A DISSERTATION ON

Isolation of polyextremophiles from high altitude hyper saline lake and formation of liposomes from heat-stable lipids

SUBMITTED TO THE
DEPARTMENT OF BIOENGINEERING
FACULTY OF ENGINEERING & INFORMATION TECHNOLOGY
INTEGRAL UNIVERSITY, LUCKNOW

IN PARTIAL FULFILMENT
FOR THE
DEGREE OF MASTER OF TECHNOLOGY
IN BIOTECHNOLOGY

BY
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M. Tech Biotechnology (IV Semester)
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UNDER THE SUPERVISION OF
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DECLARATION FORM

I, **Sujata Sharma**, a student of **M.Tech Biotechnology** (II Year/IV Semester) Integral University have completed my six months dissertation work entitled **"Isolation of polyextremophiles from hyper saline lake and formation of liposomes from heat-stable lipids"** successfully from **Integral University, Lucknow** under the able guidance of **Dr. Roohi.** I, hereby, affirm that the work has been done by me in all aspects. I have sincerely prepared this project report and the results reported in this study are genuine and authentic.

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This is to certify that Ms. **Sujata Sharma** (Enrollment Number 2100102252) has carried out the research work presented in this thesis entitled **"Isolation of polyextremophiles from high altitude hyper saline lake and formation of liposomes from heat-stable lipids"** for the award of **M.Tech Biotechnology** from Integral University, Lucknow under my supervision. The thesis embodies results of original work and studies carried out by the student himself and the contents of the thesis do not form the basis for the award of any other degree to the candidate or to anybody else from this or any other University/Institution. The dissertation was a compulsory part of her **M.Tech Biotechnlogy** degree.

I wish her good luck and bright future.

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I wish him/her good luck and bright future.

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TO WHOM IT MAY CONCERN

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I wish her good luck and bright future.

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Sujata Sharma

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LIST OF ABBREVIATIONS

ABBREVIATIONS	NAME
ATP	Adenosine tri-phosphate
DHABs	Deep hypersaline anoxic basins
Hi RISE	High-Resolution Imaging Science Experiment
PG	Phosphatidylglycerol
PGP-Me	Phosphatidyl glycerophosphate
PGS	Phosphatidylglycerosulfate
PA	Phosphatidic acid
DGD	Glucosyl-diphytanylglycerol
GPCRs	G- protein coupled receptors
SDS	Sodium dodecyl sulphate
LPS	Lipopolysaccharide
NB	Nutrient broth
PBS	Phosphate buffer saline
DDW	Double distilled water
CV	Crystal violet
BAT	Bacillus acidoterrestris
TKHSL	Tso kar hypersaline lake
HEPA	High efficiency particulate air
LB	Luria- bertani
CFU	Colony forming unit
TNTC	Too numerous to count

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1. INTRODUCTION

Extremophile microorganism which can survive in environments of high or low acidity, alkalinity, or temperature. The marine environment covers nearly three-quarters of the planet and it is here that evolution takes its first steps. Bacteria consist of three main groups: extreme, extreme halophiles therefore, many are as extremophiles as they live in extreme environments. In trying to define extreme environments, it became clear that a highly anthropocentric component emerged: Extreme environments are generally considered hostile to higher life forms and uninhabitable by other organisms.

Cold-adapted microorganisms make up the bulk of the Earth's biomass Low temperature environment predominance. Extremely cold environments mainly depend on it microbial activity due to this climate restricts higher plants and animals. Himalayas is one of the major cold environment on Earth, because it shares climate similarities with the Polar Regions. It includes a wide range of ecosystems, from moderate to very cold, spread over the higher elevations height. This region is characterized as a stressful environment due to the high level of exposure from harmful rays, nutritional deficiencies and freezing conditions. Precipitated microorganisms this region is considered cold-tolerant (psychrotolerant) and/or cold-loving (psychrophilic) microorganisms (Dalmaso *et al*, 2015).

To date, few extremophiles have found their way into large-scale use in the field of biotechnology (Elleuche *et al*, 2014); however, their potential is undeniable in many applications. Four success stories are the thermostable DNA polymerases used in the polymerase chain reaction (PCR), various enzymes used in the process of making biofuels (Barnard *et al*, 2010), organisms used in the mining process (Johnson *et al*, 2014), and carotenoids used in the food and cosmetic industries (Oren *et al*, 2010). Other potential applications include making lactose-free milk; the production of antibiotics, anticancer, and antifungal drugs and the production of electricity or, more accurately, the leaching of electrons to generate current that can be used or stored (Dopson *et al*, 2016).

Terrestrial analogues for off-Earth environments provide a natural laboratory for conducting experiments and testing instruments and operational practices for future space exploration missions. In addition to well-known analogue regions (Hipkin *et al*., 2013), efforts are being made to recognize unexplored and less studied parts of the world and introduce relevant sites to the astrobiology community (Pandey *et al*, 2019). It offers a natural setting where a diverse range of mostly near-pristine extreme environments including glacial deposits, arid regions, dune fields and intra dune ponds, hot springs, saline lakes and permafrost regions can be found. Several saline lakes present in Ladakh's high-altitude, rain shadowed region are potential analogues to lakes that existed on early Mars (>3 Ga), formed by evaporation of briny surface lakes (Osterloo *et al*., 2008; Osterloo *et al.*, 2010) and/or ground water upwelling (e.g., Glotch *et al*., 2010).

They are relevant to planetary. geomorphologists studying early Mars and have astrobiology significance in relation to the habitability of groups studying microbial life in briny environments and salt deposits. More recently, chloride salt deposits have been observed by the MRO's High-Resolution Imaging Science Experiment (HiRISE) in the Sinus Meridiani region Opportunity rover's landing site, in relation to a lake formed ca. Tso-Kar region is characterized by extreme climatic conditions. Temperature varies from −40 °C in winter to >30 °C in summer, with extreme diurnal fluctuations, i.e. 30°C (Pandey *et al*, 2019). Mean annual precipitation (rain or snow) is 50%. The lake is inhabited by several species of ostracods with high tolerance to salinity extremes. This lake is situated within the Taglang La Formation of the Tso Moriri Crystalline Complex, assigned as an accreted unit of the ISZ.

OBJECTIVES

a) Isolation and identification of polyextremophiles from high altitude Hypersaline Lake.

b) Extraction of membrane lipids from selected polyextremophiles.

c) Generation of liposomes from heat-stable lipids.

2. REVIEW OF LITERATURE

Cold-adapted microorganisms make up the bulk of the Earth's biomass Low temperature environment predominance. Extremely cold environments mainly depend on it microbial activity due to this climate restricts higher plants and animals. Ladakh is one of them the major cold environment on Earth, because it shares climate similarities with the Polar Regions. It includes a wide range of ecosystems, from moderate to very cold, spread over the higher elevations height. Due to the high stress, this region is considered a stressful environment harmful radiation, nutritional deficiencies and freezing conditions.

The lake at Tso kar is a hypersaline lake with pH 9 and salinity 26%. The pool temperature is 22 ℃ and is situated at N 33º 18.7683′ & E 78º 01.9211′ at an altitude of 4,530 metres and are easily accessible from the road (Bhattacharya *et al*, 1989) as shown in fig.1

Fig 1. **Tso kar lake situated in ladakh**

Extremophiles have many structural and functional adaptations to carry out normal life processes in a stressful environment with low temperature. Their biological activity maintains and contributes to the flow of nutrients in the environment global biogeochemical cycles. There are culturally dependent and limited cultural studies reveal their diversity in community structure and functional potential.

Extremophiles organisms are classified as living organism able sustain and grows rapidly in extreme condition. Extreme condition for example physical parameter such as temperature, pressure, radiation and geochemical parameter such as salinity, pH, and redox potential. Polyextremophile microorganisms are those that can survive in more than one of these extreme conditions.

Extremophile microorganisms are classified according to the extreme environments in which they grow and the major types are summarized in Table 1 (Horikoshi *et al*, 2011).

Table 1. **Extremophiles and their favourable environment.**

S.No.	**Extremophile microorganism**	**Favourable environment to growth**
1.	Acidophile	Optimum pH for growth below 3.
2.	Alkaliphile	Optimum pH for growth –above 10.
3.	Halophile	Requires at least 1M salt for growth.
4.	Hyperthermophile	Optimum growth at temperature above 80°C.
5.	Thermophile	Grows at temperatures between 60° and 80° C.
6.	Psychrotolerant	Grows at temperature above 25° C but also grow below 15°C.
7.	Psychrophiles	Grows at temperature between 10°C and 20°C.
8.	Piezophile	Grows under high pressure- above 400 atm.
9.	Endolithic	Grows inside rock.
10.	Oligotroph	Able to grow in environments of scarce nutrients.
11.	Radioresistant	Tolerance to high doses of radiation.
12.	Metalo-tolerant	Tolerance to high level of heavy metals.
13.	Toxi-tolerant	Tolerates to high concentration of toxic agents
14.	Xerophile	Grows in low water availability.
15.	Hipolith	Grows on rocks and cold dessert.

2.1 Lipids of extremophiles

The interactions between lipid molecules and proteins that take place in the cell membrane are primarily what shape the membrane in general. In addition to being the primary structural elements of the cell membrane, lipids are now known to be essential for a number of crucial cellular processes. Furthermore, membrane lipid shapes and patterns are particularly helpful taxonomically to distinguish and recognise representations of the many domains of phylogenetic trees, as well as between various groups within the same domain. Since the majority of phospholipids and glycolipids in severe

halophiles are anionic, their negatively charged groups would give the halophile membranes a high negative charge density.

2.2 Types of lipids

2.2.1 Neutral Lipid: In the halophilic Archaea of the family Halobacteriaceae, neutral lipids make up around 10% of the total lipid content; they are virtually entirely made up of C_{20}–C_{50} isoprenoids and compounds generated from isoprenoids. The following types have been reported: carotenoids (C_{40}-isoprenoid compounds), bacterioruberins (C_{50}-isoprenoid compounds), quinones, geranylgeraniol (C_{20}-isoprenoid lipid), neutral phytanyl ethers (Kushwaha and Kates, 1978) and C_{30} isoprenoid compounds (squalene, dihydrosqualene, tetrahydrosqualene, dehydrosqualene) (Kushwaha *et al*., 1972), and indole (Kushwaha *et al*, 1977). Quinones may account for about 9% of the total neutral lipid content of the cells (Kamekura and Kates, 1988). The major respiratory quinones in the Halobacteriaceae are MK-8 and MK-8(H2), two menaquinones with eight isoprenoid units.

2.2.2 Polar lipid: The main lipid core of archaeal extreme halophiles, which represents the hydrophobic portion of complex polar membrane lipids, is usually obtained from archaeal lipids by strong-acid methanolysis or acetolysis to remove polar groups such as phosphate esters or sugars (Kates, 1986).

2.2.3 Phospholipid: The phospholipid structures have been shown to be archaeol analogs of: phosphatidylglycerol (PG), methyl ester of phosphatidyl glycerophosphate (PGP-Me), phosphatidylglycerosulfate (PGS) and phosphatidic acid (PA). PGP-Me is the major phospholipid in all extreme halophiles and extreme haloalkaliphiles, having been identified by FAB-MS and TLC in several genera of extreme halophiles, including Halobacterium, Haloarcula, Haloferax, Halococcus, Natronobacterium and Natronococcus (Kates *et al*., 1993).

2.2.4 Glycolipids: Glycolipids have become useful taxonomic markers in the classification of different genera of halophilic Archaea. The structures of the glycolipids appear to be derived from a basic diglycosyl archaeol, mannosyl glucosyl-diphytanylglycerol (DGD), by substitution of sugar or sulphate groups at the 3 or 6position of the mannose residue (Kates *et al*, 1986)

2.2.5 Cardiolipin: Another interesting class of phospholipids consists of dimeric phospho lipids having four chains in the hydrophobic tail. In Bacteria and Eukarya, the major representative of this class is diphosphatidylglycerol, also called cardiolipin (Corcelli *et al*, 2000).

One of the most versatile and effective lipid extraction procedures is that of Bligh and Dyer (1959), as modified for extreme halophiles (Kates, 1986; Kates and Kushwaha, 1995). Briefly, this procedure uses a one phase alcoholic solvent system, namely methanol–chloroform–water (2:1:0.8, v/v) which rapidly and efficiently extracts the lipids; the extract is then diluted with one volume each of

chloroform and methanol–water (1.0:0.9), any water–soluble contaminants are thus readily partitioned into the methanol–water phase, leaving the lipids relatively free of contaminants in the chloroform phase.

Hypersaline lakes and salterns are examples of habitats where dense populations of halophilic microorganisms can provide a vivid red colour. The abundance of microbial life in hypersaline conditions raises a number of important issues regarding the mechanisms of osmoadaptation. The studies of the molecular structures of new lipids required an experimental approach which entailed hydrolysis of the polar head groups, selective cleavage of the chain linkages, identification of the pieces thus produced, and reconstruction of the starting molecule.

2.3 Liposome from extremophilic lipid

Liposomes are small bilayer vesicles build from phospholipid. Special property of these vesicles is defined based on low toxicity, biodegradable and lack of immunogens.

The creation of liposomes is possible using either natural phospholipids (egg or soya) or synthetic lipids like di-oleoylphosphatidylcholine. These treatments cost a lot of money while being quite helpful. The development of an alternative to synthetic liposomes has been the focus of numerous studies (Arzani, P., & Baserisalehi, M. (2020)).

Bacterial lipids are suitable candidates for liposome preparation even though conventional liposomes are unstable, have a brief half-life, and are expensive to produce on a large scale. This is because microorganisms are easily accessible year-round and can grow on a variety of substrates. These microbes can endure extreme pH levels, high pressures, high salt levels, and high temperatures. Compared to esters, ether connections are more resistant to oxidation and high temperatures.

2.4 Extremophiles: current applications

The abundance of organisms (extremophiles) that can survive in harsh settings makes them a great source of substitute enzymes for presently employed in these processes are mesophilic ones. Biotechnology has an unavoidable impact on how we live. Some of these effects, such as the creation of biofuels, are well known. However, there are numerous other uses for enzymes that are not well known outside of the realm of specialists and have an impact on our daily lives. For instance, lactose-free milk and bioinsecticides, how we make and wash our clothes (e.g., cellulose to create "stone-washed" jeans, lipases, and proteases in detergents), and the medications we take to stay healthy are just a few examples.

2.4.1 Industrial Applications

Extremozymes have numerous applications, as their high-temperature optima ensure that they resist degradation at elevated temperatures, with higher productivity and shorter process times (Irwin *et al*, 2020). Glycosyl hydrolases are carbohydrate-degrading enzymes, which have numerous applications in the food and animal feed industries and lignocellulose degradation (Aulitto *et al,* 2019, Cabrera *et al*, 2018). They include α- and β-amylases, glucoamylases, α-glucosidases, and pullulanase, all of which are required to fully digest starch. Cellulases and xylanases are employed in food processing (Elleuche *et al*, 2015). Many of these enzymes have also been modified by site-directed mutagenesis to optimize their catalytic properties (Irwin *et al*, 2020). Proteases are also used in various industries, e.g. alcalase, a protease used to process soy meal, and thermolysin, which is used to produce the artificial sweetener aspartame (Barzkar *et al*, 2018).

2.4.1.1 Proteases/ Lipases

More than 70% of all enzymes are proteases and lipases, along with glycosyl hydrolases, making proteases the most extensively utilised kind of enzyme. Proteases have several uses in a variety of industries, but their most significant use is in laundry detergents, where they have been an essential ingredient since 1985 and are employed to disassemble and remove protein-based stains. Proteases are also widely used in the brewing, baking, and cheese making industries. However, research has been done on employing psychrophilic proteases to improve cold water washing. Due to their poor stability at ambient temperature, the majority of psychrophilic enzymes have shown to be useless (Yang *et al*, 2012).

2.4.1.2 Glycosyl hydrolases and sugar

There are well over 100 families of glycosyl hydrolases, which break down the glycosidic connection between a carbohydrate and another molecule. In most cases, the hydrolysis only requires the utilisation of two amino acids—a proton donor and a nucleophile/base—and the resultant carbohydrate retain or invert its anomeric shape. A lack or loss of -galactosidase activity causes lactose intolerance, which affects around 70% of the world's population. For this vast majority of people, the greatest strategy to prevent the frequently consuming lactose-free milk and other dairy products, which are produced by using the lactase (-galactosidase) from organisms like *Kluyveromyces lactis*, can help alleviate humiliating symptoms of lactose intolerance. However, the temperature of the dairy product must be increased (from around 5 °C to 25 °C) in order for the enzyme to be active. The potential for pathogen growth and flavour changes in milk are both increased by this temperature increase (Messia *et al*, 2007)

2.4.1.3 Amylase

Since the industrial processes involved in hydrolyzing starch require high temperatures(95 °C for one step and 60 °C for the other) and high pH, polyextremophilic (thermophilic and alkaliphilic) enzymes would be ideal. Currently, a α-amylase from *Bacillus acidicola*, glucoamylases from *Picrophilus*, and a pullulanase from *Thermococcus kodakarensis* show great promise in replacing their mesophilic counterparts.

2.4.2 Medical application

Unexpectedly, microorganisms, especially extremophiles, are producers of a variety of antibiotics, antifungals, and anticancer molecules. Different vaccine delivery method is an extremely intriguing contribution from extremophiles to the realm of medicine. Internal gas vesicles, tiny gas-filled proteinaceous structures produced by a variety of microorganisms, are most well-studied in halophilic archaea.

In *Halobacterium* species NRC-1, these structures have been modified to produce a recombinant form that expresses parts of the simian immunodeficiency virus on the outside surface. When administered into mice after collection, these recombinant vesicles demonstrated a potent antibody response and immunological memory.

Adjuvants, such as cholera toxin B, are frequently added to vaccines created by recombinant techniques in order to stimulate a strong adequate immune response.

Membrane-lipid therapy is a novel therapeutic approach aimed at developing drugs to regulate membrane-lipid composition and/or structure. Some insight already exists into the effects of dietary lipids (Gutierrez *et al,* 1996) or certain drugs (e.g. anesthetics and alcohols) (Frangopol *et al*, 2001) on membrane structure and function, but lipids had not previously been considered as targets for drug development. Enzymes and GPCRs constitute the two most common targets for marketed and developing drugs (Swindell *et al,* 2002). Many enzymes and GPCRs, and related proteins, interact with membranes and might be modulated by specific lipid therapies. Because the type and/or composition of membrane lipids are altered in several pathologies and an important number of cellular functions occur within or around membranes, membrane-lipid therapy might have potential use for the treatment of several illnesses (Escribá *et al,* 2006).

2.5 Application of extremophilic lipids in medicine

The unusual properties of lipids found in extremophiles have led to the development of liposomes. Their properties enable the formation of liposomes at any temperature in the physiological range or lower, allowing the encapsulation of thermally labile compounds (Irwin *et al,* 2010) and thus serving as novel agents for drug delivery. They also target mononuclear phagocytes and can be taken up by these cells more readily than can liposomes made from ester lipids. This makes them good candidates

for antigen delivery, as carriers, or as adjuvants that directly stimulate the immune system (Irwin *et al*, 2010).

Lipids derived from halophilic bacteria have found uses as deliverers of drugs and vaccines. Other compounds found in halophilic archaea, such as siderophores, offer iron-chelating agents that can be used to treat iron deficiency diseases or to increase antibiotic activity against bacteria (Babu *et al*, 2015).

3. MATERIALS AND METHODS

3.1 Labwares:

- Micropipette
- Hand gloves
- Rubber bands
- Scissors
- Tape
- Inoculation loop
- Spatula

3.2 Glassware:

a) Petri plates
b) Test tubes
c) Flasks

3.3 Chemicals:-

i. Sodium chloride
ii. Potassium chloride
iii. Magnesium sulphate
iv. Sodium carbonate
v. Potassium phosphate
vi. β – mercaptoethanol
vii. Glycerol
viii. Tris HCl
ix. Sodium dodecyl sulphate
x. Bromophenol blue

3.4 Media preparation:

3.4.1 Nutrient agar: The growth of a diverse spectrum of non-fibrous organisms is supported by the general purpose medium known as nutrient agar. Because it promotes the growth of different kinds of bacteria and fungus and contains many of the nutrients required for bacterial growth, nutritional agar is widely used.

Composition of nutrient agar media:-

Ingredients	**g/L**
Peptone	10.00
Beef extract	10.00
Sodium chloride	5.00

Agar	12.00
pH	7.3 ± 0.1

3.4.2 Nutrient broth: A variety of fastidious and non-fastidious bacteria are grown in nutrient broth, a general-purpose medium with no nutritional needs. Nitrogen molecules, vitamin B complex, amino acids, and other growth-promoting elements are provided via peptone and yeast extract.

Composition of nutrient broth:-

Ingredients	**g/L**
Peptone	10.00
Beef extract	10.00
Sodium chloride	5.00
pH	7.3±0.1
Distilled water	1000

3.4.3 Halophilic agar: Extremely halophilic bacteria can be isolated and grown on halophilic agar. For the isolation and development of extremely halophilic species of Halobacterium and Halococcus from foods halophilic media have been developed. They need a high salt concentration of between 20 and 30% for the best growth.

Composition of halophilic agar:

Ingredients	**g/L**
Casein acid hydrolysate	10.00
Yeast extract	10.00
Peptone	5.00
Sodium citrate	3.00
Potassium chloride	2.00
Magnesium sulphate	25.00
Sodium chloride	250.00
Agar	20.00
Final pH	7.2± 0.2

3.4.4 Crystal violet tetrazolium agar: Gram-negative psychrophilic bacteria that cause food spoiling is found using Crystal Violet Tetrazolium Agar Base. Based on Olson's formulation and the APHA's recommendation. It can identify gram negative psychrotrophic bacteria.

Composition of CV tetrazolium agar:-

Ingredients	**g/L**

Tryptone	5.00
Yeast extract	25.00
Dextrose (glucose)	1.00
Crystal violet	0.001
Agar	15.00
Final pH	7.2 ± 0.2

3.4.5 BAT agar: BAT agar gets its name from *Alicyclobacillus acitoterrestris*, formerly known as Bacillus acidoterrestris (BAT). Glucose is used as an energy and carbon source in BAT agar. The B-group of vitamins, in particular, is found in yeast extract. A buffering technique called potassium dihydrogen phosphate is used.

Composition of BAT agar media

Ingredients	**g/L**
Yeast extract	2.000
Dextrose	5.000
Calcium chloride	0.25066
Magnesium sulphate	0.500
Ammonium sulphate	0.200
Potassium dihydrogen phosphate	3.000
Zinc sulphate	0.00018
Copper sulphate	0.00016
Manganese sulphate	0.00015
Cobalt chloride	0.00018
Boric acid	0.0001
Sodium molybdate	0.0003
pH	4.0±0.2

3.5 Buffers:

3.5.1 2X SDS Buffer: To make SDS buffer 2ml β-mercaptoethanol , 2 gm SDS, 10ml glycerol 0.1 M Tris HCl and 100 µl bromophenol blue was taken and dissolve in 50 ml distilled water.

For 1M Tris HCl

Molecular weight of Tris HCl-157.6

1M Tris HCl = 157.6 gm in 1000 ml

For 0.1 M - 157.6/10 =15.76 gm in 1000 ml

0.1M = 1.57 gm in 100ml

0.1 M= 0.78 gm of Tris HCl in 50 ml DW

3.5.2 Phosphate buffer saline: To make phosphate buffer 2.62 gm potassium phosphate, 0.8 gm sodium carbonate and 0.9 gm NaCl was dissolved in 100 ml distilled water.

4. Methods and Protocols

4.1 Objective 1: Isolation and identification of Polyextremophile from high altitude Hypersaline Lake.

4.1.1 Sample collection

The following high altitudes lake at Ladakh are being proposed for study:

Tso Kar lake: The lake at Tso kar is a hypersaline lake with pH 9 and salinity 26%. The pool temperature is 22 ℃ and is situated at N 33º 18.7683′ & E 78º 01.9211′ at an altitude of 4,530 metres and are easily accessible from the road.

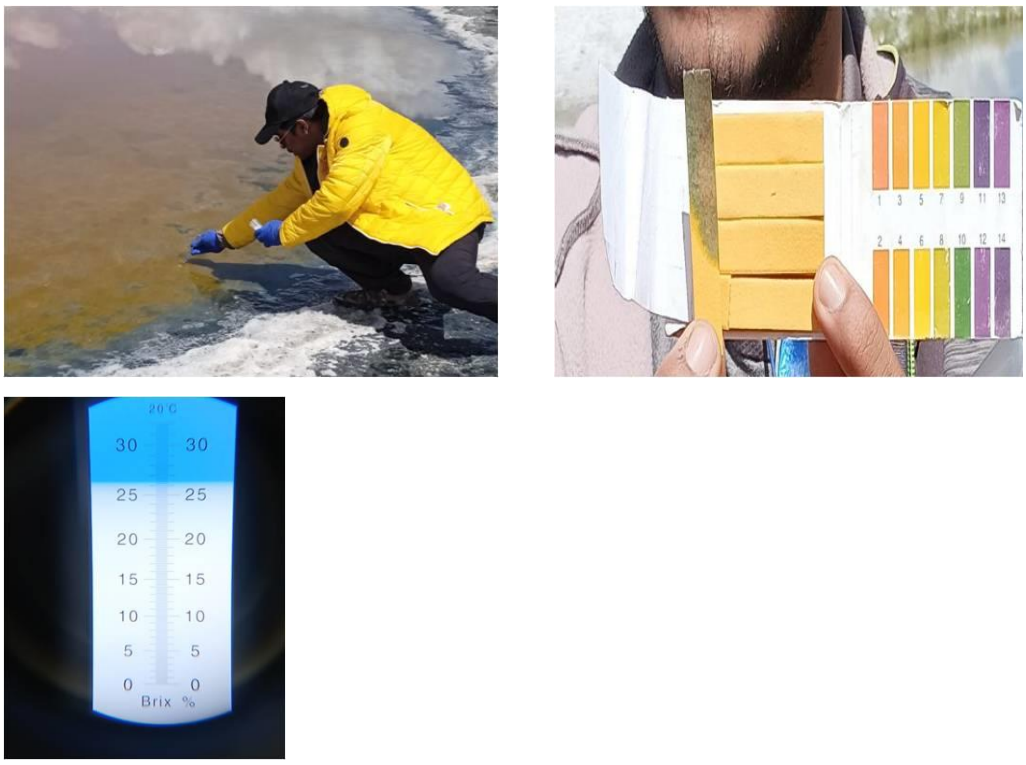


Fig. 2. Collecting soils from Tso kar hypersaline lake with pool temperature 22 °C

4.1.2 Isolation of halophilic bacteria from the soil sample collected from Tso kar Hypersaline Lake at 37 °C.

Colonies formed after enumeration of polyetxremophiles on NA media (6% NaCl) on different dilution of plates will be counted and plates having number of colonies in between 25-250 will be considered for halophiles isolation.

Briefly, nutrient broth media with 6% NaCl is prepared in distilled water, autoclaved, allowed to cool down and poured onto the test tubes. Each colony from the selected plate is picked using inoculating loop and is inoculated in the respective broth and incubated at 37 °C for 2 days.

Nutrient agar media with 6% NaCl is prepared, autoclaved, allowed to cool and poured onto the respective petri plates with the marking.

Culture from the broth is inoculated via cross streaking onto the petri-plates using inoculation loop.

Growth response is observed after 2 days at 37 °C.

4.1.3 Enumeration of bacteria by plate count after serial dilution and incubation agar plate technique

This method is based on the principle that when a material containing bacterium is cultured every viable bacteria develops into a visible colony on a nutrient agar medium.

Dilution = volume of sample/Total volume of sample in diluent

Number of cells/ml = number of colonies/Amount plated× dilution

Label the dilution blanks as10^{-1}, 10^{-2}, 10^{-3},10^{-4},10^{-5},10^{-6} and 10^{-7}. Prepare the initial dilution by adding 1 ml or 1 gm of the sample into a 9 ml dilution blank labelled 10^{-1} thus diluting the original sample 10 times. Mix the content to obtain uniform distribution by cells. From the 1st dilution transfer 1 ml of suspension while in motion to dilution blank 10^{-2} with a sterile and fresh 1ml pipette. From 10^{-2} suspension transfers 1 ml of suspension to 10^{-3} dilution blank. Repeat this procedure till the original sample has been diluted to 10^{-7} vials. From 10^{-4} to 10^{-7} dilutions, transfer 0.1 ml of suspension to sterile petri dishes. The dilution is increased 10 times three petri dishes are to be used.

Add approximately 15ml of nutrient medium, melted and cooled to 45 °C, to each petri plate containing the sample. Mix the content of each plate by rotating gently to distribute the cells throughout the medium. Allow the plate to solidify. Incubate these plates in an inverted position for 24-48 hours at 37 °C.

4.1.4 Identification of psychrophiles on CV agar media with 6% NaCl at 4 ℃

CV agar media was prepared and autoclaved it. Media was pour into each petri plates. Let the media get solidify. Streaking was done in each plates label with sample number and date. After that the plate was kept in incubator for 2 days at 37 °C. Next day it was kept in refrigerator at 4 °C. Plate was observed each day for 9 days.

4.1.5 Isolation of thermoacidophiles using BAT media with 6% NaCl, pH: 5.5

BAT agar media was pour into each petri plates. Let the media get solidify. Streaking was done in each plate with the culture sample**.** Cover the plates with parafilm. Later put each plate into incubator for 2 days at 50°C. Observe the plates for its growth for two days.

4.1.6 Identification of Thermophiles on NA plates with 6% NaCl at 50 ℃

Nutrient agar media was prepared and autoclaved it. It was pour into petri plates. Let the media get solidify. Streaking was done in each plates with the culture sample**.** Cover the plates with parafilm. Later put each plate into incubator for 2 days at 50 °C.

4.1.7 Gram staining of bacteria

It is very useful for identifying and classifying bacteria into two major groups: gram positive and gram negative bacteria. In these process four different reagents in the order listed:

- Crystal violet (primary stain)
- Iodine solution (mordant)
- Alcohol (decolorizing agent)
- Safranin (counter stain)

The bacteria which stain (appears dark blue or violet) i.e. not decolorized when stained with grams method are called gram- positive whereas those that lose the crystal violet and counter stained by safranin (appears red) are referred as gram- negative.

4.2 Objective 2: Isolation of lipids from the selected bacteria

4.2.1 Isolation of lipids

The outer membrane of gram-negative bacteria contains lipopolysaccharide (LPS), which is a low molecular weight carbohydrate with a molecular mass of 10-20 kDa. LPS is a tripartite molecule consisting of lipid A that is embedded in the outer membrane, a core oligosaccharide and repeating O-antigen units that extend outward from the surface of the cell. Lipid A has multiple fatty acids which

serve to anchor LPS into the bacterial membrane, allowing the O antigen and core oligosaccharide to protrude, and contributes to the main part of the toxicity of gram-negative bacteria.

4.2.2 Preparation of Bacteria for LPS Extraction

Grow an isolated culture of bacteria overnight on Nutrient Broth (NB) media with 6% NaCl at 37 ℃. Centrifuge the bacteria growth from nutrient broth media at 2500 x g for 10 minutes to pellet the bacteria.

Decant supernatant, then add 12 ml cold PBS, pH 7.2 and repeat centrifugation. Remove supernatant with the pipette and discard.

Resuspend the pelleted bacteria in 1 ml of 1x SDS-buffer. Ensure that the pellet is completely resuspended through pipetting the solution up and down and slowly. Boil the suspended bacteria in a water bath for 15 minutes.

4.2.3 Extraction of lipid

Allow the solution to cool at room temperature for 15 minutes. The mixture is centrifuged 2 times at 6000 rpm for 15 minutes and the supernatant is transferred to another vial. Take 200 uL of the supernatant and add 800 µL of chloroform: methanol in the ratio 2:1.

Store at -20℃ for overnight. Centrifuge the mix 2 times at 6000 rpm for 15 minutes. Three layers will be formed. Take the first and third layers and transfer them to a new vial. Vacuum dry the sample using a lyophilizer and store the sample at -20 ℃ until further use.

The sample will be used for liposomes formation.

4.3. Objective 3: Preparation of liposome

By using the thin film approach, liposomes are created from the extracted lipid. In a nutshell, 2% chloroform was introduced into the recovered lipid solution. A rotary evaporator is used to evaporate the mixture. In a round-bottom flask, a thin film is created and then hydrated with 0.1 M phosphate buffer (pH 7.4). The suspension is vortexed for 30 min., followed by 45 min. of sonication. The samples' morphology is evaluated using microscope.

4.3.1 Formulation of liposome

The creation of liposomes is possible using either natural phospholipids (egg or soya) or synthetic lipids like di-oleoylphosphatidylcholine. These treatments cost a lot of money while being quite helpful. The development of an alternative to synthetic liposomes has been the focus of numerous studies (Arzani, P., & Baserisalehi, M. (2020).

Bacterial lipids are suitable candidates for liposome preparation even though conventional liposomes are unstable, have a brief half-life, and are expensive to produce on a large scale. This is because microorganisms are easily accessible year-round and can grow on a variety of substrates.

RESULTS

For each experiment the pure culture is grown for overnight in nutrient broth with 6% NaCl and is then streaked onto the respective plates. The growth is observed after 2 days for halophiles, thermoacidophiles and thermophiles and 9 days for psychrophiles. Growth condition is measured depending on the extent of growth on the streaking area and is divided into inhibited, very less, less, good, luxuriant and overgrowth (Table.2).

Table 2. Percentage of growth and growth response shown in selected culture plate

S.No.	Percentage growth onto streaking area	Growth response
1.	0%	Inhibited
2.	1-25%	Very less
3.	25- 50%	Less
4.	50-75%	Good
5.	75-100%	Luxuriant
6.	Growth beyond the streaking area	Overgrowth

5.1 Isolation of halophiles on Nutrient Agar media with 6% NaCl

Enumeration of bacteria by plate count after serial dilution and incubation agar plate technique:

The plate with dilution factor 10^{-5} shows the best colony count as shown in the image below (Fig. 3).

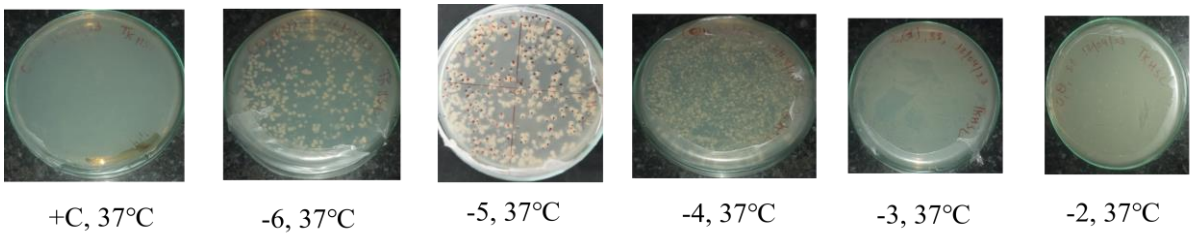


Fig. 3. Culture plate showing no. of colony at different dilution factor

Table 3. Counting of the bacteria by plate count after serial dilution at 6% NaCl

S. No.	Dilution factor	No. of colonies (CFU)
1.	10^{-3}	TNTC (too numerous to count)
2.	10^{-4}	350
3.	10^{-5}	120

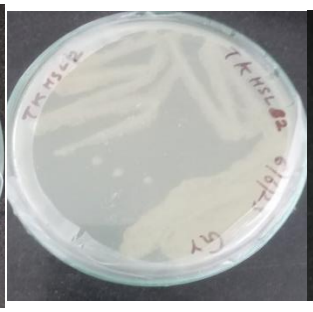
Number of cells/ml = 120/0.05× 5 = 1.2 x 10^4 cells/ml

5.2 Isolation of halophiles in Nutrient agar media with 6% NaCl at 37 ℃

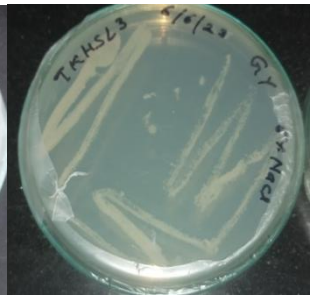
22 separate colonies showing varied morphology on 10^{-5} dilution plate are picked randomly and inoculated in Nutrient broth with 6% NaCl and is grown for 2 days at 37 ℃ and is marked with Tso Kar Hyper saline lake 1-15 (TKHSL 1-15). Growth is observed with the change in the turbidity of the broth.

Petri plates having nutrient agar with 6% NaCl is prepared and is marked with the TKHSL 1-22. Cross streaking is done from each test tube labelled with TKHSL 1-22 on the respective plates and incubated at 37 ℃ for 2 days and growth is observed as shown below (Fig. 4).

TKHSL 1

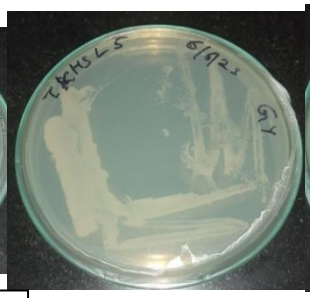


TKHSL 2

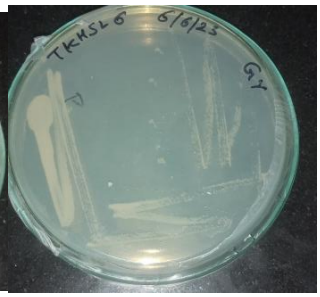


TKHSL 3

TKHSL 4



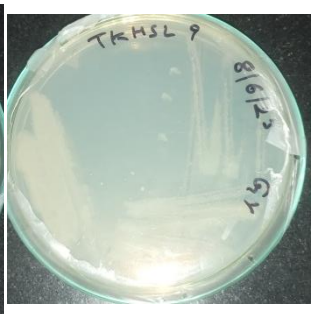
TKHSL 5



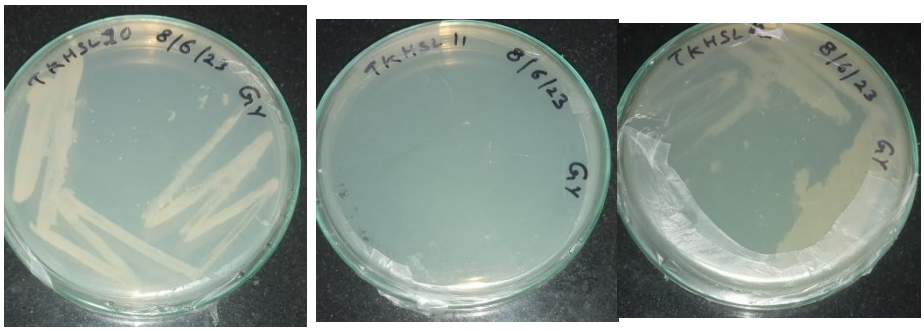
TKHSL 6

TKHSL 7

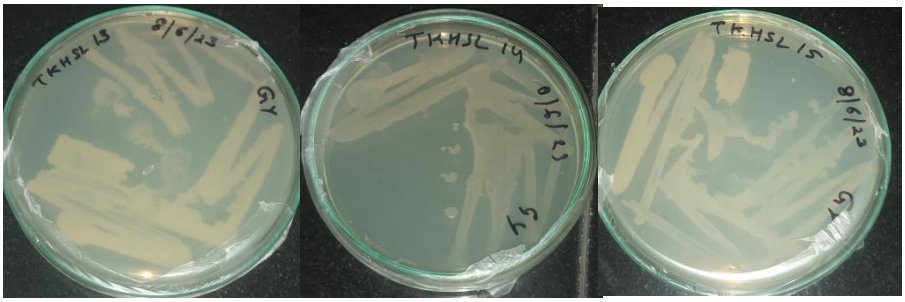
TKHSL 8



TKHSL 9



TKHSL 10 TKHSL 11 TKHSL 12



TKHSL 13 TKHSL 14 TKHSL 15

Fig 4. Growth response of selected nutrient agar media plate

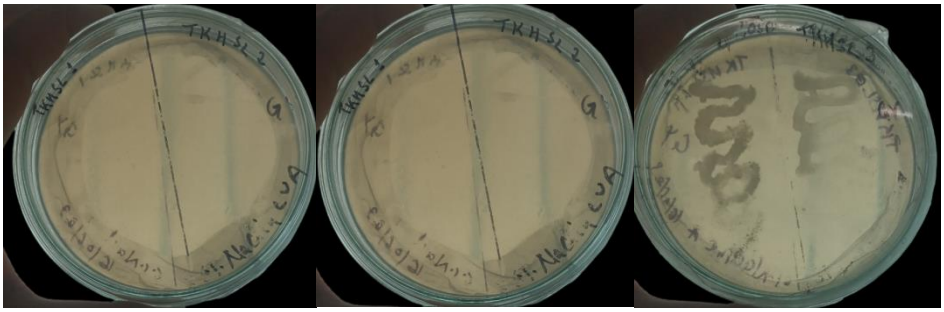
Table 4. Growth of selected polyextremophiles with 6% NaCl at 37 ℃

Sample No.	Growth reponses	Sample No.	Growth response	Sample No.	Growth response
TKHSL 1	Luxuriant	TKHSL 6	Less	TKHSL 11	Inhibited
TKHSL 2	Luxuriant	TKHSL 7	Good	TKHSL 12	Luxuriant
TKHSL 3	Good	TKHSL 8	Luxuriant	TKHSL 13	Luxuriant
TKHSL 4	Good	TKHSL 9	Luxuriant	TKHSL 14	Luxuriant
TKHSL 5	Luxuriant	TKHSL 10	Luxuriant	TKHSL 15	Luxuriant

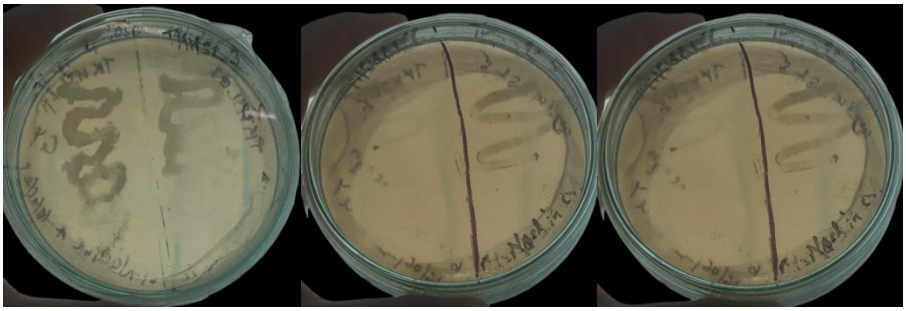
5.3 Identification of psychrophiles on crystal violet agar plate with 6% NaCl at 4 °C

CV agar media was prepared and sterilised. Streaking was done in each petri plates and kept in fridge at 4 °C for 9 days. Growth was observed each day which is shown in fig.5.

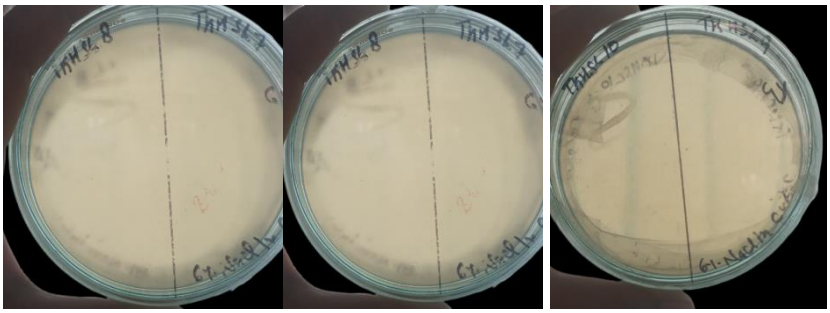
DAY 9



TKHSL 1 | TKHSL 2 | TKHSL 3



TKHSL 4 | TKHSL 5 | TKHSL 6



TKHSL 7 | TKHSL 8 | TKHSL 9

TKHSL 10 | TKHSL 13 | TKHSL 14

TKHSL 15

Fig. 5. Growth response of selected crystal violet media plate

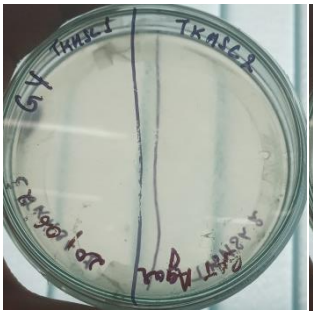
Table 5. Growth of selected polyextremophiles with 6% NaCl at 4 °C

Sample no.	Growth response	Sample no.	Growth response	Sample no.	Growth response
TKHSL 1	Inhibited	TKHSL 6	Very less	TKHSL 13	Good
TKHSL 2	Inhibited	TKHSL 7	Inhibited	TKHSL 14	Good
TKHSL 3	Very less	TKHSL 8	Very less	TKHSL 15	Good
TKHSL 4	Luxuriant	TKHSL 9	Inhibited		
TKHSL 5	Good	TKHSL 10	Inhibited		

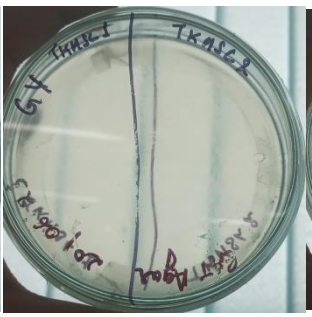
5.4 Identification of Thermoacidophiles on BAT agar media with 6% NaCl at 50 ℃, pH: 5.5

In this study, we found that in BAT agar media the growth of bacteria was inhibited.

We kept the plates in incubator at 50 °C for two days and there was no growth of bacteria was seen in fig.6



TKHSL 1



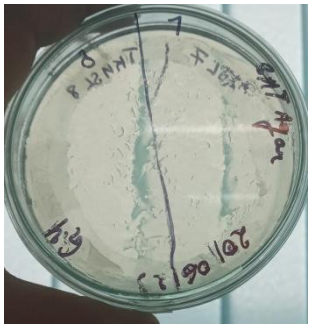
TKHSL 2

TKHSL 3

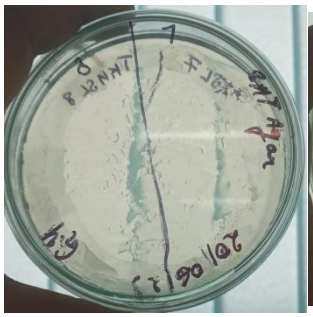
TKHSL 4

TKHSL 5

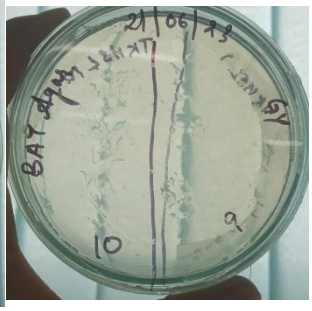
TKHSL 6



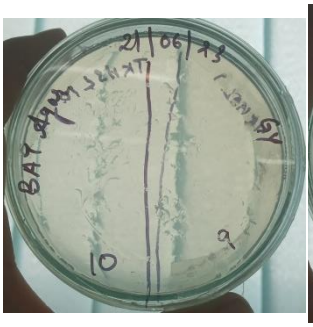
TKHSL 7



TKHSL 8



TKHSL 9



TKHSL 10

TKHSL 13

TKHSL 14

TKHSL 15

Fig 6. Growth response of selected BAT agar media plate

Table 6. Growth of Thermoacidophiles on BAT agar media with 6% NaCl at 50 °C, pH: 5.5

Sample no.	Growth response	Sample no.	Growth response	Sample no.	Growth response
TKHSL 1	Inhibited	TKHSL 6	Very less	TKHSL 13	Inhibited
TKHSL 2	Inhibited	TKHSL 7	Inhibited	TKHSL 14	Inhibited
TKHSL 3	Inhibited	TKHSL 8	Inhibited	TKHSL 15	Inhibited
TKHSL 4	Inhibited	TKHSL 9	Inhibited		
TKHSL 5	Inhibited	TKHSL 10	Inhibited		

5.5 Identification of Polyextremophiles

Below is the aggregated table for the growth conditions of bacteria isolated from high-altitude hypersaline lake of Ladakh (Tso Kar Lake).

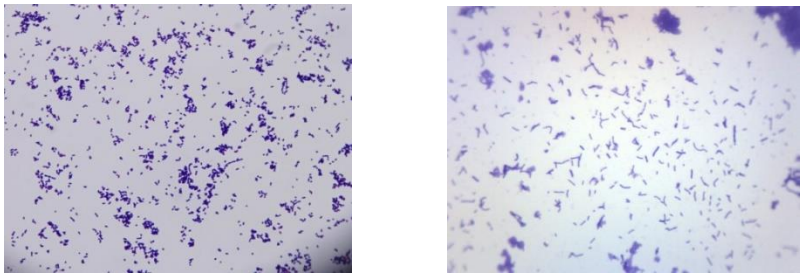
TKHSL 4 & 5 is showing best polyextremophilic properties (Highlighted entire row with purple) and therefore they are selected for lipid isolation and liposome formation.

Table 7. Identification of polyextremophiles. Row consists of the sample number and column consist of the type of extremophilic condition exhibited by the isolated bacteria. For each sample luxuriant growth condition is marked with ++, good condition is marked +, while less, very less and inhibited growth condition is marked with -.

Growth media condition → / Sample No. ↓	Halophiles	Psychrophiles	Thermoacidophiles	Thermophiles
TKHSL1	++	–	–	–
TKHSL2	++	–	–	–
TKHSL3	+	–	–	–
TKHSL4	+	++	–	–
TKHSL5	++	+	–	–
TKHSL6	–	–	–	–
TKHSL7	+	–	–	–
TKHSL8	++	–	–	–
TKHSL9	++	–	–	–
TKHSL10	++	–	–	–
TKHSL13	+	+	–	–
TKHSL14	+	+	–	–
TKHSL15	+	+	–	–

5.6 Grams staining

Gram staining was done only on sample TKHSL4 and TKHSL5 bacteria since they showed more polyextremophilic properties. In gram staining of bacteria it was observed that both the bacteria were gram positive as it retain purple colour and it is in cocci shape (Fig.7)



GP- TKHSL 4 | GP- TKHSL 5

Fig. 7. The image is showing gram positive bacteria cocci in shape

5.7 Lipid isolation and liposome formation

TKHSL 4 and 5 are incubated in nutrient broth at 6% NaCl for two days. *E. coli* is assumed to as a control. Growth is observed in TKHSL 4 and TKHSL 5with the change in turbidity of the media.

No change in turbidity was observed in *E.coli*. TKHSL 4 and TKHSL 5 is subjected for lipid isolation as described in protocol (refer to section 4.2.2)

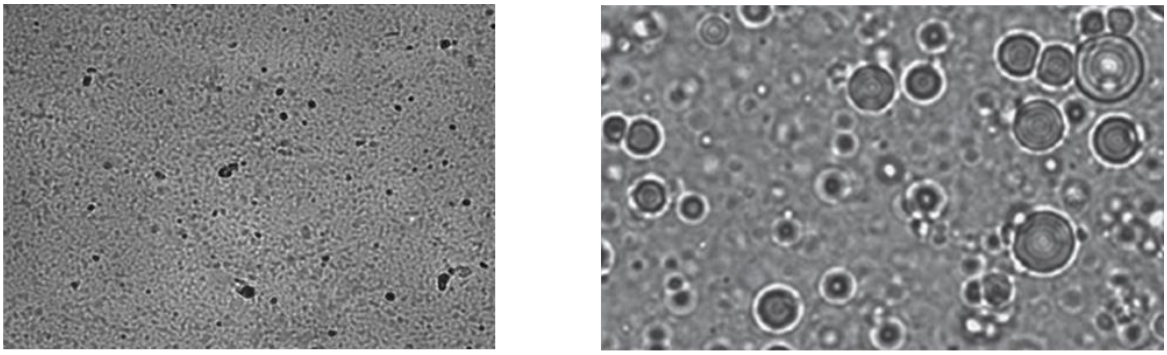
TKHSL 4 | TKHSL 5 | *E. Coli.*

Fig. 8. Lipid isolation from selected culture sample

Liposome formation:

Lipid isolated from TKHSL 4 and 5 are further selected for liposome formation as described in the protocol (refer to section 4.2.3). The Olympus microscope was used to observe the formation of liposomes at a resolution of 40X and 100X. Liposome was seen under microscope on a glass slide with

low light intensity. Only liposome prepared using TKHSL5 lipid can be seen under microscope (Fig.9) and further analysis is needed to determine the size, stability and morphology. The prepared liposome from the lipids of Polyextremophile TKHSL5 can be conjugated with different types of drugs to treat diseases like anti-microbial resistance, cancer, viral infection and various other diseases.



Liposomes under 40X

Liposomes under 100X

Fig. 9 Microscopic images of prepared liposome

Conclusion

Isolation of polyextremophiles from high altitude hypersaline lake was successfully done by the serial dilution and streaking method. Lipids of polyextremophiles have unusual properties to develop liposome. Their characteristics allow liposomes to develop at any temperature in the physiological range or lower, enabling the encapsulation of thermally labile substance and acting as innovative drug delivery system.

Lipids derived from halophilic bacteria have found uses as deliverers of drugs and vaccines. Other compounds found in halophilic archaea, such as siderophores, offer iron-chelating agents that can be used to treat iron deficiency diseases or to increase antibiotic activity against bacteria.

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