchtop. The data presented here is the average of the triplicates. The Temperature mentioned here is as of the measured on 22 December 2013.

Part B: Chemical Analysis: The HiMedia Kit was used to determine the chemical properties of the water. It was observed that the Nitrate and Sulfite concentration was maximum compared to other non metals. (Table No. 3)

Among the various chemicals found at *Laxmi Pokhari*; 0.5 ppm Fluoride, 100 ppm Nitrite, 30 ppm Chloride, 500 ppm Sulphite and 0.3ppm Iron whereas Alkalinity, Total hardness, $CaCO_3$, and Orthophosphate dissolved in sample water was below detection limit.

Table No. 3: Chemical Analysis of water

Sno.	Name of the Chemical test	Millipore Water (Control)	Water Sample *Laxmi Phokhari*
1.	Fluoride Test (ppm)	Nil	0.5
2.	Sulfite Test (ppm)	Nil	500
3.	Nitrate Test (ppm)	Nil	100
4.	Chloride Test (ppm)	Nil	30
5.	Total Hardness Test (ppm)	Nil	0
6.	Orthophosphate Test (ppm)	Nil	0
8.	Alkalinity Test (ppm)	Nil	0

Chemical Analysis of water: The data was measured by AQUA CHECK kit (HiMedia, Mumbai, India) and concentration of various chemicals were in parts per million (ppm).

3. Microbiological Observations.

Enumeration of microbial load by Total Plate Count Method:-

The microbial load in the water, food and dairy products are enumerated by Total Plate Count Method in Plate Count Agar. Initial sample was diluted from 10^1 to 10^5 in 0.85% saline. Then, from each dilution 0.1 ml of sample was spread plate in Plate Count Agar. After incubation total microbial count in form of viable bacterial colonies were enumerated. Plates showing more than 300 colonies were rejected as TNTC (Too Numerous To Count) and plates with less than 20 colonies were also rejected as TFTC (Too Few To Count). Plates with colonies ≥20 colonies and ≤300 colonies were accepted for enumeration. It was found that, *Laxmi pokhari* had 4.76 X 10^{-5} viable cells/ml or microbial load.

3.1. General Colony Morphology

The isolates were analyzed on the basis of their pigmentation and shape of the colony formed on the LB plate. The isolates L1, L2, L3, L4, L5, L6, L7, L10, L12, L15, L16, L18, L21, L22, L25, L28 and L29 form white colonies, whereas L8 form yellowish white colonies, L23, L24, L26, L27, L30 and L31form golden yellow colonies, whereas LL9 deep blooded colony, whereas L14 form off white colony (Table No. 4). The isolate L11 form yellow concentric at center, whereas L13 yellowish at center. The isolate T4 formed yellow colored colony. The colony formed by L11 was smallest in size compared to others with only 2mm as colony diameter size whereas L4 and L7 formed large colonies of 6mm in diameter size. Most of the colonies were smooth and elevated.

To understand the physical morphology of the bacteria isolated from *Pokhari*, Gram staining was performed and it was observed that the eleven isolates were gram negative, out of which 10 isolates were coccus and one isolate L8 was rod shaped. Twenty isolates were gram positive, out of them 18 isolates were coccus and 2 isolates (L16 and L17) were rod shaped (Table 4). Also, nine isolates

shown single cell arrangement whereas 13 isolates shown chain and 10 isolates shown cluster arrangement.

Table No: 4: Morphological data

Isolates	Shape	Arrangement	Gram Nature	Motility	Spore
L1	Coccus	Single	+	+	-
L2	Coccus	Single	-	+	-
L3	Coccus	Chain	+	+	+
L4	Coccus	Cluster	+	+	+
L5	Coccus	Chain	+	+	+
L6	Coccus	Single	+	+	-
L7	Coccus	Single	+	+	-
L8	Rod	Cluster	-	+	+
L9	Coccus	Cluster	+	+	+
L10	Coccus	Chain	+	+	-
L11	Coccus	Chain	-	+	-
L12	Coccus	Chain	-	+	-
L13	Coccus	Chain	+	+	+
L14	Coccus	Single	+	+	-
L15	Coccus	Cluster	+	+	+
L16	Rod	Cluster	+	+	-
L17	Rod	Cluster	+	+	-
L18	Coccus	Chain	+	+	-
L19	Coccus	Chain	+	+	+
L20	Coccus	Chain	+	+	-
L21	Coccus	Cluster	+	+	-
L22	Coccus	Chain	-	+	+
L23	Coccus	Cluster	-	+	-
L24	Coccus	Cluster	+	+	+
L25	Coccus	Chain	+	+	-
L26	Coccus	Chain	+	+	+
L27	Coccus	Chain	-	+	-
L28	Coccus	Single	-	+	-
L29	Coccus	Single	-	+	-
L30	Coccus	Single	-	+	+
L31	Coccus	Single	-	+	-

Table No. 5. Morphology data: Bacterial isolate were observed under Olympus compound microscope with/without Gram staining. Similarly for spore formation and motility were checked under compound microscope.

3.2 Broth Morphology

All the isolates formed heavy clouding but none of them formed sediments when grown in broth conditions (Table No. 5).

Table No. 5. Morphology data

Isolates	Surface Growth	Clouding	Sediment
L1	Ring	Slight	None
L2	None	Slight	None
L3	Ring	Slight	None
L4	Ring	Slight	None
L5	Ring	Slight	None
L6	Ring	Slight	None
L7	Ring	Slight	None
L8	Ring	Slight	None
L9	None	Slight	None
L10	None	Slight	None
L11	Ring	Slight	None
L12	Ring	Slight	None
L13	Ring	Slight	None
L14	Ring	Heavy	None
L15	None	Slight	None
L16	Ring	Slight	None
L17	Ring	Slight	None
L18	Ring	Slight	None
L19	Ring	Heavy	None
L20	Ring	Slight	None
L21	Ring	Slight	None
L22	Ring	Slight	None
L23	Ring	Heavy	None
L24	Ring	Slight	None
L25	Ring	Slight	None
L26	Ring	Slight	None
L27	Ring	Slight	None
L28	Ring	Heavy	None
L29	Ring	Slight	None
L30	Ring	Slight	None
L31	Ring	Slight	None

Morphology data: The data was observed as per the growth in agar and broths.

4. Identification of bacterial samples

4.1. Carbohydrate Fermentation Test:--

According to Bergey's classification of bacteria, carbohydrate utilization is an important characteristic feature of bacteria through which differentiation and group classification can be done. Thus, carbohydrate fermentation test was carried out with 14 different carbohydrates (arabinose, galactose, lactose, maltose, rhamnose, xylose, cellobiose, inisitol, trehalose, dextrose, fructose, sucrose, Melibiose and sorbitol) and each of them were substituted and checked as a carbohydrate substrate for the different isolates. It was observed that out of 31 isolates 10 isolates (L2, L4, L8, L9, L12, L14, L18, L21, L22 and L31) were galactose positive and rest 21 isolates were negative (Table No. 6), one isolate (L7) was arabinose positive and others were negative, 13 isolates (L1, L5, L13, L15, L16, L19 and L24-L30) were galactose negative, whereas 6 isolates were lactose positive (L17, L18, L14, L26, L27 and L30), 9 isolates (L13, L15, L16, L19, L24, L25 and L28-L30) fructose negative, 14 isolates (L5, L6, L13, L15-L17, L19, L23-L25 and L27-L30) mannose negative, 12 isolates (L1, L13, L15-L17, L19, L24, L25 and L27-L30) maltose negative, 12 isolates (L1, L13, L16, L17, L19, L20, L24, L25 and L27-L30) sucrose negative, 13 isolates (L1, L5, L13, L15-L17, L19, L24, L25 and L27-L30) rhamnose negative, 17 isolates (L1, L5, L6, L8, L13-L20, L25 and L27-L30) inisitol negative, 8 isolates (L1, L16, L17, L20, L25 and L28-L30) mellibiose negative, 9 isolates (L1, L16, L17, L19, L20, L25 and L28-L30) mennitol negative, 11 isolates (L2, L4, L9, L18, L19, L21-L24, L26 and L31) cellobiose positive and finally 9 isolates (L1, L5, L16, L17, L19, L20 and L28-L30) were xylose negative.

Table No. 6: Carbohydrate Fermentation Data:

Carbohydrate	Isolates								
	L1	L2	L3	L4	L5	L6	L7	L8	L9
D (-) Arabinose	–	–	–	–	–	–	+	–	–
D (+) Dextrose	–	+	–	+	–	–	–	+	+
D (-) Fructose	+	+	+	+	+	+	+	+	+
D (+) Galactose	–	+	+	+	–	+	+	+	+
D (+) Lactose	–	–	–	–	–	–	–	–	–
D (+) Mannose	+	+	+	+	–	–	+	+	+
D (+) Maltose	–	+	+	+	+	+	+	+	+
D (+) Rhamnose	–	+	+	+	–	+	+	+	+
D (+) Sucrose	–	+	+	+	+	+	+	+	+
D (+) Xylose	–	+	+	+	–	+	+	+	+
D (+) Cellobiose	–	+	–	+	–	–	–	–	+
D (+) Melibiose	–	+	+	+	+	+	+	+	+
D (-) Mannitol	–	+	+	+	+	+	+	+	+
D (-) Inositol	–	+	+	+	–	–	+	–	+

Carbohydrates	Isolates								
	L10	L11	L12	L13	L14	L15	L16	L17	L18
D (-) Arabinose	–	–	–	–	–	–	–	–	–
D (+) Dextrose	+	–	+	–	+	–	–	–	+
D (-) Fructose	+	+	+	–	+	–	–	+	+
D (+) Galactose	+	+	+	–	+	–	–	+	+
D (+) Lactose	–	–	–	–	–	–	–	+	+
D (+) Mannose	+	+	+	–	+	–	–	–	+
D (+) Maltose	+	+	+	–	+	–		–	+
D (+) Rhamnose	+	+	+	–	+	–	–	–	+
D (+) Sucrose	+	+	+	–	+	+	–	–	+
D (+) Xylose	+	+	+	+	+	+	–	–	+
D (+) Cellobiose	–	–	–	–	–	–	–	–	+
D (+) Melibio-se	+	+	+	+	+	+	–	–	+
D (-) Mannitol	+	+	+	+	+	+	–	–	+
D (-) Inositol	+	+	+	–	–	–	–	–	–

Carbohydrates	Isolates								
	L19	L20	L21	L22	L23	L24	L25	L26	L27
D (-) Arabinose	–	–	–	–	–	–	–	–	–
D (+) Dextrose	–	–	+	+	–	–	–	–	–
D (-) Fructose	–	+	+	+	+	–	–	+	+
D (+) Galactose	–	+	+	+	+	–	–	–	–
D (+) Lactose	–	–	–	–	–	+	–	+	+
D (+) Mannose	–	+	+	+	–	–	–	+	–
D (+) Maltose	–	+	+	+	+	–	–	+	–
D (+) Rhamnose	–	–	+	+	+	–	–	+	–
D (+) Sucrose	–	–	+	+	+	–	–	+	–
D (+) Xylose	–	–	+	+	+	+	+	+	+
D (+) Cellobiose	+	–	+	+	+	+	–	+	–
D (+) Melibiose	+	–	+	+	+	+	–	+	+
D (-) Mannitol	–	–	+	+	+	+	–	+	+
D (-) Inositol	–	–	+	+	+	+	–	+	–

Carbohydrates	Isolates			
	L28	L29	L30	L31
D (-) Arabinose	–	–	–	–
D (+) Dextrose	–	–	–	+
D (-) Fructose	–	–	–	+
D (+) Galactose	–	–	–	+
D (+) Lactose	–	–	+	–
D (+) Mannose	–	–	–	+
D (+) Maltose	–	–	–	+
D (+) Rhamnose	–	–	–	+
D (+) Sucrose	–	–	–	+
D (+) Xylose	–	–	–	+
D (+) Cellobiose	–	–	–	+
D (+) Melibiose	–	–	–	+
D (-) Mannitol	–	–	–	+
D (-) Inositol	–	–	–	+

Carbohydrate Fermentation Data: The data was analyzed by the biochemical changes of the culture broths observed in the form of color change. The entire individual sugar/carbohydrate fermentation test has been carried out in triplets. The result shown here is on the basis of the observation of acid production or reduction of phenol red as per the triplets.

4.2. Enzyme Activity:-

The further key to identify a bacterium and distinguish it from the others, is the detection of various enzyme activities of the isolates. Depending on the enzyme activity, they can be classified into their respective groups as per the Bergey's Systemic Classification.

Isolates L1, L2, L3, L4, L5, L6, L9, L10, L12, L13, L14, L15, L20, L21, L22, L23, L24, L25, L26, L29, L30 and L31 were catalase positive whereas L1, L5, L8, L9, L10, L11, L12, L14, L15, L17, L20, L23, L24, L25, L26, L27, L29 and L31 isolates showed protease positive and finally none of the isolates showed amylase activity (Table No. 7).

Table No. 7: Enzyme Activity Data

Isolates	Catalase Activity	Protease Activity	Amylase Activity
L1	+	+	–
L2	+	–	–
L3	+	–	–
L4	+	–	–
L5	+	+	–
L6	+	–	–
L7	–	–	–
L8	–	+	–
L9	+	+	–
L10	+	+	–
L11	–	+	–
L12	+	+	–
L13	+	–	–
L14	+	+	–
L15	+	+	–
L16	–	–	–

L17	–	+	–
L18	–	–	–
L19	–	–	–
L20	+	+	–
L21	+	–	–
L22	+	–	–
L23	+	+	–
L24	+	+	–
L25	+	+	–
L26	+	+	–
L27	–	+	–
L28	+	–	–
L29	+	+	–
L30	+	–	–
L31	+	+	–

Enzyme Activity Data: The data was analyzed by the biochemical changes of the agar plates observed in the form of color change. The entire individual enzyme activity test has been carried out in triplets. The result shown here is on the basis of the observation as per the triplicates.

5. Growth profile at various Physical parameters

5.1. Temperature Dependent Growth Profile:-

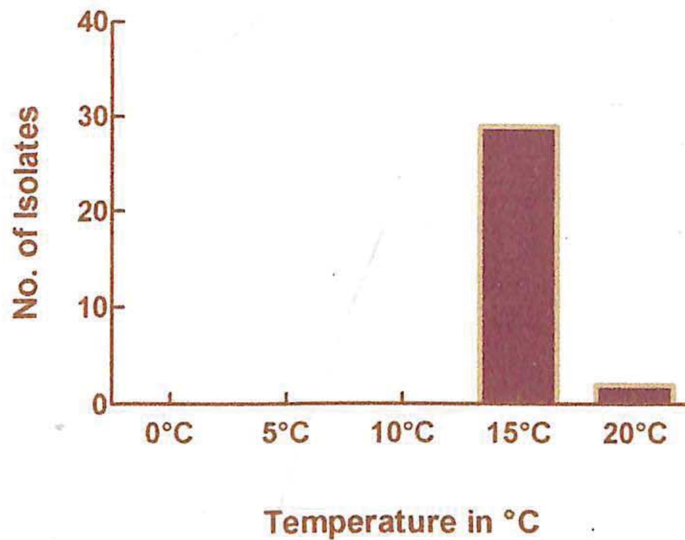
Laxmi Pokhari isolates were investigated for their growth at various temperature ranges so as to find out the optimum temperature required for their growth. All the isolates showed good growth at 15°C (Table No. 8), but among all the isolates, L25 isolate showed the highest growth at 15°C and thus it might be the optimum temperature for the bacteria. For other isolates it was found that they could tolerate a wide range of temperature from 0°C to 20°C as they showed growth within this range but the optimum temperature requires further investigation.

Table 8. Temperature dependent growth profile

	Temperature				
Isolates	0°C	5°C	10°C	15°C	20°C
L1	0.209	0.445	0.332	**0.976**	1.154
L2	0.377	0.691	0.696	**1.757**	1.109
L3	0.346	0.372	0.695	1.588	**1.787**
L4	0.137	0.346	0.625	**1.564**	1.181
L5	0.064	0.082	0.617	**1.636**	0.802
L6	0.103	0.368	0.505	**1.729**	0.849
L7	0.115	0.302	0.26	0.118	**0.826**
L8	0.128	0.238	0.397	**1.554**	1.067
L9	0.145	0.413	0.512	**1.647**	0.083
L10	0.097	0.13	0.112	**1.559**	0.689
L11	0.129	0.376	0.918	**1.853**	0.717
L12	0.172	0.656	0.5088	**1.499**	0.776
L13	0.083	0.079	0.322	**1.918**	0.837
L14	0.146	0.317	0.507	**1.868**	1.28
L15	0.113	0.444	0.514	**1.906**	1.206
L16	0.172	0.449	0.695	**1.791**	1.393
L17	0.179	0.254	0.486	**1.717**	1.09
L18	0.147	0.423	0.515	0.515	**1.274**
L19	0.192	0.252	0.414	**1.585**	1.054
L20	0.189	0.405	0.349	**1.592**	0.955
L21	0.433	0.852	0.774	**1.771**	1.224
L22	0.445	0.845	1.182	**2.113**	1.146
L23	0.458	0.574	0.678	**2.167**	1.035
L24	0.489	1.009	1.266	**2.137**	1.446
L25	0.443	0.832	1.363	**2.302**	1.131
L26	0.441	0.525	0.654	**2.108**	0.905
L27	0.477	0.774	0.773	**2.108**	1.085
L28	0.477	0.791	0.869	**2.087**	0.718
L29	0.466	0.732	0.941	**2.071**	0.911
L30	0.502	0.371	0.723	**1.523**	1.109
L31	0.364	0.199	1.078	**2.04**	0.925

Table 8. Temperature Dependent Growth Profile. Growth of the isolates were measured at 600 nm in terms of Optical Density (O.D) with UV/Visible spectrophotometer. The data shown here represents the mean average value. Numbers in bold letters indicates maximum growth. Similar results were also obtained at 660 nm.

Graph 3



Graph: 3.Temperature Dependant growth profile of isolates

9.2. NaCl Dependent Growth Profile:-

Saline conditions are most important in deciphering the physiology of the bacteria. A halophilic bacterium requires high concentration of saline and this same characteristic feature was investigated for the isolates isolated from the *Laxmi Pokhari.* The maximum isolates showed good growth when grown at 2.5% NaCl condition, whereas seven isolate show good growth at 0% NaCl or negligible amount of NaCl concentration for its optimum growth conditions (Table No. 9).

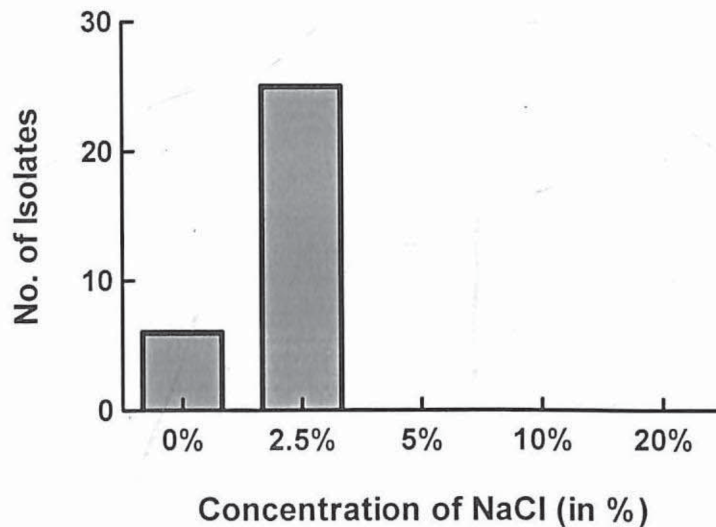
Table 9. NaCl Dependent Growth Curve.

Isolates	Concentration of NaCl				
	0%	2.5%	5%	10%	20%
L1	**0.381**	0.134	0.229	0.079	0.018
L2	**0.6215**	0.5656	0.5334	0.0945	0.103
L3	0.617	**0.697**	0.501	0.103	0.081

L4	0.626	**0.886**	0.398	0.081	0.178
L5	0.478	**0.779**	0.476	0.081	0.19
L6	**0.692**	0.588	0.457	0.087	0.273
L7	0.531	**0.735**	0.476	0.091	0.581
L8	0.638	**0.823**	0.491	0.106	0.538
L9	0.749	**0.994**	0.663	0.097	0.718
L10	**0.767**	0.437	0.229	0.075	0.689
L11	0.725	**0.968**	0.475	0.088	0.721
L12	**0.656**	0.637	0.388	0.089	0.615
L13	0.734	**1.022**	0.438	0.154	1.036
L14	0.745	**0.934**	0.383	0.368	0.319
L15	**0.849**	0.657	0.322	0.106	0.243
L16	0.753	**0.89**	0.468	0.176	0.693
L17	0.535	**0.907**	0.376	0.092	0.483
L18	0.688	**1.196**	0.702	0.089	0.697
L19	0.741	**1.186**	0.686	0.088	0.684
L20	0.694	**0.659**	0.368	0.095	0.558
L21	0.722	**1.265**	0.602	0.117	0.652
L22	0.817	**1.307**	0.617	0.108	0.629
L23	0.686	**0.783**	0.607	0.099	0.474
L24	0.348	**0.836**	0.707	0.101	0.651
L25	0.727	**0.933**	0.564	0.104	0.502
L26	0.662	**1.099**	0.697	0.103	0.503
L27	0.637	**1.148**	0.65	0.095	0.654
L28	0.783	**1.103**	0.977	0.094	0.503
L29	0.663	**1.185**	0.959	0.104	0.653
L30	0.989	**0.992**	0.988	0.105	0.093
L31	0.83	**1.391**	0.755	0.11	0.661

Table 9. NaCl Dependent Growth Profile: Growth of isolates were measured at 600 nm in terms of Optical Density (O.D) with UV/Visible spectrophotometer. The data shown here represents the mean average value. Numbers in bold letters indicates maximum growth. Similar results were also obtained at 660 nm.

Graph 1



Graph: 1. NaCl Dependant growth profile of isolates

9.3. pH Dependent Growth Profile:-

pH conditions are also important in deciphering the physiology of bacteria. Depending on the bacterial optimum pH conditions, they are classified as Acidophiles and Alkaliphiles. Acidophiles requires acidic conditions and Alkaliphiles requires basic conditions. This same characteristic feature was investigated for the isolates of *Laxmi Pokhari*.

It was found that 10 isolates (L25, L27, L28, L15, L16, L18, L19, L20, L21 and L22) required pH 5.0 for its highest growth and 8 isolates (L2, L3, L7, L10, L13, L23, L30 and L31) show good growth at pH 6.0, which suggest that possible these isolates are acidophiles. It was observed that only 2 isolates (L17 and L1) show good growth at pH 9.0 and thus can be considered as alkaliphiles (Table No. 10). Further, it was found that one isolate (L6) show good growth at pH 7.0, whereas 7 isolates (L4, L5, L8, L9, L12, L14 and L24) preferred pH 8.0 for its growth.

Table 10. pH Dependent Growth Profile.

Isolates	pH 3	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9
L1	0.089	0.074	0.215	0.894	0.917	0.754	**1.213**
L2	0.189	0.745	1.076	**1.503**	0.964	1.315	1.364
L3	0.171	0.606	0.782	**1.379**	1.004	1.243	1.091
L4	0.116	0.087	0.089	1.238	0.771	**1.624**	1.148
L5	0.025	0.008	0.031	0.121	0.603	**1.183**	0.417
L6	0.059	0.026	0.037	0.495	**0.876**	0.393	0.364
L7	0.175	0.827	0.967	**1.379**	0.995	1.489	1.314
L8	0.152	0.516	0.888	1.419	1.114	**1.664**	1.199
L9	0.356	1.161	1.223	1.371	1.412	**1.598**	1.154
L10	0.429	1.393	1.33	**1.719**	1.236	1.628	1.318
L11	**1.628**	1.318	1.184	1.498	1.348	1.334	1.343
L12	0.124	0.444	0.901	**1.354**	1.137	1.43	1.179
L13	0.173	0.508	0.859	1.491	1.215	0.061	**1.507**
L14	0.328	1.56	1.373	1.238	1.228	**1.698**	1.342
L15	0.137	1.199	1.359	0.271	0.798	**1.465**	1.214
L16	0.239	1.384	**1.753**	1.324	0.998	1.383	1.526
L17	0.161	1.13	1.204	1.315	0.986	1.276	**1.395**
L18	0.148	1.631	**1.686**	1.502	0.978	1.444	1.318
L19	0.146	1.267	1.273	**1.529**	0.842	1.443	1.354
L20	0.131	0.851	**1.592**	1.367	0.777	1.196	1.285
L21	0.145	1.122	**1.291**	1.254	0.813	1.238	1.131
L22	0.178	1.365	**1.539**	1.419	1.228	1.381	1.316
L23	0.131	0.584	0.728	1.206	1.110	**1.231**	1.118
L24	0.184	1.486	1.188	1.179	1.298	**1.547**	1.278
L25	0.1634	1.235	**1.517**	1.186	0.909	0.896	1.351
L26	0.263	**1.514**	1.303	1.316	1.067	1.378	1.081
L27	0.158	0.643	**1.443**	1.197	0.882	1.384	0.587
L28	0.176	1.502	**1.781**	1.127	1.253	1.336	1.355
L29	0.345	1.537	1.373	**1.397**	1.243	1.395	1.351
L30	0.247	1.332	1.271	**1.626**	1.346	1.481	1.48
L31	0.348	0.756	1.555	**1.777**	1.392	1.597	1.469

Table 10. pH Dependent Growth Profile: Growth of isolates were measured at 600 nm in terms of Optical Density (O.D) with UV/Visible spectrophotometer. The data shown here represents the mean average value. Numbers in bold letters indicates maximum growth. Similar results were also obtained at 660 nm.

Graph 2

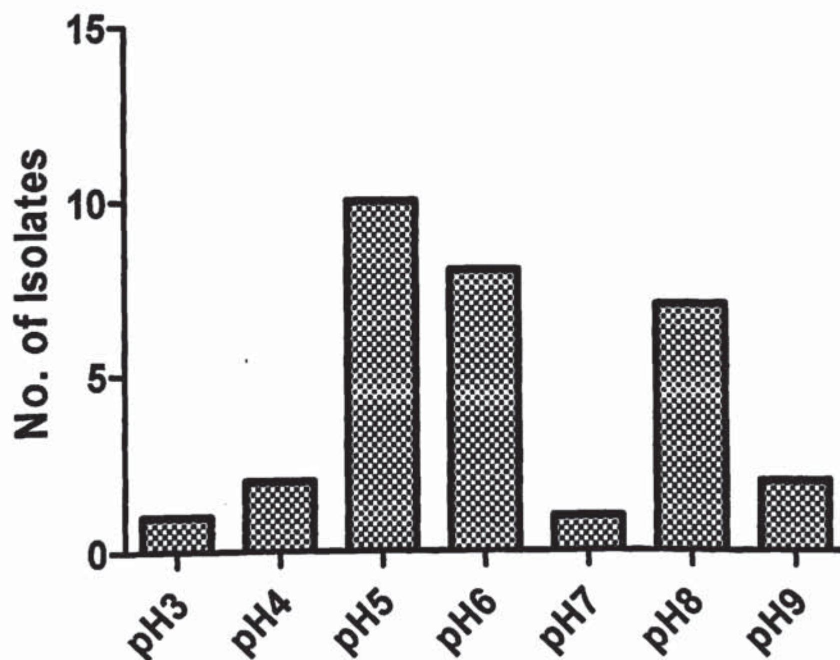


Fig: 2. pH Dependant growth profile of isolates

10. Antibiotic Sensitivity Test:- (Table No. 7.7)

27 isolates were resistant and 5 isolates showed sensitive response to the Ampicillin antibiotics at the concentration of 25 mcg, whereas 18 isolates showed resistant, 6 isolates were sensitive and only 4 isolates showed intermediate response or resistance to the Amoxicillin concentration of 30 mcg. 10 isolates showed resistant, 15 isolates show sensitive and only 6 isolates showed intermediate response or resistance to the Chloramphenicol antibiotic concentration of 30 mcg, but the 18 isolates show resistant, 5 isolates were sensitive and only 9 isolates were intermediate response to the antibiotic Erythromycin antibiotic concentration of 15 mcg. 10 isolates showed sensitive, 14 isolates were intermediate and only 4 isolates were resistant to the Tetracycline antibiotic used in the Antibiotic Sensitivity Test.

Table 7.7: Antibiotic Sensitivity test:-

	Amp 25	Amx 30	C30	E15	TE30
Isolates	Z.I (Int)	Z.I (Int)	Z. I (Int)	Z. I (Int)	Z. I (Int)
L1	0 (R)	18 (S)	20 (R)	0 (R)	23 (S)
L2	0 (R)	0 (R)	34 (S)	23 (S)	28 (I)
L3	0 (R)	0 (R)	28 (I)	12 (S)	28 (I)
L4	21 (R)	32 (S)	34 (S)	18 (I)	>40 (S)
L5	>40 (S)	>40 (S)	27 (I)	40 (S)	>40 (S)
L6	31 (S)	>40 (S)	16 (I)	22 (I)	40 (S)
L7	14 (R)	0 (R)	13 (I)	11 (R)	33 (I)
L8	0 (R)	0 (R)	36 (S)	14 (I)	14 (R)
L9	29 (R)	0 (R)	0 (R)	0 (R)	28 (I)
L10	>40 (S)	16 (I)	0 (R)	0 (R)	29 (S)
L11	0 (R)	14 (I)	12 (R)	0 (R)	26 (I)
L12	18 (R)	0 (R)	36 (S)	19 (I)	32 (I)
L13	0 (R)	14 (I)	0 (R)	0 (R)	28 (I)
L14	0 (R)	14 (I)	34 (S)	0 (R)	26 (I)
L15	>40 (S)	0 (R)	26 (I)	0 (R)	28 (I)
16	0 (R)	21 (S)	15 (I)	0 (R)	30 (R)
L17	0 (R)	0 (R)	40 (S)	0 (R)	30 (R)
L18	0 (R)	14 (I)	29 (S)	0 (R)	28 (I)
L19	0 (R)	22 (S)	0 (R)	0 (R)	32 (I)
L20	0 (R)	0 (R)	0 (R)	18 (I)	30 (R)
L21	0 (R)	0 (R)	30 (S)	15 (I)	28 (I)
L22	0 (R)	0 (R)	36 (S)	19 (I)	28 (I)
L23	0 (R)	0 (R)	21 (S)	24 (S)	20 (I)
L24	0 (R)	0 (R)	32 (S)	0 (R)	24 (S)
L25	0 (R)	0 (R)	31 (S)	0 (R)	26 (S)
L26	15 (R)	0 (R)	28 (S)	0 (R)	26 (S)
L27	0 (R)	0 (R)	12 (R)	17 (I)	24 (S)
L28	0 (R)	0 (R)	0 (R)	20 (I)	28 (S)
L29	0 (R)	13 (R)	29 (S)	0 (R)	27 (S)
L30	0 (R)	15 (I)	26 (S)	0 (R)	32 (S)
L31	0 (R)	14 (I)	0 (R)	0 (R)	29 (S)

Antibiotic Sensitivity: Data was measured by comparing the Antibiotic Assay values with the Zone Size Interpretative Chart Antibiotics as per CLSI. (Int: Interpretation). Each value is mean of three independent experiments. Z. I (Zone of Inhibition in cm).

11. Detection of *coli – aerogenes* contamination in water.

As water from the *Laxmi Pokhari* is utilized for both drinking and bathing purpose and therefore, it is important to check if these *Pokhari* have presence of fecal contamination. Water from these *Pokhari* was checked for *coli-aerogenes* contamination on Violet Red Bile Agar. It was found few colonies appeared on Violet Red Bile Agar which indicates the presence of *coli-aerogenes* contamination.

12. Detection of *Salmonella sp.* contamination in water.

The water from *Laxmi Pokhari* was also check for pathogenic contamination or the presence of *Salmonella sp.* The water sample was grown on *Salmonella Shigella agar* and it was found that it is free of *Salmonella* Contamination.

Discussions

DISCUSSION

The earth's geology determines the fate and composition of the lakes. Geothermal areas are limited to few places around the world where the volcanic activity is present. Sikkim lies in The Great Himalayan Range, which is considered as a relatively young range of mountain in the world and it nurtures numerous lakes and rivers and most of them are of sacred value to the locals. It is also regarded as a hot spot having huge diversity in flora, fauna and microbial community. On the basis of temperature, the snow cover areas are categorized under cryosphere. The cryosphere are the snow cover area of the alpine region which cover glaciers and lakes in the Himalayan regions. For the present study we selected one alpine Lake of Sikkim, i.e., *Laxmi pokhari* (lake). Although people regard the Himalayan lakes as scared, the *Laxmi Pokhari* stands out among the rest in its sacredness. There are many stories associated with it and in these stories the faith and the respect of the people stays with the lake till date. Since, no literature is available on these lake, we decided to do a preliminary study related to its location or mapping, Physicochemical and Microbiological analysis of these lake.

1. Mapping, Geography and Earth Science of the site:-

Laxmi pokhari is physically located at the base Dzori, West Sikkim between 27°29'58.4" N and 088°09'48.2"E longitude. The elevation of from the sea level was 4145m. West Sikkim district covers an area of about 1166 sq. km. Geyzing is the district headquarters. The characteristic geological feature of this district is that it is entirely of mountainous terrain.

2. Physicochemical Analysis of the water samples.

Although, the cold lake water at *Dzongri* is used for drinking and bathing purpose, the physicochemical analysis in detail is required to know about the dissolved metal and trace elements concentration which will indicate if the water from this lake can be utilized for drinking and bathing.

The Physicochemical parameters used to describe the physical state of the water i.e., temperature and pH. Acidity and basicity of any test substance depends on the most important chemical property describe as pH which is the measure of free hydrogen ions and hydroxyl ions in water. Due to various dissolved chemical constitutes, pH acts as an important indicator of water. The pH of the *Laxmi Pokhari* was observed to be 6.7 which is safe for bathing as per APHA Standards.

The chemical analysis was done by using HiMEDIA water testing kits. The result showed that the presence of fluoride, Nitrite, Chloride, Alkalinity and Suphite. Among the various chemicals found in *Laxmi Pokhar* has an average of 0.5 ppm Fluoride, 100 ppm Nitrite, 30 ppm Chloride, 0 ppm Alkalinity, 0 ppm Total hardness, 500ppm Sulphite, 0ppm $CaCO_3$, 0.3ppm Iron and 0 ppm Orthophosphate dissolved within it. Fluoride compounds are salts of inorganic compounds found in minerals, soil or rocks. Fluoride exposure leads to fluorosis which is a dental degrading disease and might also lead to bone anomalies accompanied by weakening of the bones. Water having high concentration of nitrate can develop symptoms like shortness of breath and blue baby disease especially in children less than six months. The *Pokhari* water, although, the concentrations vary among different elements but all of them fall within the maximum permissible limits and highest desirable limits recommended by WHO for drinking purpose.

3. Microbiological Analysis of *Pokhari*

Lakes in Himalayas meet the need of the people, the economy and religious tourism. However, human interference on lake water may cause the water shortage in a locality and villages far and near, also it may lead to water pollution, as a result of chemical and biological pollutants which are derived from human activities. Further, the microbial communities of any ecosystem are important determinants of its characteristics and therefore, it is very important to

analyze water from Microbiological point of view. In the present study we isolated total 31 isolates from *Laxmi Pokhari*. These isolates were both Gram negative and Gram negative and most of them were coccus whereas as few rod shaped bacteria were also present. According to their morphology, carbohydrate utilization and enzymatic activity, possibly, they can be placed in Group I (L17), Group III (l16), Group V (L3, L4, L5, L9, L13, L15, L19, L24 and L26), Group VI (L1, L6, L10, L14, L20, L21 and L25, Group VII (L7 and L18), Group IX (L8), Group XI (L2, L11, L12, L22, L23, L27, L28, L30 and L31), according to the Bergey's Manual for Bacterial Classification. Since these bacteria were isolated from a psychrophilic lake it was not surprising that the isolates were psychrophilic and most of the isolates grow well at 15°C. They all required low NaCl concentration for their optimum growth. Most of the isolates were (18) were acidophiles whereas as only two isolates were alkaliphiles. They were motile and none of them had protease activity. They were resistant to many antibiotics, however most of the isolates showed intermediate level of sensitivity to Tetracycline and Chloramphenicol antibiotics.

The present studies of *Laxmi Pokhari* preliminary; in nature, however these studies suggest that it is rich in many minerals and chemicals; however none of them is present at alarming level. The microbiological studies showed the maximum number of gram positive bacteria and some were negative bacteria. Most of these isolates were slow growing and can utilize wide variety of carbon source.

Conclusion

CONCLUSION

Sikkim which is considered as "The Blessed Land" harbours several lakes which meets the need of its local people and considered as sacred. In the present study we aimed at studying *Laxmi Pokhari* (a lake situated in west Sikkim) through Physico-chemical and Microbiological analysis. We found that the Sociologically importance of this *Pokhari* is tremendous as it defines the culture of the local people. It binds together the society and the traditional customs of the culture. Also many people had a strong internal belief that any diseased individual can be cured completely with this Holy water. For others it is a customs associated with culture to visit, pray and protect the natural elixir.

This study, being the first in its Physicochemical and Microbiological aspect of study, may have shed light into the understanding of bacterial load and water quality analysis. The pH, temperature, and other chemical analysis of the *Pokhari* water were done and it was found that all of them fall within the maximum permissible limits recommended by WHO for drinking and bathing purpose. In the present study 31 bacterial isolates were isolated and it was found that majority of isolates were Gram positive, coccus, motile and not spore forming. Further, these isolates were mainly acidophiles which require low NaCl concentration and 15^{0}C for optimal growth. Also, most of these isolates were sensitive to different kinds of antibiotics. Although the present study is preliminary in nature, further research such as genetic sequencing has to be done in order to identify the bacterial isolates property.

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Appendices

APPENDIX I

Composition of the used Microbiological Media.

1. Carbohydrate Fermentation Broth

Ingredients	Gms/Liter
Trypticase / Peptone	10.00
Carbohydrate*	5.00
Sodium Chloride	15.00
Phenol Red	0.018

Final pH (at 25°C) 7.3 ± 0.1

2. Luria Bertani Agar

Ingredients	Gms/Liter
Casein enzyme hydrolysate	10.00
Yeast extract	5.00
Sodium Chloride	10.00
Sodium Chloride	5.00
Agar	15.00

Final pH (at 25°C) 7.5 ± 0.2

3. Luria Bertani Broth

Ingredients	Gms/Liter
Casein enzyme hydrolysate	10.00
Yeast extract	5.00
Sodium Chloride	10.00
Sodium Chloride	5.00
Agar	15.00

Final pH (at 25°C) 7.5 ± 0.2

4. Mueller Hinton Agar

Ingredients	Gms/Liter
Beef Infusion	300.00
Casein acid hydrolysate	17.50
Starch	1.50
Agar	17.00

Final pH (at 25°C) 7.3 ± 0.1

5. Plate Count Agar

Ingredients	Gms/Liter
Casein enzymic hydrolysate	5.00
Yeast Extract	2.5
Dextrose	1.00
Agar	15.00

Final pH (at 25°C) 7.0 ± 0.2

6. SS Agar (Salmonella Shigella Agar)

Ingredients	Gms/Liter
Beef extract	5.00
Peptic digest of animal tissue	5.00
Lactose	10.00
Bile salt mixture	8.50
Sodium thiosulphate	10.00
Sodium citrate	8.50
Ferric citrate	1.00
Brilliant green	0.00033
Neutral red	0.025
Agar	15.00

Final pH (at 25°C) 7.4 ± 0.2

7. Trypticase Soy Agar

Ingredients	Gms/Liter
Trypticase	15.00
Soy Peptone	5.00
Sodium Chloride	5.00
Agar	15.00

Final pH (at 25°C) 7.4 ± 0.1

8. Violet Red Bile Agar

Ingredients	Gms/Liter
Peptic Digest of animal tissue	7.00
Yeast Extract	3.00
Lactose	10.00

Bile salts mixture	1.50
Sodium Chloride	5.00
Neutral Red	0.03
Crystal Violet	0.002
Agar	15.00

Final pH (at 25°C) 7.4 ± 0.1

APPENDIX –II

Illustration No.1: Map of Sikkim

Illustration No. 2: Map of Sikkim.

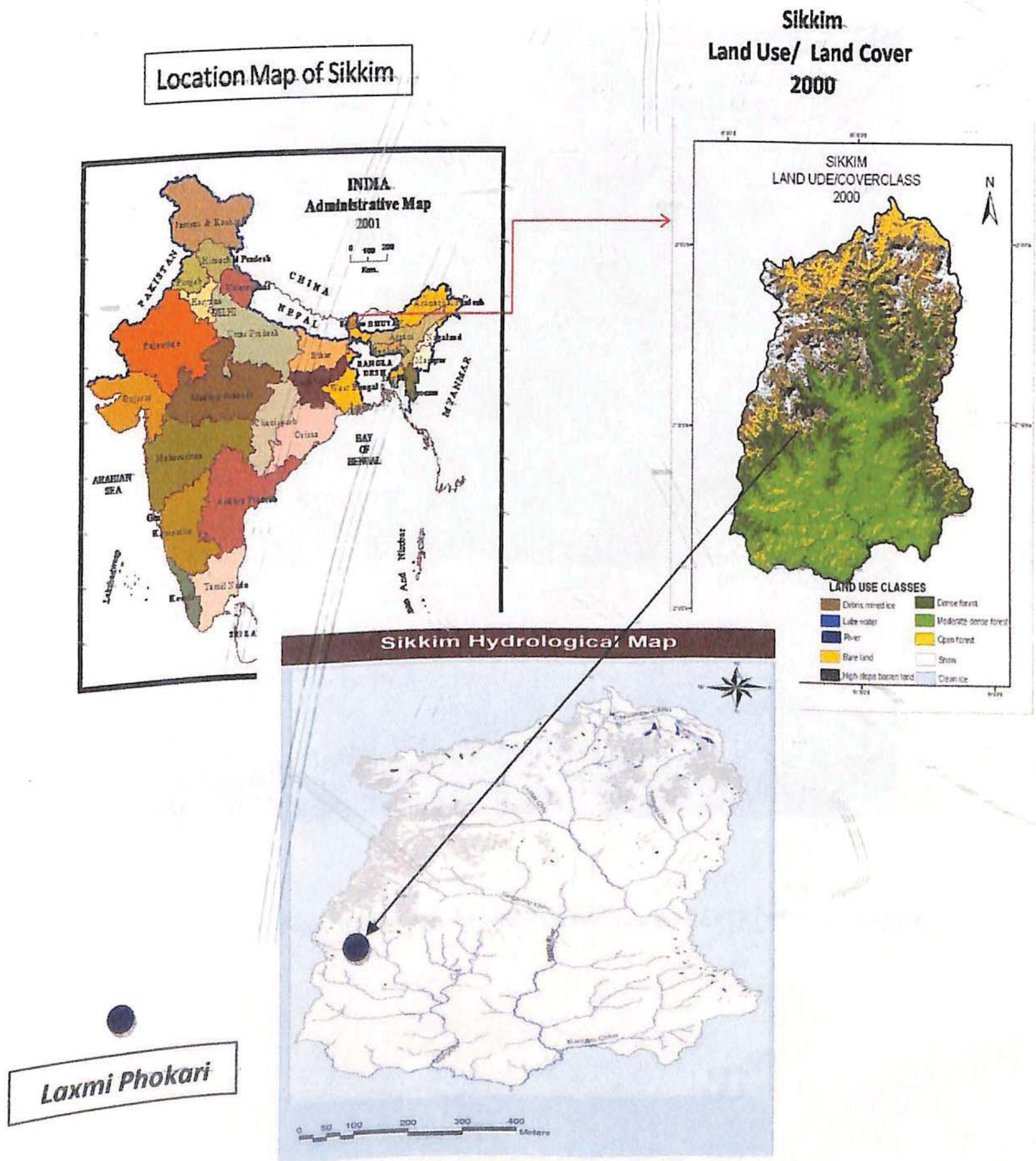

Illustration No. 2: Map showing the Geographical Location of *Laxmi Pokhari* Spot in Sikkim, India

Illustration No. 3: ***Laxmi Pokhari***

Illustration No. 3: Figure showing *Laxmi Pokhari* (*Laxmi Lake*) of west Sikkim, India

Illustration No. 4: Sampling Point of *Laxmi Pokhari*

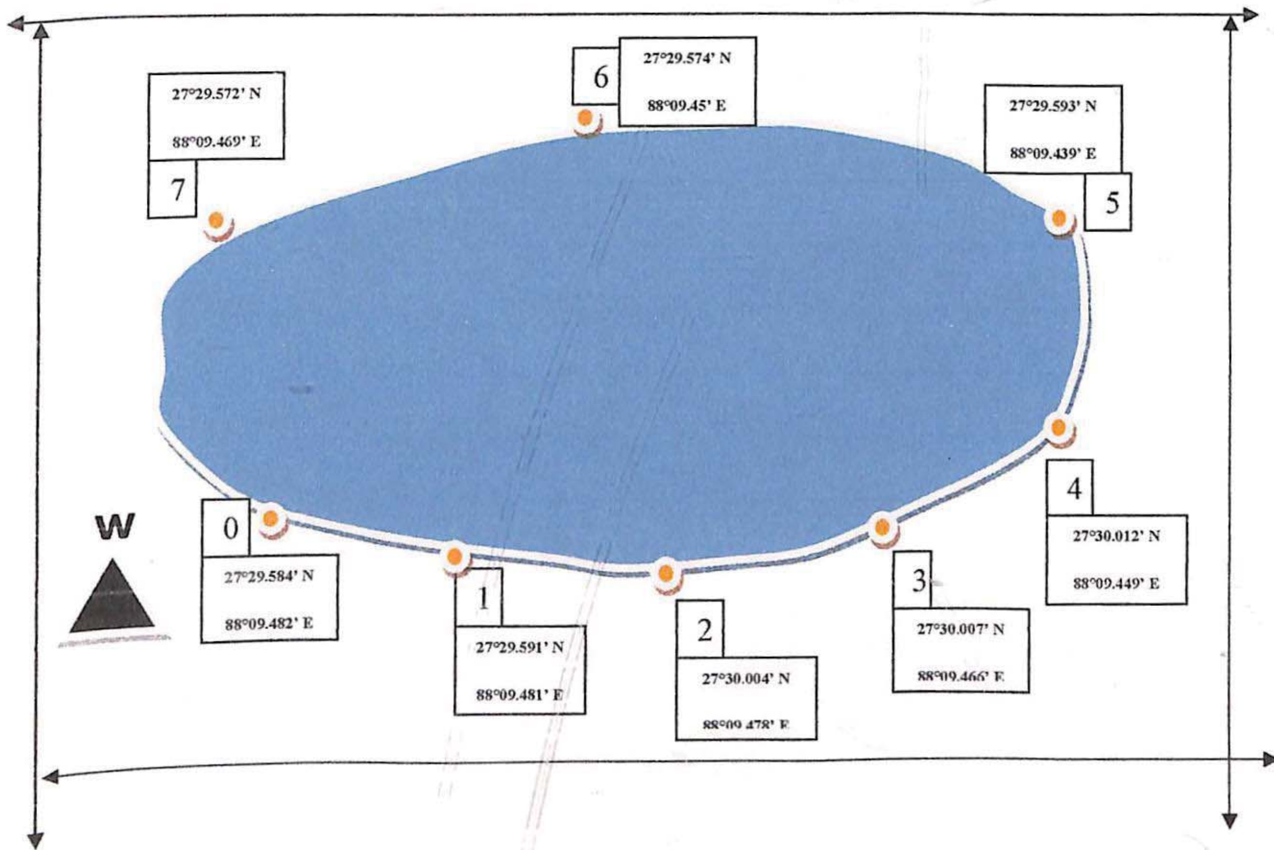

Illustration No. 4: Rough Outline Boundary Sketch of *Laxmi Pokhari*, West Sikkim, India