POLYPHASIC TAXONOMIC CHARACTERIZATION OF BACTERIA ISOLATED FROM CHILKA LAKE, ODISHA, INDIA

Submitted to

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Master of Science in Biotechnology

By

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June 28, 2013

CERTIFICATE

This is to certify that the dissertation entitled **"Polyphasic taxonomic characterization of bacteria isolated from Chilka Lake, Odisha, India"** submitted to the **Department of Biotechnology and Environment Science, Thapar University,** Patiala, Punjab in partial fulfillment for the award of Degree of **Master of Sciences in Biotechnology** is a bonafide record of the project work done by **Ms. Mansi Kwatra** at **Laboratory for the Conservation of Endangered species (LaCONES), Centre for Cellular and Molecular Biology, Hyderabad** under my guidance. It is further certified that the dissertation or any part thereof has not been submitted elsewhere for any other degree or diploma.

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CERTIFICATE

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DECLARATION

I, Mansi Kwatra hereby declare that the project entitled "**Polyphasic taxonomic characterization of bacteria isolated from Chilka Lake, Odisha, India**" was done by me under the guidance of **Dr. S. Shivaji** and supervision of **Dr. G.S.N. Reddy** at the Laboratory for Conservation of Endangered Species (LaCONES), a constituent unit of the Centre for Cellular and Molecular Biology (CCMB), Hyderabad. This dissertation thesis has not been submitted elsewhere for the award of any degree or diploma by other universities.

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Date: 28th June, 2013

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Place: Hyderabad **Mansi Kwatra**

Date: 28th June, 2013

List of Abbreviations:

DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
16S rRNA	Small subunit ribosomal ribonucleic acid
Weight and Measures	
g	Gram
mg	Milligram
ng	Nanogram
^{0}C	Degree centrigrade
Concentrations	
L	Litre
ml	Milliliter
µl	Microliter
mM	Millimolar
µM	Micromolar
M	Molarity
ppt	Parts per thousand
ppm	Parts per million
Chemicals	
EDTA	Ethylene Diamine Tetra Acetic acid
NaCl	Sodium chloride
TAE	Tris Acetate EDTA buffer
TE	Tris EDTA buffer
ZMA	Zobell Marine Agar
NA	Nutrient Agar
Miscellaneous	
bp	Base pair
pH	-log[H+]
Tm	Melting temperature
MQ	MilliQ water

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ABSTRACT

Cultivable bacterial diversity of brackish water from Chilka Lake, Odisha was explored in the present investigation. The total bacterial cell count of water samples from Chilka Lake ranged from 1.5 X 10^5 to 4.5 X 10^5 cells per milliliter and the cultivability of the bacteria was found to be 9.3%. Close to 189 bacteria were isolated and purified on Zobell marine agar and of which, 52 were of importance as they represented different species belonging to 25 genera, based on the sequences of 16S rRNA gene, and showed 95.0 to 98.0% similarity with their nearest phylogenetic neighbours. These isolates were found to survive at pH 7.0 to 12.0, temperature of 4.0 to 45.0°C and 0-10.0% salinity. Majority of the isolates produced enzymes such as gelatinase, caseinase, urease, DNase, catalase, oxidase, lipase etc. which could be beneficial for biotechnology industry. Amongst the selected strains, ten (CL1-16, CL1-17, CL3-4, CL3-9, CL4a-14, CL4b-2, CL4b-8, CL1R-2, CL2R-4 and CL3R-6) were found to be potentially novel species as they showed ≤98.0% similarity with their phylogenetic neighbours. Further, two isolates-CL1-17 and CL3R-6 showed ≤97.0% similarity with their phylogenetic neighbours and confirmed their species status and named them as *Streptomyces indica* sp. nov. for CL1-17 and *Rheinheimera chilikensis* sp. nov. for CL3R-6.

1. INTRODUCTION

1.1. GENERAL INTRODUCTION:

Microbes are the most ubiquitous organisms on the entire planet. They are found in air, soil, water, on/inside organisms etc. Since ages, microbes have been useful to mankind, being the source of various antibiotics, enzymes, metabolic products and used in food, healthcare and other industries. The economic importance of microbes and their application in the field of biotechnology renders them as the pillars of current biological research.

Bacteria have been known to thrive in all kinds of environment. Though, ideal growth conditions found in freshwaters, soil, and air are most suitable for their survival, certain bacteria have also adapted to live in extreme conditions. This adaption to survive under stress and the phenomenon behind it has been questioned over the years. Hence, it becomes important to identify microbes occupying extreme environments since they contain enzymes or possess certain metabolic processes to sustain in such harsh habitats. Study of bacterial biodiversity of extreme environment will help to shed light on adaptability of these microorganisms and helps in establishing the presence of certain endemic bacteria.

Microbial diversity is studied through two approaches: the culture dependent and the culture independent approach. Culture independent method involves analysis of microbial genomes present in an environmental sample (Handelsman *et al.,* 1998). This method is deemed better as culture dependent method yields only 1.0% or less of actual population density (Amaan *et al.,* 1990a). It involves extraction of genomic DNA from environmental sample which is subjected to 16S rRNA amplification and cloned into suitable host to obtain sequences from profiling microbial communities (Figure 1). Non-cultivable approach uses both quantitative and qualitative methods. Quantitative techniques include DGGE, FISH, T-RFLP and QPCR to estimate the microbial diversity of an environmental sample.

1.1.1. Denaturing Gradient Gel Electrophoresis (DGGE): Denaturing gradient gel electrophoresis (DGGE) is a molecular fingerprinting method. In DGGE, PCR generated amplicons from a microbial community with the same length but with different nucleotide compositions are separated in a denaturing gradient gel of polyacrylamide, based on their differential denaturation profile (Fischer & Lerman, 1983; Muyzer *et al*., 1993). The denaturing conditions are provided by urea and formamide (100% of denaturant solution consists of 7M urea and 40% formamide). Low and high denaturing solutions are prepared, mixed with an acrylamide solution and poured in a gel casting using a gradient former to generate a linear denaturing gradient (Muyzer *et al*., 2004). During denaturation, the two strands of a DNA molecule separate at a specific denaturant concentration, and the DNA sequence stops its migration in the gel. The optimal resolution of DGGE is obtained when molecules do not completely denature, To prevent the complete denaturation of PCR amplicon and continue to run through the gel as single stranded DNA, a GC clamp (a stretch of DNA of 40-60 nucleotides composed by guanine and cytosine) is attached to the 5' end of one of the PCR primers, resulting in a product with one end having a very high melting domain (Muyzer *et al*., 1993). The fragment containing the GC clamp when running through the gel will form a Y-shaped piece of DNA that will stick firmly on the gel when attaining its denaturing point. At the end, fragments with different melting points will migrate to different positions. After gel staining, the number of bands on the gel will be indicative of the genetic diversity of the original sample (Muyzer *et al*., 1993, 2004).

1.1.2. Fluorescent *in-situ* hybridization (FISH): Fluorescence in situ hybridization (FISH) with rRNA-targeted probes is a staining technique that allows phylogenetic identification of bacteria in mixed assemblages without prior cultivation by means of epifluorescence and confocal laser scanning microscopy, or by flow cytometry

(Giovannoni *et al*., 1988; DeLong *et al*., 1989; Amann *et al*., 1990a; Amann *et al*., 1990b; Amann *et al*., 1996). Probes labelled with fluorescent dyes such as Cy3 or Fluorescein are used to hybridize with the specific intracellular target site in the ribosomes (Amann *et al*., 1995). The ratio of the specific binding of different probes may be used to quantify the ratio of different microbial groups in the environmental samples.

1.1.3. Terminal- Restriction Fragment Length Polymorphism (T-RFLP): Analysis by T-RFLP is based on a differential display of restriction sites in bacteria of different taxonomical affiliations. T-RFLP involves amplication of 16S rRNA gene using set of primers with one of the primers labeled with a fluoro probe, followed by restriction digestion with a frequently cutting enzyme and separation on a gene scanner. The relative abundance of community and their diversity is represented in the form of peaks, where each signal is related to a distinct bacterial taxon (Liu *et al*., 1997)**.**

1.1.4. Quantitative PCR (Q-PCR): The qPCR is a highly sensitive tool to quantify microbial populations within a sample, since it is based on detecting specific sequences of nucleic acids, and estimating the amount in a sample (Lacava *et al*., 2008). This technique uses either intercalating fluorescent dyes such as SYBR Green or fluorescent probes (TaqMan) to measure the accumulation of amplicons in real time during each cycle of the PCR. Software records the increase in amplicon concentration during the early exponential phase of amplification which enables the quantification of genes (or transcripts) and indirectly, the bacterial diversity.

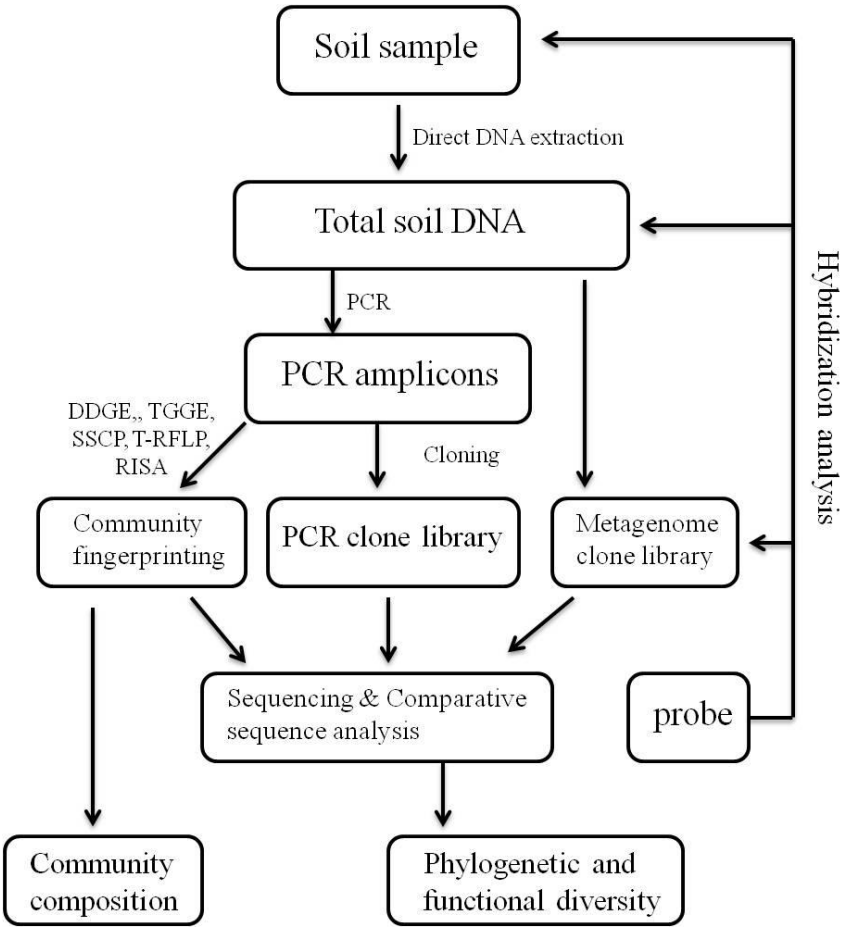


Figure 1: Culture- independent approach to study microbial diversity

The culture dependent methods are reliable only for studying diversity of cultivable microorganisms which have to be isolated by growing on artificial media. This approach involves isolation of bacteria from environmental sample followed by polyphasic taxonomical characterization (Colwell, 1970). Polyphasic taxonomy is a consensus type of taxonomy and is a method of integration of phenotypic, genotypic and phylogenetic methods of characterization. It involves morphological, physiological, chemotaxonomic and phylogenetic characterization (Figure 2).

1.1.5. Morphological characterization: Based on colour, shape, size, opacity, elevation etc.

1.1.6. Physiological characterization: Based on salt, temperature and pH tolerance.

1.1.7. Chemotaxonomic characterization: Based on chemical analyses of Fatty acid methyl ester (FAMES), lipids, menaquinones, ubiquinones, cell wall peptidoglycan, mycolic acids, polyamines etc.

1.1.8. Phylogenetic characterization: The phylogenetic analysis uses 16S rRNA gene amplification followed by identification of bacterial isolate by BLAST (http://www.ncbi.nlm.nih.gov/BLAST) or EzTaxon (http://www.eztaxon.org/) similarity search.

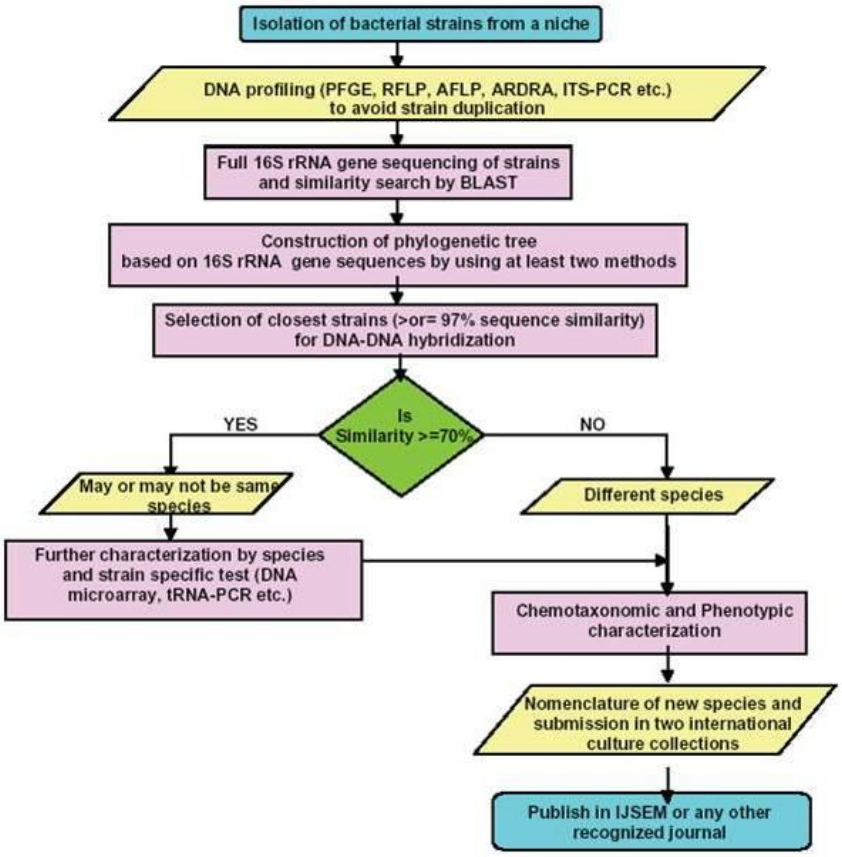


Figure 2: Species identification by Polyphasic taxonomy

1.2. REVIEW OF LITERATURE:

Biodiversity has always been the base of development of civilizations. According to International Union for Conservation of Nature (IUCN), biological biodiversity is defined as the variability among living organisms from all sources including, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are a part; this includes diversity within species, between species and of ecosystems.

Out of earth's biodiversity, bacteria have proved to be of high economic importance, playing a vital role in natural processes like nutrient cycling, bioaugmentation, bioremediation and bioprospecting. The diversity in an ecosystem establishes its functionality and the processes mediated by these bacteria maintain the balance of the ecosystem. Bacteria were classified as a domain in three-domain system (Woese, 1977). Bacterial diversity is organized into various phyla, class, order, families, genera and species. Table 1 provides the numbers of taxa into which bacteria are classified till date.

Table 1: Taxonomical classification of bacteria

Domains	2
Phyla	25
Class	40
Order	92
Family	210
Genera	2702
Species	11275

The bacterial diversity has been speculated to consist of 10 billion species (Dykhuizen, 1998), but only 1.0% of species have been described till date (Torsvik *et al.,* 1990; Ward *et al.,* 1990; Amann *et al.,* 1995). The quantification of bacterial diversity has remained restricted owing to constraints such as small proportion of bacterial species that can be cultured (Brock, 1987), the large number of individuals that may be present per environmental sample (Torsvik *et al.,* 1990), the high diversity that may be present at a small

scale (Klug & Tiedje, 1993) and the difficulty of defining a bacterial species (Goodfellow & O'Donnell 1993; Rossello-Mora & Amann, 2001).

Species is the basic unit of taxanomy and in case of bacteria, the genetic definition of a species is quantifiable as (i) less than 70.0% DNA-DNA relatedness or (ii) less than 5°C or less *Tm* for the stability of heteroduplex molecules or (iii) less than 97.0% similarity at 16S rRNA gene sequence (Stackebrandt & Goebel, 1994). The current definition of bacterial species relies on the genomic-phylogenetic species concept (GPSC) (Stayley, 2006). The GPSC is a methodological species concept that concentrates on DNA, RNA or protein sequence, ecological niche and evolutionary history of an organism through phylogeny of a gene or protein sequence.

The species diversity is intricately linked to ecological diversity. Bacteria, being ubiquitous, occupy various niches, from ideal living conditions to extreme physiological conditions. Their habitat ranges from environments such as soil, oceans, lakes and other organisms to extremes such as hot springs (Brock, 1978), salt brines (Anton *et al.,* 2000), acid mine waters at very low pH (Baker & Banfield, 2003), deep in Antarctic ice (Price, 2000; Christner *et al.* 2001) and kilometres below the Earth's surface (White *et al.,* 1998). The environmental parameters tend to influence the bacterial diversity, the general consensus being that aquatic niches hold less diversity than the soil environment (Nold & Zwart 1998; Curtis *et al.,* 2002; Torsvik *et al.,* 2002).

Bacterial species surviving in extreme conditions such as mentioned above are called as 'extremophiles'. These organisms have adapted to these niches, thus contributing significantly to evolution. Such bacteria show expression of certain enzymes or metabolites, helping them to survive in harsh environments. Identification of these bacteria promises a greater prospect for industrial growth and helps in minimizing the input. Hence, isolation and characterization of bacteria from various environments serves for betterment of human life. One of the extreme environments is marine waters which harbour bacteria adapted to high

salt concentrations. Marine environment constitutes 70.0% of surface of earth where bacteria are found in surface waters and also in depths. Marine bacterial biodiversity is considered commercially important as they are sources of bioactive compounds such as antibiotics, anti-virals, anti-tumorals, anti-oxidant and anti-inflammatory (Okami, 1982, *Kamei et al.,* 1987; Nunez *et al.,* 2006; Uzair *et al*., 2009; *Shankar et al.,* 2010).

Diversity of saline lakes has been considered worthy of interest as microbes of industrial application could be isolated from them. These lakes are habitat of halotolerant and halophilic bacteria, which are potential sources for enzymes which can function at high salt concentrations. In India, several bacteria have been characterized from soda lakes like Lonar Lake (Wani *et al*., 2006; Joshi *et al.,* 2008) and Pulicat Lake (Sahay *et al.,* 2011). The bacterial species identified from these lakes include *Halomonas salina, H. Shengliensis, H. salifodinae, H. pacifica, H. aquamarina, H. halophila, Bacillus flexus, B. megaterium, B. thuringiensis, B. horikoshii, B. boroniphilus, B. granadensis, Salimicrobium halomicrobium* (Sahay *et al.,* 2011), *Bacillus subtilis, B. licheniformis, B. cohnii, Planococcus maritimus, Alcanivorax borkumensis* and *Oceanobacillus ineyensis* (Deshmukh *et al.,* 2011). Many novel genera have been isolated from Lonar lake *viz. Nitritalea halalkaliphila* and *Indibacter alkaliphilus* (Kumar *et al.,* 2010a, b). Besides the above studies, one of the lakes less studies was Chilka from Odisa, India. From this lake, so far, novel bacteria like *Shewanella chilikensis* and *Thirhodococcus modestalkaliphilus* (Sucharita *et al.,* 2009; Sucharita *et al.,* 2010) have been isolated. *S. chilikensis* has been shown to have urease, catalase and oxidase activity (Sucharita *et al.,* 2009) while *T. modestalkaliphilus* is a phototrophic purple sulphur bacterium (Sucharita *et al.,* 2010). Not much work has been done on the lake's bacterial diversity. Hence, the focus of this study is on bacterial diversity of Chilka Lake.

Chilka Lake is a brackish water lagoon situated in the state of Odisha, on the east coast of India. It is the largest lake in Indian subcontinent located between latitude 19˚28' and 19˚54' N, and longitude 85˚05' and 85˚38'E. The average length and breadth of the lagoon

are about 65km and 16km. It covers an area of 815 km^2 which extends to 992 km^2 during rainy season (Mohanty *et al.*, 2001) (Figure 3). This lake has been formed by silting action of Mahanadi River. It is separated from the Bay of Bengal by sand bar of 60 km in length (Arya & Lakhotia, 2006). The lagoon has been classified into four different sectors: northern sector, central sector, southern sector and outer channel according to its physico-chemical properties and supports a combination of marine, brackish water and fresh water biodiversity. It has been designated as "Ramsar site" to identify it as wetland of importance in 1991. The physico-chemical characteristics of Chilka Lake are given in Table 2.

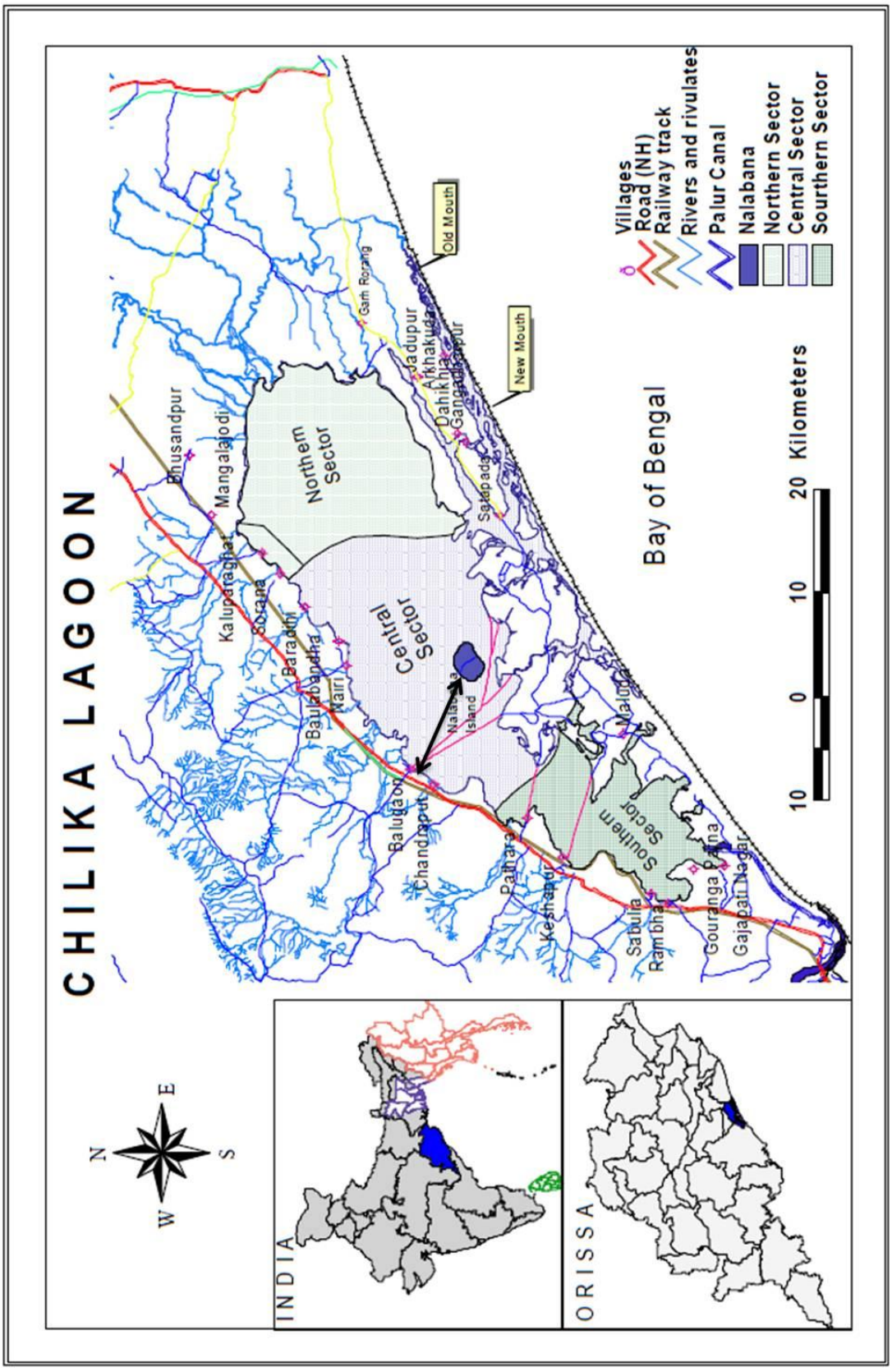


Figure 3: Map of Chilka Lake showing various sectors, rivers and inlet mouth to Bay of Bengal (⟷ represents the transect of sample collection)

(Source: Panda and Mohanty, 2008)

The physico-chemical properties of Chilka Lake show that the waters pose extreme conditions of survival due to high range of salinity and pH. The bacteria habituating these brackish waters can be expected to possess salt stress tolerance or other novel traits.

Table 2. Physico-chemical properties of Chilka Lake*

Temperature (°C) Surface water	 17.5 – 32.0
Salinity (ppt) Range	 Traces – 36.0
pH Range	 7.6-10.0
Dissolved oxygen (mg/litre)	1.3-13.4
Transparency (Secchi dic depth in mts.)	Minimum – 0.32 Maximum – 1.40
Nutrients (mg/litre) Nitrate Phosphate Silicate	 Traces – 0.19 Traces – 0.18 0.10 – 0.60
Trace elements (ppm) Copper Zinc Iron	 0.02 – 0.04 0.025 – 0.19 0.12 – 0.32
Sediments Carbonate contents (%)	 1.6 – 3.8
Trace metals (ppm) in sediments Copper Nickel Chromium Lead	 5 – 66 150 – 270 30 – 270 72 – 122

*Data from: Asthana (1978); Banerjee & Choudhury (1966); Jhingran & Natarajan (1966, 1969); Misra *et al.*, (1988); Mohanty (1981); Mohapatro *et al.*, (1988); Patnaik (1973); Patnaik (1988); Patnaik & Sarkar (1976); Panda (1988); Panigrahy (1985); Raman *et al.*, (1990); Sarma (1988).

This present investigation is aimed at studying the bacterial diversity of Chilka Lake and characterization of novel bacterial isolates up to the species by polyphasic taxonomic methods.

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Sample collection: 9 water samples and 1 sediment sample were collected in a straight line at an interval of 1km from Eco-Tourism boat centre to Nalaban Islands. The samples were collected in sterile falcon tubes under aseptic conditions and stored at 4 °C**.**

2.1.2. Chemicals and Reagents: PCR red dye master mix (Merck Genei™; contains unique red dye, Taq DNA polymerase, dNTPs and rection buffer with 1.5mM $MgCl_2$), PCR primers (Bioserve), mercuric chloride, iodine solution (1% Iodine and 20% potassium iodide in distilled water)**.**

2.1.3. Buffers:

Acetate buffer: 0.1M acetic acid and 0.1M sodium acetate. Mix to 357 ml acetic acid and 643 ml sodium acetate to get pH 5.0 and 52.2 ml acetic acid and 947.8 ml sdodium acetate to get pH 6.0.

Potassium Chlroride- Sodium hydroxide buffer: 0.2M potassium chloride and 0.2M sodium hydroxide. Mix to 50 ml of potassium chloride and 8.2 ml of sodium hydroxide to get pH 11.0, 50 ml of KCl and 12 ml of NaOH to get pH 12.0 and 50 ml of KCl and 132 ml of NaOH to get pH 13.0.

TAE (Tris-acetate-EDTA) buffer: 40 mM Tris Base and 2 mM EDTA. pH adjusted to 8.0 using glacial acetic acid**.**

Tris buffer (pH 10.0): 1M Tris, pH adjusted to 10.0 with 1N HCl.

1X Tris-EDTA buffer: 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA.

50X TAE (Tris- acetic acid-EDTA) buffer: 40 mM Tris-acetate and 2 mM. Working concentration of the buffer is 1X.

2.1.4. Media:

2.1.4.1. Zobell Marine Agar (HiMedia cat. #M384-500G)

Components	Quantity (g/L)
Peptone	5.00
Yeast extract	1.00
Ferric citrate	0.10
Sodium chloride	19.45
Magnesium chloride	8.80
Sodium sulphate	3.24
Calcium chloride	1.80
Potassium chloride	0.55
Sodium bicarbonate	0.16
Potassium bromide	0.08
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluorate	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
Agar	15.00

55.25 g of the readymade media was dissolved in 1000 ml of distilled water. After dissolving, sterilization was done by autoclaving at 121°C for 15 min.

2.1.4.2. Urease agar (HiMedia cat. #M112):

Components	Quantity (g/L)
Peptone	1.00
Dextrose	1.00
Sodium chloride	5.00
Disodium phosphate	1.20
Monopotassium phosphate	0.80
Phenol red	0.012
Agar	15.00

24 g of readymade Urease agar media was dissolved in 950 ml of distilled water. Urea solution (40% w/v) was prepared separately and sterile filtered. 50 ml of this solution was added to cooled media prior to pouring.

2.1.4.3. Arginine dihydrolase test media (HiMedia cat. #M619):

Components	Quantity (g/L)
Peptone	1.00
Sodium chloride	5.00
Dipotassium phosphate	0.30
L-Arginine	10.00
Bromocresol purple	0.016
Agar	3.0

19.3 g of readymade arginine dihydrolase media was dissolved in 1000 ml of distilled water and autoclaved at 121°C for 15 min.

2.1.4.4. DNase Test Agar w/ Toluidine blue (HiMedia cat. #M1041-100G):

Components	Quantity (g/L)
Tryptose	20.00
Deoxyribonucleic acid (DNA)	2.00
Sodium chloride	5.00
Toluidine blue	0.10
Agar	15.00

42.1 g of readymade DNase test media was dissolved in 1000 ml of distilled water and autoclaved at 121°C for 15 min.

2.1.4.5. Decarboxylase broth:

Components	Quantity (g/L)
Peptone	5.00
Yeast extract	3.00
Dextrose	1.00
Amino acid	5.00
Bromocresol purple	0.02

The above components were weighed and dissolved in distilled water. The media was autoclaved at 121°C for 15 min.

2.1.4.6. Triple sugar iron agar:

Components	Quantity (g/L)
Peptone	10.00
Casein enzyme hydrolysate	10.00
Yeast extract	3.00
Beef extract	3.00
Lactose	10.00
Sucrose	10.00
Dextrose	1.00
Sodium chloride	5.00
Ferrous sulphate	0.20
Sodium thiosulphate	0.30
Phenol red	0.024
Agar	12.00

All the above components were dissolved in distilled water and autoclaved. After sterilization, the media was poured into test tubes and allowed to solidify in slanting position.

2.1.4.7. Gelatinase agar: Gelatin (0.4% w/v) was dissolved in warm water first and then added to ZMA (55.25 g/L). The media was autoclaved at 121°C for 15 min.

2.1.4.8. Starch agar: Soluble starch (0.2% w/v) was added to ZMA (55.25 g/L). The media was autoclaved at 121°C for 15 min.

2.1.4.9. Motility agar: Zobell Marine Broth (40.25 g/L) is supplemented with agar (0.4%w/v) and autoclaved at 121°C for 15 min. After autoclaving, the media is poured into test tubes and allowed to solidify in vertical position.

2.1.4.10. Lipase test media: Tween-80 (1% v/v) and $CaCl_2$ (0.01% w/v) were added to ZMA (55.25 g/L) and the media was autoclaved at 121°C for 15 min.

2.1.4.11. Aesculin test media: Aesculin (0.1% w/v) and Ferric acitate (0.05% w/v) were added to ZMA (55.25 g/L) and then autoclaved at 121°C for 15 min.

2.1.4.12. Phosphatase test media: 55.25 g of ZMA was dissolved in 1000 ml of distilled water and then filter sterilized phenolphthalein diphosphate (0.01% w/v) was added to it. The media was autoclaved at 121°C for 15 min.

2.2. METHODS

2.2.1. Isolation of bacteria: 20 mg of the sediment sample was dissolved in 1ml sterile water. Then, 100µl of the water samples and sediment sample were spread on ZMA (Zobell, 1946), ZMA 1/10 and ZMA 1/100 plates and incubated at 20°C for 5-7 days. Colonies were picked according to morphological variation and characteristics of each morphotype were recorded.

2.2.2. Total and viable count: Total counts of water and sediment samples were estimated by epifluorescence microscopy using Molecular Probes' Live/Dead® Baclight™ bacterial viability kit (Cat # L7007). This kit utilizes mixtures of SYTO® 9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide. The SYTO® 9 stain generally labels all bacteria in a population — those with intact membranes and those with damaged membranes. In contrast, propidium iodide penetrates only bacteria with damaged membranes, causing a reduction in the SYTO 9 stain fluorescence when both dyes are present. The dye components of kit are Component A (1.67 mM Syto 9 dye and 1.67 mM Propidium iodide) and Component B (1.67 mM Syto 9 dye and 1.83 mM Propidium iodide) and were mixed in 1:1 ratio. For water samples, 250 µl of water was stained with 0.75 µl of dye mix, incubated for 15 min at room temperature and cells were counted on haemocytometer in Zeiss AxioPlan 2 imaging microscope using the lamps HAL 100 (halogen) and HBO 100 (mercury). The excitation/emission maxima for these dyes are about 480/500 nm for Syto 9 and 490/635 nm for propidium iodide. Viable counting was done by counting the number of colonies on the plates and expressed as colony forming units per millilitre of the sample (CFU/ml).

2.2.3. Morphological Characterization:

2.2.3.1. Colony morphology: The colour, form, elevation, opacity and margin characteristics of each bacterial isolate were recorded.

2.2.3.2. Gram staining/KOH string test: Generally, staining method developd by Hans Christian Gram in 1884 is most commonly used method of differentiating between Gram positive and Gram negative strains. Another method called KOH string test is used for same. A bacterial colony was placed on a slide, a drop of potassium hydroxide was put on it and mixed. After a minute, the inoculation loop was touched to the surface of the slide and lifted. If a string formed while lifting the loop, the bacterium was scored as Gram negative.

2.2.4. Physiological characterization: Physiological characterization includes, physiological response of bacteria to varying pH, temp and salinity

2.2.4.1. Temperature tolerance: This involves determining the optimum growth at specified temperatures. The cultures were inoculated on ZMA plates and incubated at different temperatures between 4 and 55°C and the growth was observed from 2 to 10 days.

2.2.4.2. pH tolerance: The study of tolerance to different pH can help classify them as acidophiles, neutrophiles and basophiles. For determination of growth of bacteria at different pH, ZMA medium buffered with sodium acetate buffer for pH 5.0 and 6.0, Tris buffer for pH 8.0 to 10.0 and potassium chloride- sodium hydroxide buffer for pH 11.0 and 12.0 was used. A single bacterial colony was streaked on ZMA plates with different pH and incubated at room temperature for 5-7 days. Appearance of at least a single colony was recorded as positive for the growth.

2.2.4.3. Salt tolerance: Determining the tolerance to different salt concentrations helps in classifying the bacteria as halophilic or moderately halophilic or halophobic. The salinity of Chilka Lake ranges from traces to 36 parts per thousand. Due to continuous mixing of lake water with sea water, the salinity of water is different in different sectors of the lake. Growth of bacterial isolates was also checked on nutrient agar (0% and 0.5% NaCl) plates. Since all the bacteria grew on nutrient agar, it was used for salt tolerance test. Salt tolerance was checked by supplementing NA with NaCl concentration from 0-10%. The plates were inoculated and incubated at 25°C for 5-7 days.

2.2.4.4. Motility assay: Bacterial motility was checked by stabbing bacterial cultures into the motility test medium, ZMB with 0.4% agar, using a sterile inoculation loop and incubated at 25°C for 4-5 days. The motility was recorded on the basis of diffusion of bacterial growth away from site of inoculation or not (Figure 4).

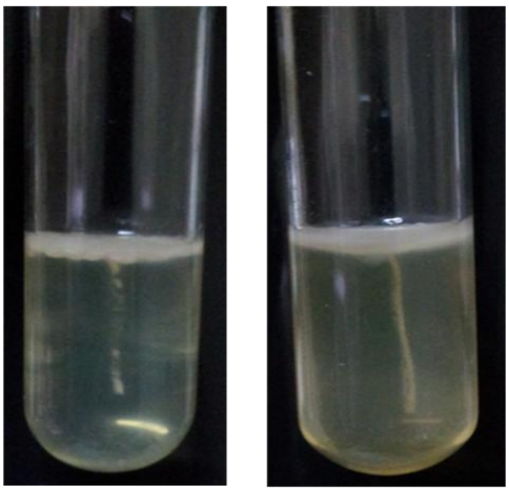


Figure 4: Motility test. a) Negative (non-motile) and b) Positive (motile)

2.2.5. Biochemical characterization

These studies help in determining presence of certain enzymes and utilization of substrates.

2.2.5.1. Arginine Dihydrolase test: Three enzymes namely arginine deiminase, ornithine transcarboxylase and carbamate phosphotransferase, convert arginine to ornithine and ammonia with the production of ATP. Consequently, organisms possessing these enzymes are capable of utilizing arginine as an energy source for growth under anaerobic conditions and liberate NH_3 and thus the pH of the medium becomes alkaline.

$$\text{Arginine} + H_2O + \text{ADP} + \text{Pi} \longrightarrow 2NH_3 + \text{Ornithine} + \text{ATP}$$

The method is normally used to detect arginine dihydrolase activity in bacteria. In this method two tubes containing 3 ml of arginine broth (HiMedia Cat. #M619) were stab inoculated with the culture and one of the tubes was overlaid with 1 ml of liquid paraffin oil. The tubes were incubated for 5 to 10 days and positive reaction was indicated by a colour change from yellow to red in both the tubes.

2.2.5.2. Catalase test: The enzyme catalase is present in most cytochrome containing aerobic bacteria and facultative anaerobic bacteria and they are capable of degrading H_2O_2 which is produced as an end product of aerobic break down of sugars. The reduced flavoprotein directly reacts with gaseous oxygen and is capable of decomposing H_2O_2 by liberating oxygen.

$$2\ H_2O_2 \longrightarrow 2H_2O + O_2$$

A loopful of bacteria was placed on a drop of H_2O_2 and mixed with the inoculation loop. Catalase positive bacteria produced gas bubbles due to the oxygen liberated from H_2O_2, whereas catalase negative bacteria did not produce any bubbles (Figure 5).

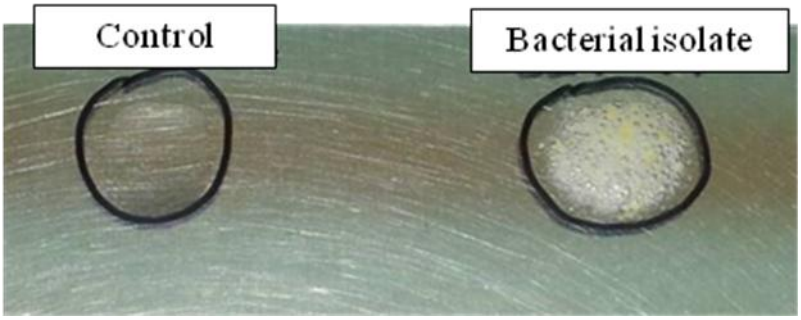
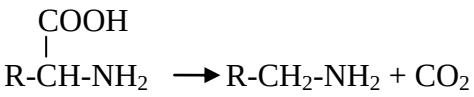


Figure 5: Catalase production as indicated by bubbling

2.2.5.3. Decarboxylase test: These enzymes bring about decarboxylation of lysine, arginine, ornithine or glutamic acid to their corresponding amines. The enzymes are normally produced under anaerobic conditions at low pH (6.0) and in a rich medium containing a specific substrate and require pyridoxal phosphate for their action. Due to carboxylase activity, an amine is formed and CO_2 is released. The formation of the amine increases the pH of the medium and this serves as an indicator of enzyme activity.



In this test, sterile decarboxylase medium (peptone 0.5%, yeast extract 0.3%, dextrose 0.1%, amino acid-arginine or glutamic acid- 0.5%, bromocresol purple 0.002%) was prepared, 3 ml of the medium was dispensed into test tubes and allowed to solidify. Subsequently, the bacterial culture was inoculated as streak, overlaid with 1 ml of sterile paraffin oil and incubated for 5 to 10 days at 22°C. Cultures, which changed the medium colour to yellow due to decarboxylation and then to violet colour due to alkalinisation, were scored as positive for decarboxylase activity. The control medium remained yellow.

2.2.5.4. Aesculin hydrolysis test: In this test, the ability of an organism to hydrolyze the glycoside aesculin to aesculetin and glucose is evaluated. Aesculetin (6,7- hydroxy coumarin) which is formed following hydrolysis reacts with ferric citrate and forms a brown to dark complex which could be monitored as an end product of the reaction. In this test, the ZMA medium is supplemented with 0.1% aesculin and 0.05% ferric citrate, the cultures were streaked and incubated for 4 to 7 days at room temperature. Appearance of dark brown colouration around the colony was taken as positive for aesculin hydrolysis.

2.2.5.5. β-galactosidase test: The enzyme β-galactosidase hydrolyses lactose into its monosaccharide constituents glucose and galactose. The activity of this enzyme can

be demonstrated by its ability to hydrolyze colourless, ONPG (*o*-nitrophenyl- β -D-galactopyranoside) to yellow-coloured *o*-nitrophenol. In this test, the medium was prepared by adding 25 ml of filter sterilized ONPG (0.6 g in 100 ml of 0.01M Na2HPO4 buffer) solution to 75 ml of Zobell Marine broth. The bacterial culture was inoculated into 3 ml of medium and the tubes were incubated for 4-10 days at 25°C. Cultures positive for β-galactosidase changed the colour of the medium to yellow (Figure 6).

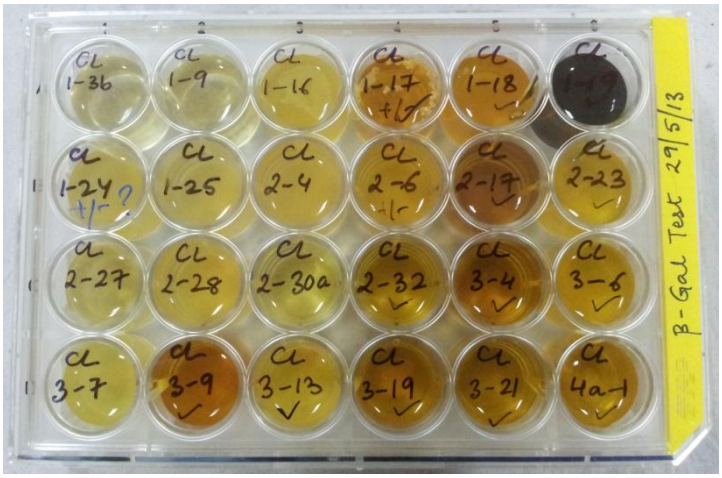


Figure 6: β-galactosidase activity as indicated by dark yellow colouration in the wells.

2.2.5.6. Gelatinase test: The method was originally described by Kohn (1953) to test for the ability of an organism to produce proteolytic enzymes capable of decomposing gelatin. The hydrolysis of gelatin can be detected by adding acidic mercuric chloride. Acidic mercuric chloride precipitates the non hydrolysed gelatine by giving it an opaque appearance and a clear zone is formed around the colony due to hydrolysis of gelatine. The ZMA medium was supplemented with 0.4% gelatine, plates were streak inoculated and incubated for 5 to 6 days at 25^{o}C. The plates were flooded with 15% mercuric chloride solution prepared in 20% HCl solution. The clear zone around the streak due to hydrolysis of gelatin was scored as positive (Figure 7).

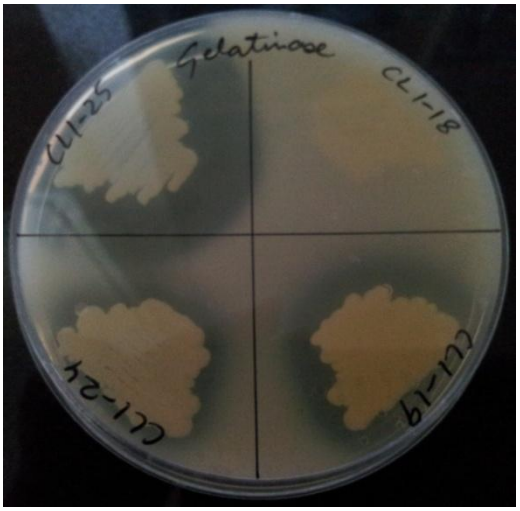


Figure 7: Gelatinase activity as indicated by clear zone around the colonies

2.2.5.7. Lipase test: Lipase activity was indicated by the presence of crystals around the colony which is formed due to the precipitation by calcium salt of the fatty acid liberated by lipolysis (Sierra, 1957). The production of extracellular lipase activity was checked by supplementing ZMA plates with 1% Tween-80 and 0.01% $CaCl_2$. Sterile plates were streak inoculated and incubated at 25°C till the growth was visible. Bacteria producing lipases formed opaque crystals around the colonies due to the formation of calcium crystals of fatty acids.

2.2.5.8. Oxidase test: This test is useful for differentiating aerobic and facultatively anaerobic groups of bacteria. The cytochrome oxidase system present in oxidase positive bacteria catalyses the conversion of reduced cytochrome C to the oxidized form. The oxidized cytochrome C passes the electrons to the molecular oxygen that in turn, by accepting hydrogens, is reduced to hydrogen peroxide. In this test, the oxidized form of cytochrome C oxidizes tetramethyl-*p*-phenylenediamine (TMPH) to a coloured compound generating the reduced cytochrome C. Oxidase discs (HiMedia) were smeared in centre with the bacterial culture and allowed to dry. A blue to purple colouration within 60 sec is taken as positive for oxidase activity (Figure 8).

Figure 8: Oxidase production as indicated by changing the colour of TMPH coated dics to purple colour.

2.2.5.9. Amylase test: The presence of amylase can be confirmed by growing the isolates on a media supplemented with starch and then flooding the plate with Iodine solution. Iodine solution causes brown colouration due to presence of starch except in clear zones around the colony due to activity of amylase. The ZMA medium was supplemented with 0.2% soluble starch and the culture was streak inoculated and incubated for 5 to 10 days at 25^{o}C. The plates were flooded with iodine solution (1% iodine and 20% KI in water) and appearance of clear zones around the colonies was taken as positive for starch hydrolysis.

2.2.5.10. Triple sugar iron test: This test is performed to test the ability of an organism to ferment lactose, sucrose and dextrose with or without production of gas and H_2S under both aerobic and anaerobic conditions. In the aerobic region of the medium (surface of the slant), glucose at the relatively low concentration is rapidly oxidized to CO_2 and H_2O. Anaerobically in the butt, the glucose is fermented to organic acids and sometimes to CO_2 and H_2 and therefore, gas can be detected. Lactose and sucrose may be catabolised but only after glucose has been consumed. Since they are present at a high concentration, their metabolism results in high levels of acids throughout the slant and butt. Gas also may be produced from lactose and/or sucrose. Slants containing TSI medium was heavily inoculated with a loop along the surface of the

medium and then stab inoculated deep down into the agar of the butt and incubated at 22°C for 7 to 10 days. The results were assessed as follows:

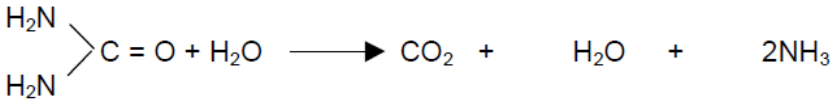
i. Organisms fermenting glucose produced yellow colour only in the butt,

ii. Organisms fermenting glucose and lactose and/or sucrose produced yellow colour on the slope and butt,

iii. Organisms producing gas formed bubbles and occasionally disturbed the medium, and

iv. Organisms producing H_2S caused blackening of the medium along the line of the stab.

2.2.5.11. Urease test: Urease is an enzyme which cleaves urea into two molecules of ammonia and one molecule of carbon dioxide. The ammonia produced by cleavage of urea by urease would increase the pH of the medium and change the indicator colour to pink.



The ability of the bacterium to produce urease was tested by inoculating the bacteria on Urease agar (HiMedia cat. #M112) plates. The ammonia produced by cleavage of urea by urease would increase the pH of the medium and change in indicator colour to pink was considered as positive for urease.

2.2.5.12. Caseinase test: Casienase causes hydrolysis of the protein casein, skim milk can be used as a substrate and a clear zone around colonies indicated activity of caseinase enzyme. Casein hydrolysis was tested on ZMA supplemented with 5% (v/v) sterilized skim milk (100 g/L). The skim milk was sterilized separately (100°C for 40 min) and added to the cooled (50°C) ZMA agar. Bacterial isolates were spot inoculated at the centre of the plate, incubated at 25°C for 5-7 days and zone of clearance around the colony was recorded as positive.

2.2.5.13. DNase test: It is usually performed to confirm the ability of bacteria to breakdown DNA. Modified DNase test agar containing toluidine blue (HiMedia cat. #M1041-100G) was used for the detection of deoxyribonuclease activity of bacteria. This test involved the inoculation of cultures on the surface of DNase test agar medium. The plates were incubated at 25°C for 5-7 days and observed for change in colour from blue to rose pink (Figure 9).

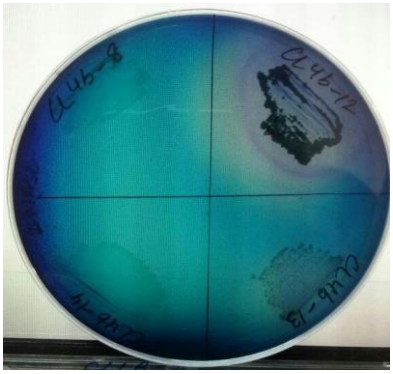


Figure 9: DNase activity as indicated by pink colouration around the colony.

2.2.5.14. Sensitivity to antibiotics: To check the sensitivity of the bacteria to various antibiotics, an overnight grown culture was spread uniformly on a ZMA plate and the antibiotic discs (HiMedia) were placed on the plate after which the plates were incubated for 5 to 7 days at 25°C. Cultures sensitive to the antibiotic failed to grow in the vicinity of the discs thus, producing a clear zone of inhibition around the colony (Figure 10).

Figure 10: Antibiotic susceptibility as indicated by clear zones around the antibiotic discs.

2.2.5.15. Biochemical characterization using HiMedia kits: KB009 HiCarbohydrate™ kit and KB003 Hi25™ Enterobacteriaceae identification kit were used to study the biochemical profile of organisms showing ≤98.0% similarity to existing bacterial species. KB009 contains 3 strips (part A, part B and part C) (Figure 13) with 35 tests including one control and the results are based on pH change resulting in utilization of a substrate. The kit KB003 contains 2 strips (strip I and strip II) (Figure 13) to perform 24 different tests. 50µl of freshly grown culture was inoculated in each well under aseptic conditions and incubated at 25°C for a week. Results were recorded based on the interpretation chart provided by the manufacturers (Figure 11, 12 and 14).

Result Interpretation chart

Test	Principle	Original colour of the medium	Positive reaction	Negative reaction
Carbohydrate utilization	Detects carbohydrate utilization	Red	Yellow	Red / Pink
ONPG decarboxylase	Detects β–galactosidase activity	Colourless	Yellow	Colourless
Esculin hydrolysis	Detects esculin hydrolysis	Cream	Black	Cream
Citrate utilization	Detects capability of organism to utilize citrate as a sole carbon source	Yellowish-green	Blue	Yellowish-green
Malonate utilization	Detects capability of organism to utilize sodium malonate as a sole carbon source	Light green	Blue	Light green

Figure 11: Result interpretation chart for KB009

Strip I — Result Interpretation chart

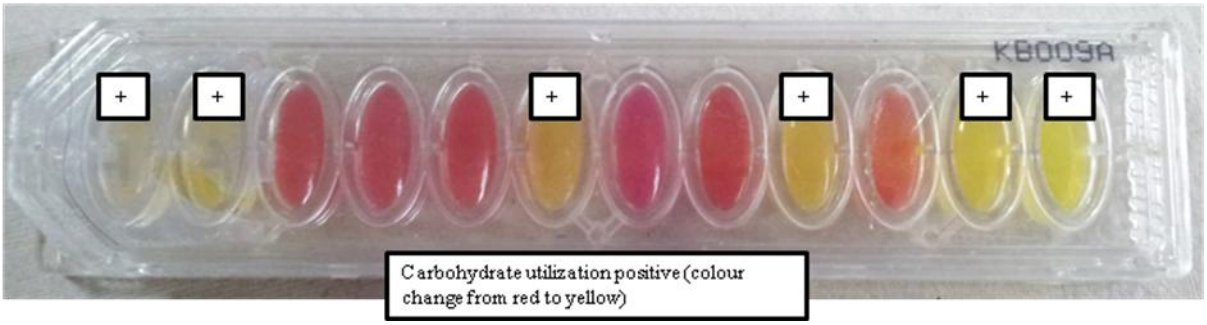
No.	Test	Reagents to be added after incubation	Principle	Original colour of the medium	Positive reaction	Negative reaction
1	ONPG	—	Detects β–galactosidase activity	Colourless	Yellow	Colourless
2	Lysine utilization	—	Detects Lysine decarboxylation	Olive green to Light Purple	Purple / Dark Purple	Yellow
3	Ornithine utilization	—	Detects Ornithine decarboxylation	Olive green to Light Purple	Purple / Dark Purple	Yellow
4	Urease	—	Detects Urease activity	Orangish yellow	Pink	Orangish yellow
5	Phenylalanine Deamination	2-3 drops of TDA reagent	Detects Phenylalanine deamination activity	Colourless	Green	Colourless
6	Nitrate reduction	1-2 drops of sulphanilic acid and 1-2 drops of N, N-Dimethyl-1-Napthylamine	Detects Nitrate reduction	Colourless	Pinkish Red	Colourless
7	H_2S production	—	Detects H_2S production	Orangish yellow	Black	Orangish yellow
8	Citrate utilization	—	Detects capability of organism to utilize citrate as a sole carbon source	Green	Blue	Green
9	Voges Proskauer's	1-2 drops of Baritt reagent A and 1-2 drops of Baritt reagent B	Detects acetoin production	Colourless / Light Yellow	Pinkish red	Colourless/ slight copper
10	Methyl red	1-2 drops of Methyl red reagent	Detects acid production	Colourless	Red	Yellowish- orange
11	Indole	1-2 drops of Kovac's red reagent	Detects deamination of tryptophan	Colourless	Pinkish Red	Colourless
12	Malonate utilization	—	Detects capability of organism to utilize sodium malonate as a sole carbon source	Light green	Blue	Light green

Strip II — Result Interpretation chart

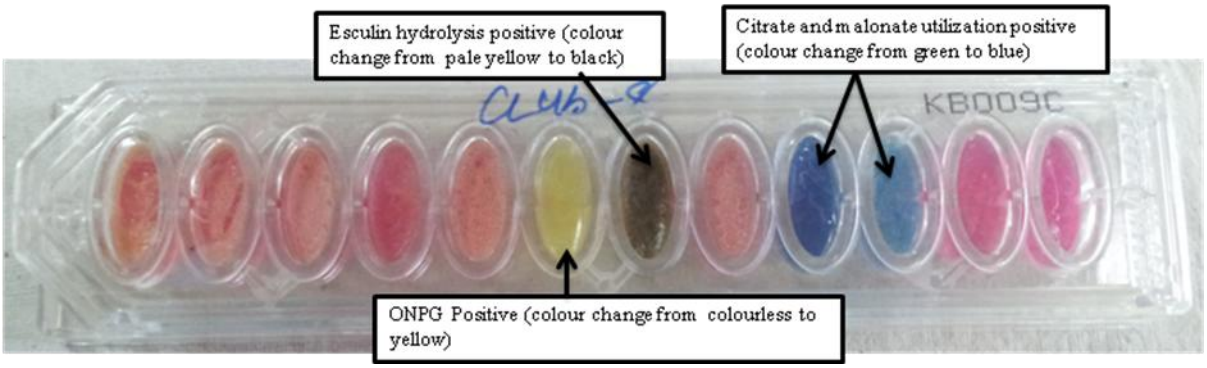
No.	Test	Principle	Original colour of the medium	Positive reaction	Negative reaction
13	Esculin hydrolysis	Esculin hydrolysis	Cream	Black	Cream
14	Arabinose	Arabinose utilization	Pinkish Red / Red	Yellow	Red / Pink
15	Xylose	Xylose utilization	Pinkish Red / Red	Yellow	Red / Pink
16	Adonitol	Adonitol utilization	Pinkish Red / Red	Yellow	Red / Pink
17	Rhamnose	Rhamnose utilization	Pinkish Red / Red	Yellow	Red / Pink
18	Cellobiose	Cellobiose utilization	Pinkish Red / Red	Yellow	Red / Pink
19	Melibiose	Melibiose utilization	Pinkish Red / Red	Yellow	Red / Pink
20	Saccharose	Saccharose utilization	Pinkish Red / Red	Yellow	Red / Pink
21	Raffinose	Raffinose utilization	Pinkish Red / Red	Yellow	Red / Pink
22	Trehalose	Trehalose utilization	Pinkish Red / Red	Yellow	Red / Pink
23	Glucose	Glucose utilization	Pinkish Red / Red	Yellow	Red / Pink
24	Lactose	Lactose utilization	Pinkish Red / Red	Yellow	Red / Pink
25	Oxidase	Done on Oxidase disc separately. Detects cytochrome oxidase production	Colourless	Deep purple within 10 seconds	White/ Purple after 60 seconds

Figure 12: Result interpretation chart for KB003

Figure 13: Biochemical Kits. KB009 Part A: This strip contains 12 wells with an appropriate medium containing lactose, xylose, maltose, fructose, dextrose, galactose, raffinose, trehalose, melibiose, sucrose, L-arabinose and mannose respectively. Part B: strip contains 12 wells and each well contains inuline, sodium gluconate, glycerol, salicin, glucosamine, dulcitol, inositol, sorbitol, mannitol, adinitol, α-methyl D- glucoside and rabinose. Strip part C contains again 12 wells with an appropriate media and represents rhamnose, cellobiose, melezitose, α-methyl D-mannoside, xylitol, ONPG, esculin, D-arabinose, citrate, malonate and sorbose. a) Shows the colour change due to utilization of carbohydrate. KB003 Strip 1: this contains 12 wells with an appropriate media and each well represents ONPG, lysine decarboxylase, ornithine decarboxylase, urease, phenyl alanine deamination, nitrate reduction, H_2S production, citrate utilization, Voges Proskauers test, methyl red, indole, and malonate respectively and the Strip 2 contains 12 wells with an appropriate media with each well containing esculin, arabinose, xylose, adonitol, rhammose, cellobiose, melibiose, saccharose, raffinose, trehalose, glucose, lactose, respectively and the last well is control.



a)



b)

Figure 14: Examples of results of HiMedia kits. a)KB009-partA and b) KB009-partC.

2.2.6. Molecular characterization: This includes comparative identification of bacterial species using 16S rRNA sequences and construction of phylogenetic trees.

2.2.6.1. PCR amplification of 16S rRNA: Comparison of 16S rRNA sequences has been the most successful method for identification of bacteria (Woese *et al.,* 1985; Woese *et al.,* 1987). 16S rRNA was PCR amplified by using two primers 16S1 (5'- GAG TTT GAT CCT GGC TCA-3') and 16S2 (5'-ACG GCT ACC TTG TTA CGA CTT-3'), complementary to the conserved regions at the 5' and 3' ends of the 16S rDNA of *E. coli* corresponding to positions 9 to 27 and 1498 to 1477, respectively. The components of reaction mixture are given in Table 3. The PCR conditions were as follows: Initial denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1 min; final extension at 72°C for 10 min. The quality and quantity of PCR products were assessed on 1.0% agarose gel along with 1Kb ladder (NEB molecular weight marker). The electrophoresis was carried out at a voltage of 90-100 V.

Table 3: Composition of PCR mix

Components	20 µL reaction volume
2X PCR Master mix (pink dye; supplied by Merck Genei™)	10 µl
Forward primer (pA)	1 µl (10 pM)
Reverse primer (pH)	1 µl (10 pM**)**
Millipore water	7.8 µl
DNA template	0.2 µl (50 ng)

2.2.6.2. Purification of PCR product: After amplification, excess primers and nucleotides act as contaminants and the PCR product cannot be used directly for sequencing purposes. The PCR product is purified using ExoSAP-IT. ExoSAP-IT is used to clean-up PCR products ranging in size from less than 100 bp to over 20 kb. This uses two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphates, to remove unwanted dNTPs and primers. ExoSAP treatment was given by adding 1 µl of the enzyme mix to 10 µl of the PCR amplicon and incubating at 37^0C for 15 min followed by enzyme inactivation at 80^0C for 15 min.

2.2.6.3. Sequencing of 16S rRNA: The 16S rRNA gene was sequenced with the primers given in Table 2. The sequencing PCR reaction mix consisted of 1µl purified PCR product of 16S rDNA (50 ng), 0.5µl of 2-5 pmoles of a single primer (Table 4), and 1.5 µl of Big Dye in a total volume of 3µl. PCR was carried out in a 96 well plate in an Eppendorf PCR machine for 30 cycles. Each cycle consisted of three steps with a denaturation of 96 ^{0}C for 10s, an annealing of 55 ^{0}C for 10s and an extension of 60^0 C for 4 min. Following PCR, 25µl of ethanol: sodium acetate mix (3 ml of absolute ethanol and 120 µl of 3M sodium acetate of pH 5.2) was added to each well, centrifuged at 4000 rpm for 15 min at 15^0 C and supernatant was discarded. The pellets were washed with 100 µl of 80% ethanol, centrifuged at 4000 rpm for 12 min at 20 ^{0}C and dried in a dark environment. Prior to submission for sequencing, 10 µl of

50% (v/v) formamide (500µl of formamide and 500 µl of sterile water) was added to each well and loaded in sequencer. The raw data obtained after sequencing was assembled manually, edited as necessary and then converted to FASTA format in a text file.

Table 4: Primers for 16S rRNA sequencing

Primers	Sequence of the primer	Binding positions of the primer
Forward primers		
pA	5'-AGA GTT TGA TCC TGG CTC AG-3'	8 to 28
pB	5'-TAA CAC ATG CAA GTC GAA CG-3'	50 to 70
pC	5'-CTA CGG GAG GCA GCA GTGGG-3'	341 to 361
pD	5'-CAG CAG CCG CGG TAA TAC-3'	518 to 536
pE	5'-AAA CTC AAA GGA ATT GAC GG-3'	908 to 928
pF	5'-CAT GGC TGT CGT CAG CTC GT-3'	1053 to 1073
Reverse primers		
pC*	5'-CCC ACT GCT GCC TCC CGT AG-3'	361 to 341
pD*	5'-GTA TTA CCG CGG CTG CTG-3'	536 to 518
pE*	5'-CCG TCA ATT CCT TTG AGT TT-3'	928 to 918
pF*	5'-ACG AGC TGA CGA CAG CCA TG-3'	1073 to 1053
pG*	5'-ACG GGC GGT GTG TAC-3'	1407 to 1392
pH*	5'-AAG GAG GTG ATC CAG CCG CA-3'	1542 to 1522

2.2.6.4. Phylogenetic analysis: Phylogenetic analysis is used for inferring or estimating the evolutionary relationships among different life forms. 16S rRNA gene is an ideal molecule, since extremely large amount of data is present in various databases and facilitates the comparison of evolutionary relationships among bacteria. For the purpose of comparison, the identification of phylogenetic neighbors was initially carried out by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1997) and Eztaxon (www.eztaxon.org) (Chun et al., 2007) against the database of type strains with validly published prokaryotic names. A phylogenetic tree, to infer the evolutionary history of the bacterial isolates, was constructed using the Molecular genetic analysis software Mega 5.0 (Tamura et al. 2011) based on the Neighbour-joining method.

3. RESULTS

3.1. Total cell count of bacteria: The total bacterial count in water samples varied from 1.5 X 10^5 to 4.5 X 10^5 cells per ml while the dead count was in the range of 1.0 X 10^4 to 3.0 X 10^4 cells per ml (Table 5). The total bacterial count of single sediment sample (Sample 4b) was 7.5 X 10^6 cells per gram.

Table 5. Total and Dead count of water and sediment samples from Chilka Lake

S.No.	Sample	Total count per ml	Dead count per ml
1.	CL1	3.3 X 10^5	2 X 10^4
2.	CL2	2.8 X 10^5	2 X 10^4
3.	CL3	2.7 X 10^5	3 X 10^4
4.	CL4a	2.3 X 10^5	1 X 10^4
5.	CL4b (Sediment sample)	1.5 X 10^5 (7.5 X 10^6 cells per gram)	3 X 10^4
6.	CL1R	3.0 X 10^5	2 X 10^4
7.	CL2R	2.4 X 10^5	1 X 10^4
8.	CL3R	4.5 X 10^5	2 X 10^4
9.	CL4R	2.4 X 10^5	3 X 10^4
10.	CL5R	1.5 X 10^5	2 X 10^4

3.2. Isolation of bacteria: Spreading of water and sediment samples on ZMA plates (Figure 15) yielded 4.19 X 10^3 to 1.37 X 10^4 colonies per ml of water and 3.93 X 10^4 colonies per gram of sediment. Close to 189 different morphotypes were picked, clonally purified by repeated streaking on ZMA plates. Out of 189 colonies, only 152 bacteria could be purified and rest 37 colonies were lost as they could not grow in pure form. The cultures were designated as CL (sample number)-(isolate number) e.g. CL1-16.

3.3. Colony morphology: The bacterial colonies showed diversity in their morphology. Their shape varied from circular to irregular, texture ranged from smooth to rough

and some colonies were raised or convex while others were flat. Some bacterial colonies were also pigmented, colours being yellow, orange, red, maroon, pink, grey and pale white. The pie diagram (Figure 16) depicts the percentage of variety in colony morphology of different bacterial isolates.

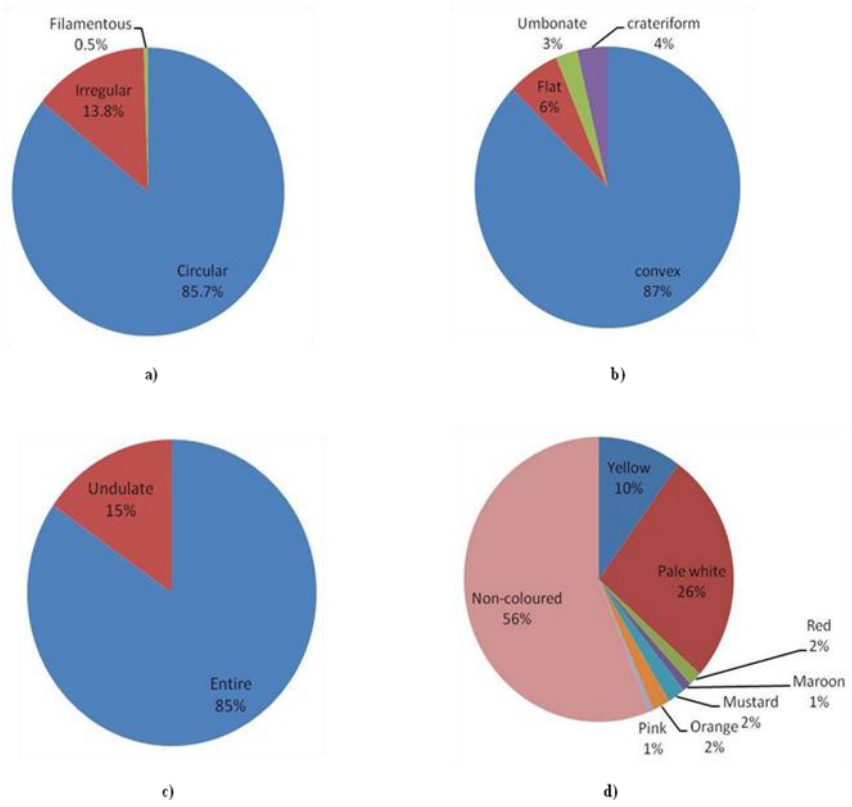


Figure 15: Morphological characteristics [a) Shape, b) elevation, c) margin and d) pigmentation] of colonies of bacteria isolated from Chilka Lake.

3.4. 16S rRNA gene sequencing: 16S rRNA gene from all the 151 bacterial isolates was amplified by colony PCR using the primers set 16S1 and 16S2. Each PCR product belonging to 151 isolates was partially sequenced using 16S1 primer and the chromatograms (opened in Geospiza's Finch TV) obtained were converted to FASTA format. These sequences were subjected to BLAST sequence similarity search. The similarity of these query sequences ranged from 96-100% with the subject sequences in NCBI database (Table 5). The BLAST results indicated that the *Gammaproteobacteria* was the most dominating community (65.0%) followed by *Firmicutes* (23.0%) and other

communities such as *Alphaproteobacteria*, *Betaproteobacteria*, *Acenetobacter* and *Bacteriodetes* were least dominating (Figure 17). All the 151 bacteria belonged to 57 species representing 25 genera which were then categorized, on the basis of 16S rRNA gene sequence, according to their phylum, class, genus and species (Table 6). Among the 25 genera identified in the present study, the genera *Pseudomonas* (34.4%), *Bacillus* (20.5%), *Rheinheimera* (11.9%) and *Pseudoalteromonas* (6.6%) were the most predominating and rest of the genera were represented by less than 5 isolates. Among all the genera, the genus *Bacillus* seems to be more diverse and represented by 11 species followed by the genus *Pseudomonas* which contained 10 species (Table 5 and 6). For the purpose of complete characterization, 52 bacteria exhibiting 98.0% or less were sequenced for full length (approx. 1.5 kb) 16S rRNA gene and also studied some of the important enzyme that can be of use in bioctechnology industries. Most of the strains exhibited a 16S rRNA gene sequence similarity of more than 98.0% after sequencing complete 16S rRNA gene with their nearest phylogenetic neighbor except strains CL1-16, CL1-17, CL3-4, CL3-9, CL4a-14, CL4b-2, CL4b-8, CL1R-2, CL2R-4 and CL3R-6 wherein they exhibited less than or equal to 98.0% sequence similarity at 16S rRNA gene, indicating that they could be potential novel species (Stackebrandt & Geobel, 1994). The affiliation of all the species to their nearest neighbor was further supported by phylogenetic analysis performed using neighbor joining option of MEGA 5 (Tamura *et al.,* 2011) (Figure 18 a-y).

Table 6. Affiliation of bacteria isolated from Chilka lake, Odisha to their nearest phylogenetic neighbour, based on partial 16S rRNA gene sequence

S.No.	Sample Designation	Nearest phylogenetic neighbour	Percentage similarity
Phylum: *Proteobacteria*			
Class: *Alphaproteobacteria*			
1	CL4b-17	*Paracoccus homiensis spH-35*	99
2	CL5R-15	*Paracoccus marcusii YUAB-SO-38*	99
3	CL3-4	*Phaeobacter gallaeciensis A90a_4k*	98
4	CL3-9	*Phaeobacter gallaeciensis A90a_4k*	98
5	CL1R-8	*Sphingobium yanoikuyae D1AR13*	100
6	CL3R-16	*Sphingobium yanoikuyae D1AR13*	99
Class: *Betaprotobacteria*			
7	CL1-3b	*Limnobacter thioxidans TSWCSN35*	98
Class: *Gammaproteobacteria*			
8	CL2-29	*Acinetobacter venetianus L21*	99
9	CL3R-6	*Alishewanella jeotgali MS1*	97
10	CL4R-4	*Alishewanella tabrizica RCRI4*	97
11	CL4R-10	*Alteromonas macleodii AD45*	100
12	CL2R-6	*Alteromonas macleodii NH87-23*	99
13	CL1-1	*Arsukibacterium ikkense K1-118*	98
14	CL1-2	*Arsukibacterium ikkense K1-118*	98
15	CL3-12	*Marinomonas communis AN32*	99
16	CL1-9	*Pseudoalteromonas ganghwensis*	99
17	CL2-20	*Pseudoalteromonas ganghwensis*	99
18	CL2R-8	*Pseudoalteromonas lipolytica VSW332*	99
19	CL2R-15	*Pseudoalteromonas mariniglutinosa BH28*	100
20	CL3R-5	*Pseudoalteromonas mariniglutinosa BH33*	99
21	CL3R-7	*Pseudoalteromonas mariniglutinosa BH33*	100
22	CL4R-9	*Pseudoalteromonas mariniglutinosa BH33*	100
23	CL1R-5	*Pseudoalteromonas mariniglutinosa BH33*	100
24	CL1R-3	*Pseudoalteromonas prydzensis CAIM 381*	99
25	CL3R-13	*Pseudoalteromonas ulvae H34q-5a*	99
26	CL2-4	*Pseudomonas alcaligenes EPAn5*	98
27	CL3-3	*Pseudomonas alcaligenes EPAn5*	99
28	CL4R-3	*Pseudomonas alcaligenes EPAn5*	98
29	CL4R-7	*Pseudomonas alcaligenes EPAn5*	99
30	CL2-30b	*Pseudomonas alcaliphila D11*	99
31	CL2R-4	*Pseudomonas azotoformans EN4*	100
32	CL2-32	*Pseudomonas fluorescens 1573*	99
33	CL3-13	*Pseudomonas mendocina CHN12*	99
34	CL1-16	*Pseudomonas mendocina PMLR-1*	98
35	CL2-5	*Pseudomonas mendocina PMLR-1*	98
36	CL2-7	*Pseudomonas mendocina PMLR-1*	98
37	CL2-8	*Pseudomonas mendocina PMLR-1*	98
38	CL2-19	*Pseudomonas mendocina PMLR-1*	99
39	CL2-22	*Pseudomonas mendocina PMLR-1*	98
40	CL2-25	*Pseudomonas mendocina PMLR-1*	98
41	CL2-26	*Pseudomonas mendocina PMLR-1*	98
42	CL4a-8	*Pseudomonas mendocina PMLR-1*	98
43	CL2-28	*Pseudomonas oleovorans HNS030*	99
44	CL5R-13	*Pseudomonas peli W1AP13*	100
45	CL2-6	*Pseudomonas pseudoalcaligenes A3*	99
46	CL1-10	*Pseudomonas pseudoalcaligenes GGRJ77*	99
47	CL1-12	*Pseudomonas pseudoalcaligenes GGRJ77*	99
48	CL2-11	*Pseudomonas pseudoalcaligenes GGRJ77*	99
49	CL2-13	*Pseudomonas pseudoalcaligenes GGRJ77*	99
50	CL2-31	*Pseudomonas pseudoalcaligenes GGRJ77*	99
51	CL3-22	*Pseudomonas pseudoalcaligenes GGRJ77*	99

S.No.	Sample Designation	Nearest phylogenetic neighbour	Percentage similarity
52	CL3R-1	*Pseudomonas pseudoalcaligenes GGRJ77*	98
53	CL3R-4	*Pseudomonas pseudoalcaligenes GGRJ77*	98
54	CL3R-12	*Pseudomonas pseudoalcaligenes GGRJ77*	99
55	CL3R-14	*Pseudomonas pseudoalcaligenes GGRJ77*	99
56	CL3R-18	*Pseudomonas pseudoalcaligenes GGRJ77*	99
57	CL3R-19	*Pseudomonas pseudoalcaligenes GGRJ77*	99
58	CL3R-21	*Pseudomonas pseudoalcaligenes GGRJ77*	99
59	CL5R-17	*Pseudomonas pseudoalcaligenes GGRJ77*	99
60	CL3R-10	*Pseudomonas pseudoalcaligenes GGRJ77*	99
61	CL5R-11	*Pseudomonas pseudoalcaligenes GGRJ77*	99
62	CL2-3	*Pseudomonas pseudoalcaligenes PPS*	99
63	CL2-21	*Pseudomonas pseudoalcaligenes PPS*	99
64	CL2-23	*Pseudomonas pseudoalcaligenes PPS*	98
65	CL1-11	*Pseudomonas stutzeri DQ-1*	100
66	CL1-15	*Pseudomonas stutzeri DQ-1*	100
67	CL2-10	*Pseudomonas stutzeri DQ-1*	100
68	CL2-12	*Pseudomonas stutzeri DQ-1*	100
69	CL2-14	*Pseudomonas stutzeri DQ-1*	100
70	CL2R-11	*Pseudomonas stutzeri DQ-1*	99
71	CL3R-8	*Pseudomonas stutzeri DQ-1*	99
72	CL3R-9	*Pseudomonas stutzeri DQ-1*	99
73	CL3R-20	*Pseudomonas stutzeri DQ-1*	99
74	CL5R-10	*Pseudomonas stutzeri DQ-1*	100
75	CL5R-12	*Pseudomonas stutzeri DQ-1*	100
76	CL4a-12	*Pseudomonas stutzeri P3*	99
77	CL3-6	*Pseudomonas toyotomiensis GL19*	99
78	CL1R-2	*Rheinheimera aquimaris ML-2*	98
79	CL1-4	*Rheinheimera aquimaris ML-2*	98
80	CL1-5	*Rheinheimera aquimaris ML-2*	98
81	CL1-7	*Rheinheimera aquimaris ML-2*	98
82	CL1R-6	*Rheinheimera aquimaris ML-2*	99
83	CL2R-13	*Rheinheimera aquimaris ML-2*	99
84	CL3-8	*Rheinheimera aquimaris PM02*	99
85	CL1R-1	*Rheinheimera aquimaris PM02*	100
86	CL2R-7	*Rheinheimera aquimaris PM02*	99
87	CL4R-1	*Rheinheimera aquimaris PM02*	100
88	CL4R-2	*Rheinheimera aquimaris PM03*	98
89	CL1-3a	*Rheinheimera aquimaris SW-369*	98
90	CL4a-2	*Rheinheimera aquimaris SW-369*	99
91	CL4a-4	*Rheinheimera aquimaris SW-369*	98
92	CL4a-6	*Rheinheimera aquimaris SW-369*	98
93	CL4R-11	*Rheinheimera aquimaris SW-369*	99
94	CL3R-11	*Rheinheimera aquimaris SW-369*	99
95	CL5R-7	*Rheinheimera nanhaiensis E407-8*	99
96	CL1-6	*Shewanella amazonensis IH7-17*	97
97	CL4R-5	*Shewanella amazonensis SB2B*	99
98	CL3-7	*Simiduia agarivorans SA1 = DSM 21679*	96
99	CL2R-3	*Vibrio diazotrophicus N6*	100
100	CL3-14	*Vibrio fluvialis LCB1*	99
101	CL2R-10	*Vibrio fluvialis LCB1*	100
102	CL3-10	*Vibrio furnissii NCTC 11218*	99
103	CL3-18	*Vibrio furnissii M1*	99
104	CL3-20	*Vibrio rotiferianus C154*	99
105	CL3-5	*Vibrio vulnificus CMCP6*	100
106	CL3-16	*Vibrio vulnificus CMCP6*	99

S.No.	Sample Designation	Nearest phylogenetic neighbour	Percentage similarity
Phylum: *Actinobacteria*			
107	CL4b-8	*Brachybacterium faecium DSM 4810*	99
108	CL1-19	*Brachybacterium paraconglomeratum 4M1SA*	100
109	CL1-21	*Brachybacterium paraconglomeratum 4M1SA*	96
110	CL1-20	*Brachybacterium paraconglomeratum SS263*	99
111	CL2-17	*Kocuria palustris XFB-BH*	100
112	CL5R-3	*Kocuria rosea zhy40*	99
113	CL5R-5	*Kocuria rosea zhy40*	100
114	CL1-18	*Microbacterium testaceum PCSB7*	99
115	CL1-17	*Streptomyces vinaceusdrappus ASU 179*	99
Phylum: *Firmicutes*			
116	CL4a-10	*Bacillus aryabhattai AIMST Aie10*	99
117	CL1R-10	*Bacillus aryabhattai KIIT BE1*	99
118	CL1-24	*Bacillus aryabhattai SCSGAB0134*	99
119	CL4a-9	*Bacillus aryabhattai SCSGAB0134*	99
120	CL2-27	*Bacillus cereus F2*	100
121	CL4b-2	*Bacillus humi BMGAE8*	100
122	CL4b-13	*Bacillus hwajinpoensis JC127*	97
123	CL4b-12	*Bacillus infantis CS6050*	100
124	CL4b-18	*Bacillus infantis isolate 1.19*	99
125	CL4b-6	*Bacillus jeotgali CCMM B670*	99
126	CL5R-8	*Bacillus marisflavi C095*	100
127	CL1-26	*Bacillus megaterium 3AP1*	99
128	CL4a-14	*Bacillus megaterium AIMST Lce46*	98
129	CL1-22	*Bacillus megaterium BAB-2448*	99
130	CL4b-9	*Bacillus megaterium H2*	99
131	CL2R-16	*Bacillus megaterium KUDC1718*	100
132	CL4a-13	*Bacillus megaterium WS19*	99
133	CL2R-17	*Bacillus megaterium X14*	100
134	CL4a-11	*Bacillus megaterium y35-17*	99
135	CL4b-14	*Bacillus megaterium ZSC-2*	95
136	CL4R-12	*Bacillus pumilus DM-B23*	100
137	CL4b-5	*Bacillus pumilus JN31*	99
138	CL4R-15	*Bacillus safensis CSR-B-5*	100
139	CL4R-16	*Bacillus safensis CSR-B-5*	100
140	CL1-25	*Bacillus subtilis 224*	99
141	CL2R-18	*Bacillus subtilis DMRB-4*	100
142	CL1R-12	*Bacillus subtilis nsp13*	99
143	CL1R-11	*Bacillus subtilis RS2*	100
144	CL4R-14	*Bacillus subtilis V26*	100
145	CL3-17	*Bacillus subtilis VKMM066*	99
146	CL3-19	*Bacillus subtilis VP13*	99
147	CL2-30a	*Carnobacterium maltaromaticum MMF-23*	99
148	CL4b-7	*Halobacillus trueperi KBM KVKPC22*	99
149	CL3-21	*Paenibacillus illinoisensis F24*	99
150	CL4a-3b	*Staphylococcus equorum KS7*	99
Phylum: *Bacteriodetes*			
151	CL4a-7	*Flavobacterium cauense NBRC 104929*	96

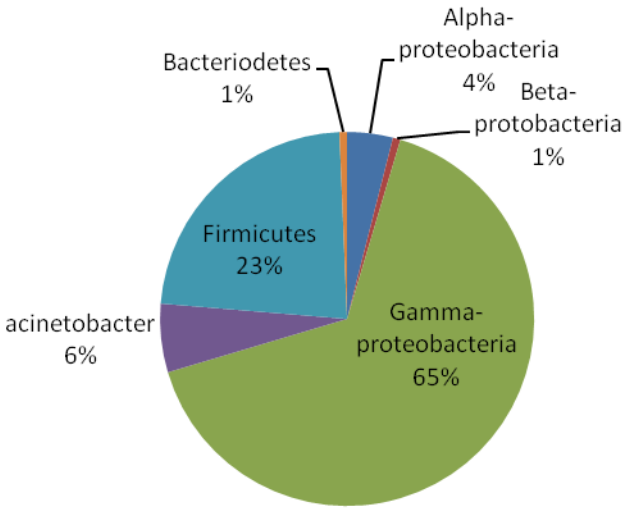


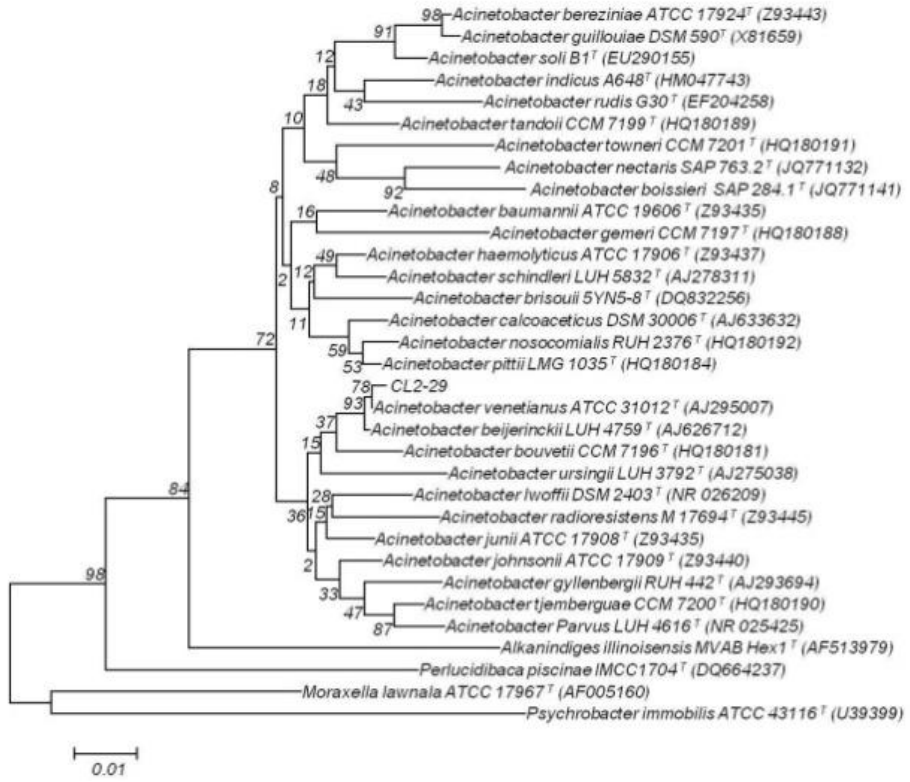
Figure 16: Pie chart depicting diversity of bacteria isolated from Chilka Lake.

Table 7. Classification of bacterial isolates from Chilka Lake, Odisha into different taxa*.

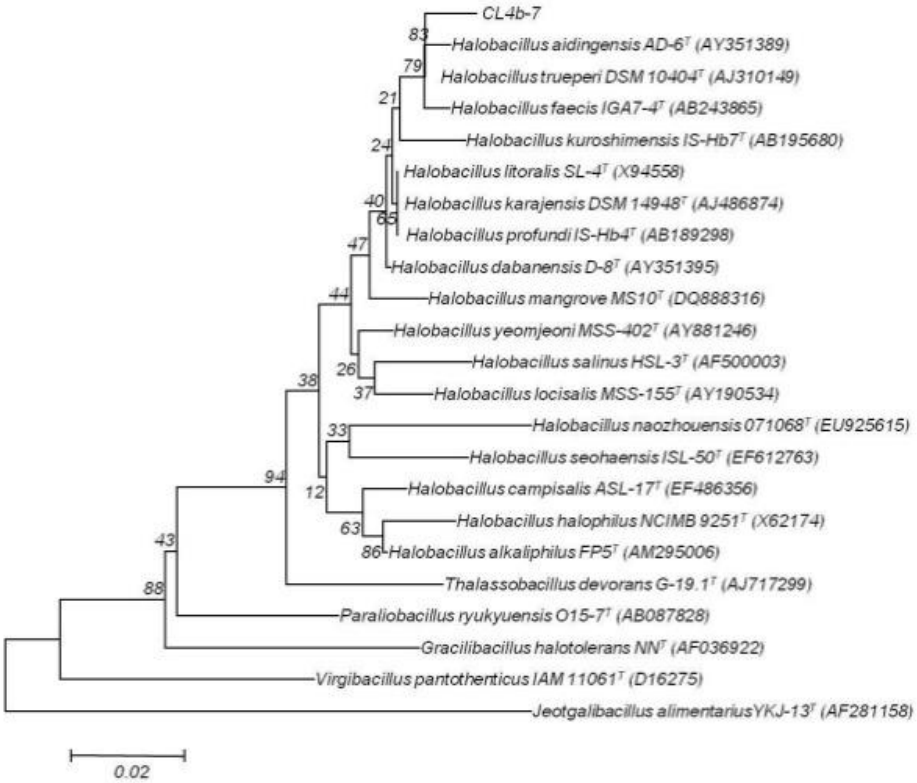
S.No.	Genera	Species	No. of isolates
Phylum: *Gammaproteobacteria*			
Class: *Alphaproteobacteria*			
1.	*Paracoccus* (2)	*P. homiensis*	1
		P. marcusii	1
2.	*Phaeobacter* (2)	*P. gallaeciensis*	2
3.	*Sphingobium* (2)	*S. yanoikuyae*	2
Class: *Betaproteobacteria*			
4.	*Limnobacter* (1)	*L. thiooxidans*	1
Class: *Gammaproteobacteria*			
5.	*Acinetobacter* (1)	*A. venetianus*	1
6.	*Alishewanella* (2)	*A. jeotgali*	1
		A. tabrizica	1
7.	*Alteromonas* (2)	*A. macleodii*	2
8.	*Arsukibacterium* (2)	*A. ikkense*	2
9.	*Marinomonas* (1)	*M. communis*	1
10.	*Pseudoalteromonas* (10) (5 speceis)	*P. ganghwensis*	2
		P. lipolytica	1
		P. mariniglutinosa	5
		P. prydzensis	1
		P. ulvae	1
11.	*Pseudomonas* (52) (10 speceis)	*P. alcaligenes*	4
		P. alcaliphila	1
		P. azotoformans	1
		P. fluorescens	1
		P. mendocina	10
		P. oleovorans	1
		P. peli	1
		P. pseudoalcaligenes	20
		P. stutzeri	12
		P. toyotomiensis	1
12.	*Rheinheimera* (18) (2 speceis)	*R. aquimaris*	17
		R. nanhaiensis	1
13.	*Shewanella* (2)	*S. amazonensis*	2

S.No.	Genera	Species	No. of isolates
14.	*Simiduia* (1)	*S. agarivorans*	1
15.	*Vibrio* (2)	*V. diazotrophicus*	1
		V. fluvialis	2
		V. furnissii	2
		V. rotiferianus	1
		V. vulnificus	2
Phylum: *Actinobacteria*			
16.	*Brachybacterium* (4)	*B. faecium*	1
		B. paraconglomeratum	3
17.	*Kocuria* (3)	*K. palustris*	1
		K. rosea	2
18.	*Microbacterium* (1)	*M. testaceum*	1
19.	*Streptomyces* (1)	*S. vinaceusdrappus*	1
Phylum: *Firmicutes*			
20.	*Bacillus* (31) (11 speceis)	*B. aryabhattai*	4
		B. cereus	1
		B. hwajinpoensis	1
		B. humi	1
		B. infantis	2
		B. jeotgali	1
		B. marisflavi	1
		B. megaterium	9
		B. pumilus	2
		B. safensis	2
		B. subtilis	7
21.	*Carnobacterium* (1)	*C. maltaromaticum*	1
22.	*Halobacillus* (1)	*H. trueperi*	1
23.	*Paenibacillus* (1)	*P. illinoisensis*	1
24.	*Staphylococcus* (1)	*S. eqorum*	1
Phylum: *Bacteriodetes*			
25.	*Flavobacterium* (1)	*F. cauense*	1

*Numbers in brackets indicate number of strains and species in respective genera

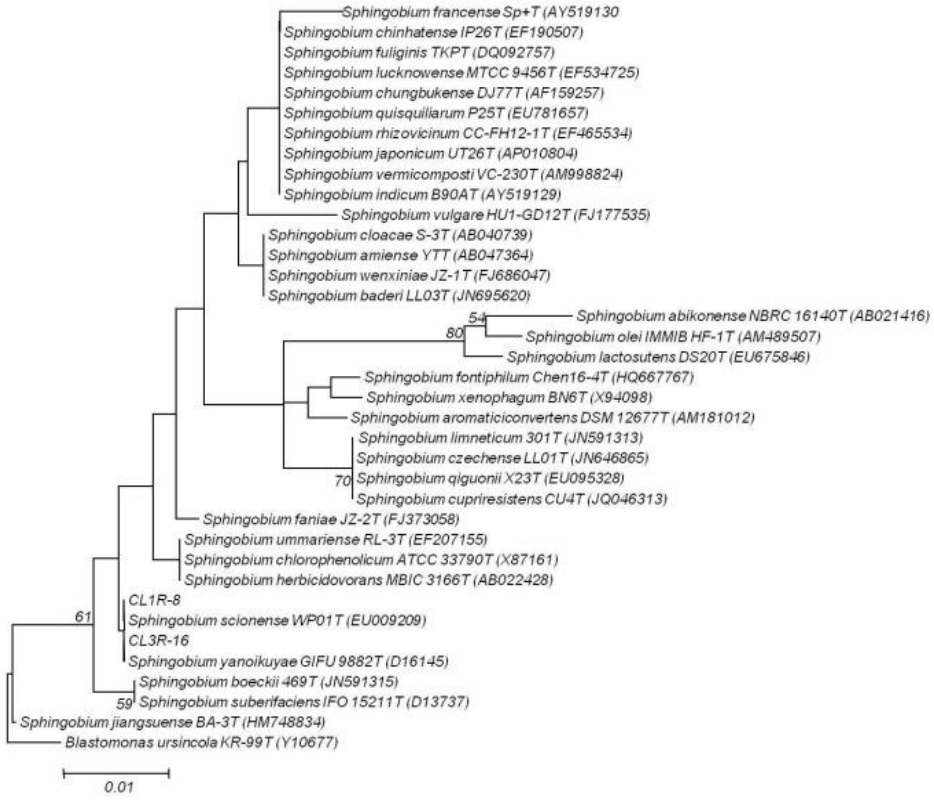


a)



b)

Figure 17: Phylogenetic analysis using neighbour-joining of MEGA 5, based on 16S rRNA gene sequences of bacterial isolates from Chilka Lake, Odisha with their respective genera a) *Acinetobacter* and b) *Halobacillus.* Percentage bootstrap values are indicated at node.



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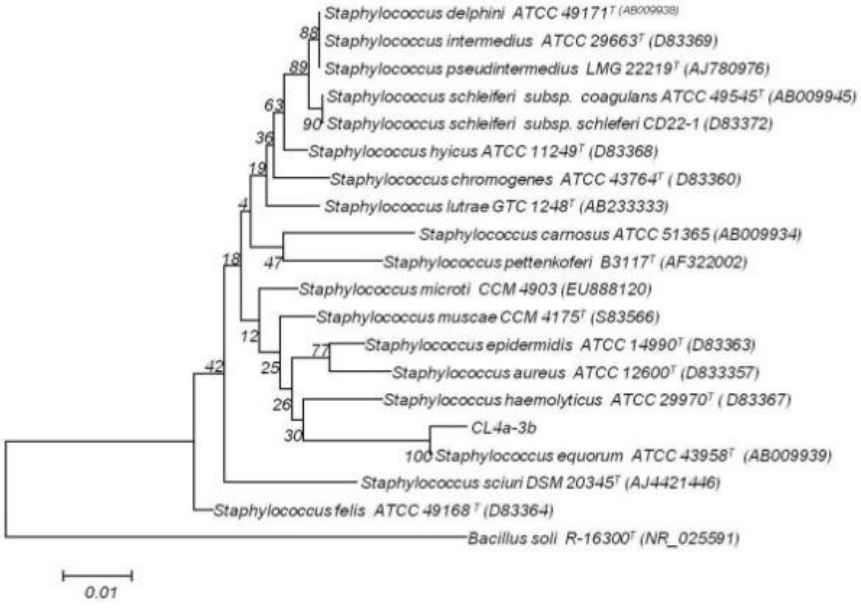
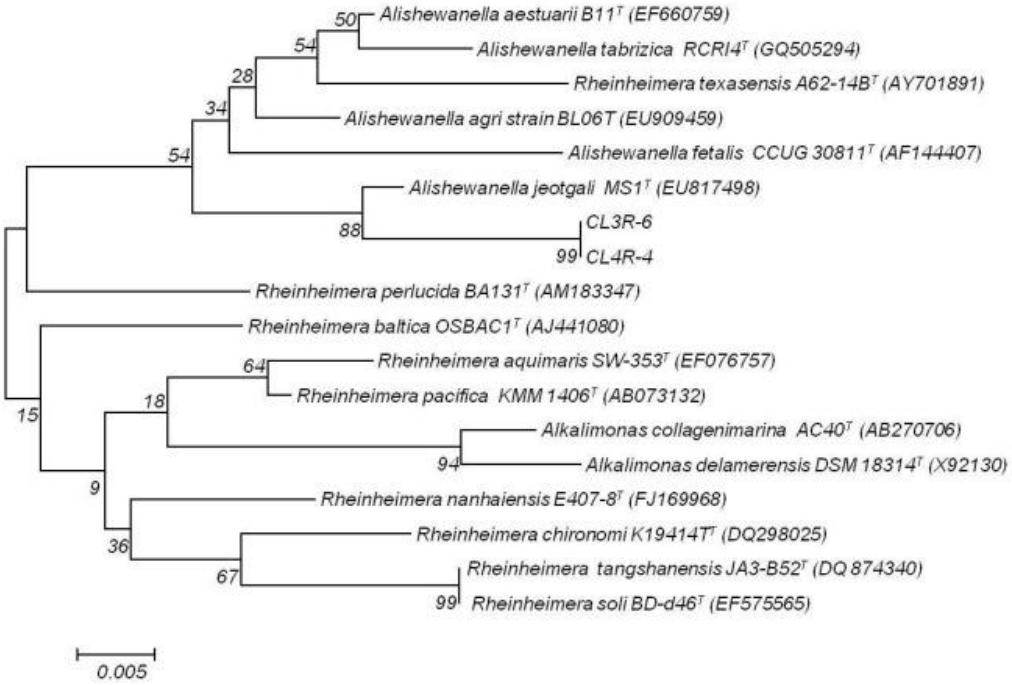
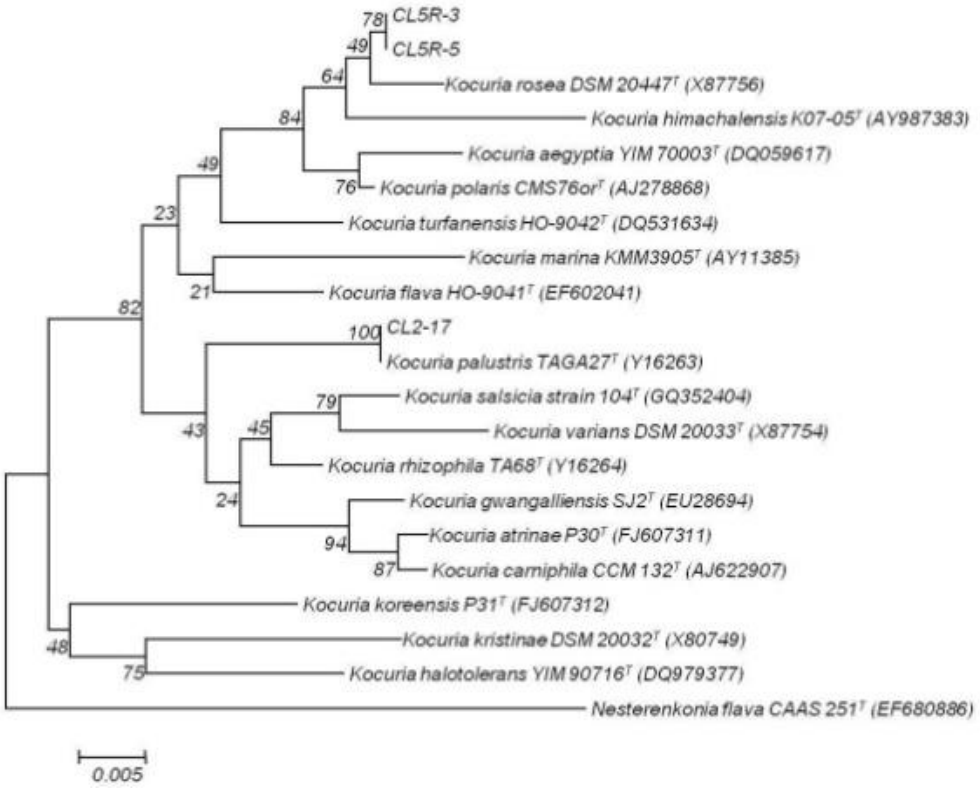


b)

Figure 18: Phylogenetic analysis using neighbour-joining of MEGA 5, based on 16S rRNA gene sequences of bacterial isolates from Chilka Lake, Odisha with their respective genera a) *Sphingobium* and b) *Staphylococcus.* Percentage bootstrap values are indicated at node.

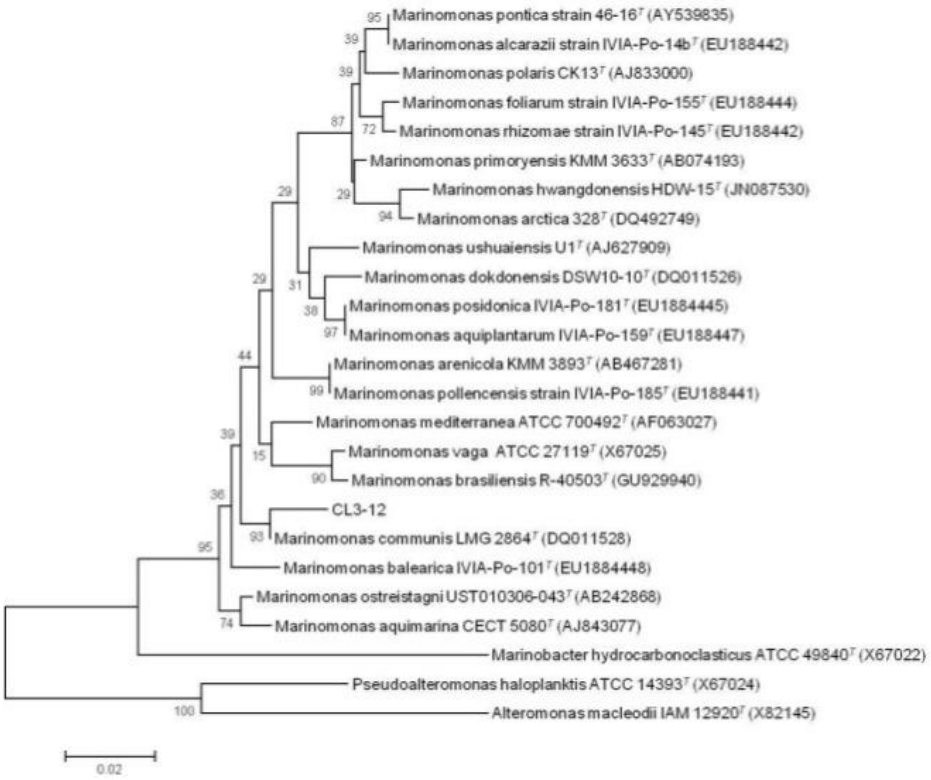


a)

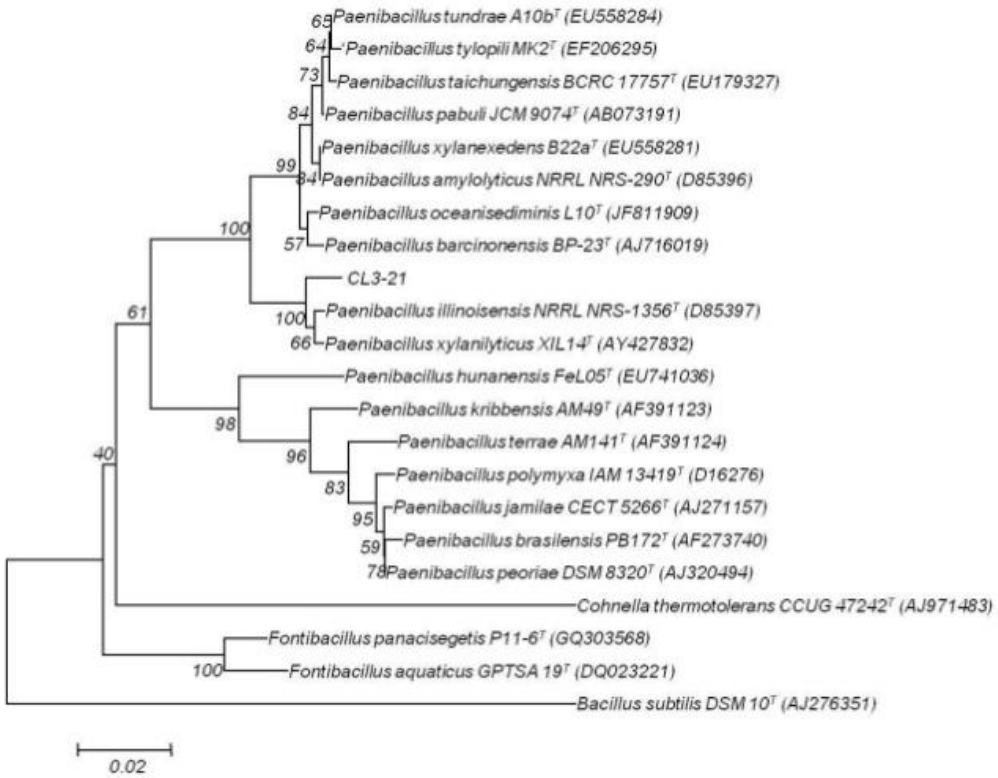


b)

Figure 19: Phylogenetic analysis using neighbour-joining of MEGA 5, based on 16S rRNA gene sequences of bacterial isolates from Chilka Lake, Odisha with their respective genera a) *Alishewanella* and b) *Kocuria.* Percentage bootstrap values are indicated at node.

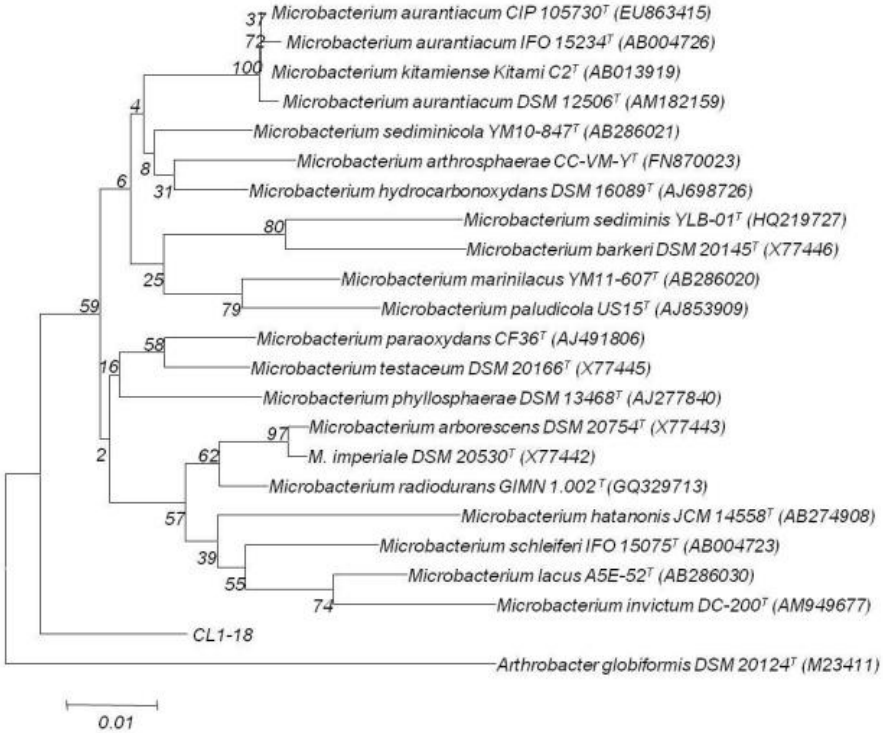


a)



b)

Figure 20: Phylogenetic analysis using neighbour-joining of MEGA 5, based on 16S rRNA gene sequences of bacterial isolates from Chilka Lake, Odisha with their respective genera a) *Marinomonas* and b) *Paenibacillus.* Percentage bootstrap values are indicated at node.



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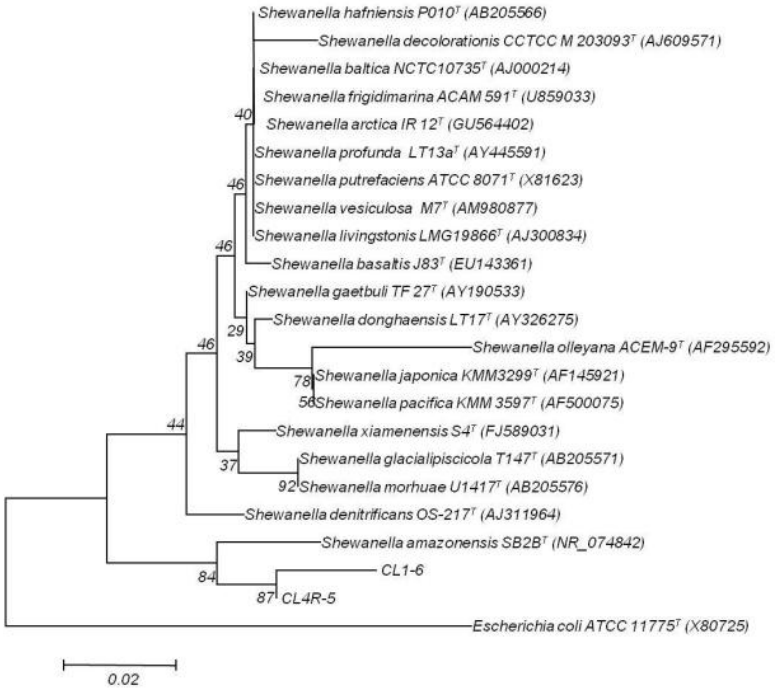
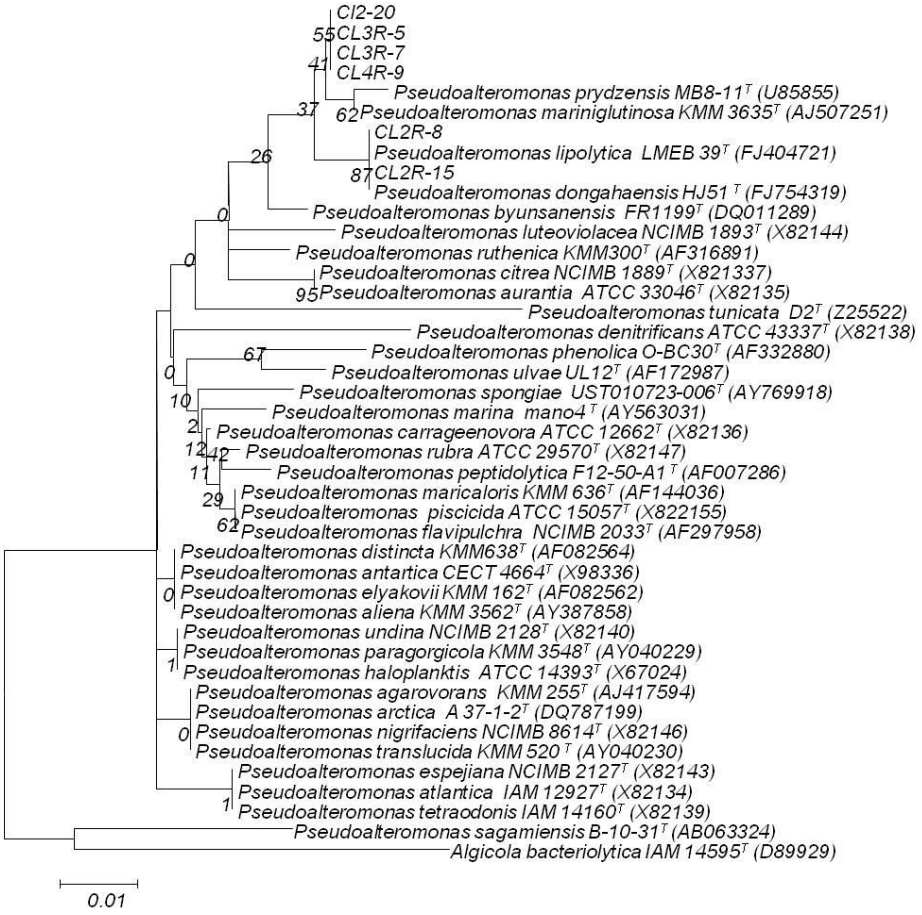


b)

Figure 21: Phylogenetic analysis using neighbour-joining of MEGA 5, based on 16S rRNA gene sequences of bacterial isolates from Chilka Lake, Odisha with their respective genera a) *Microbacterium* and b) *Shewanella.* Percentage bootstrap values are indicated at node.



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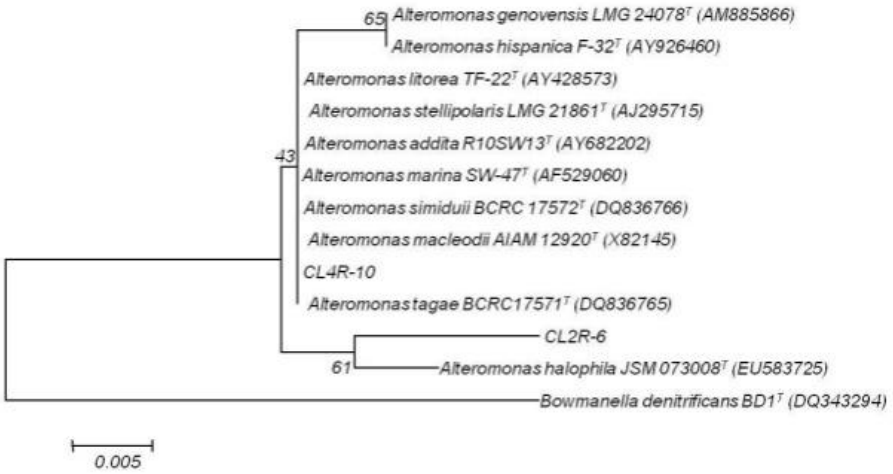
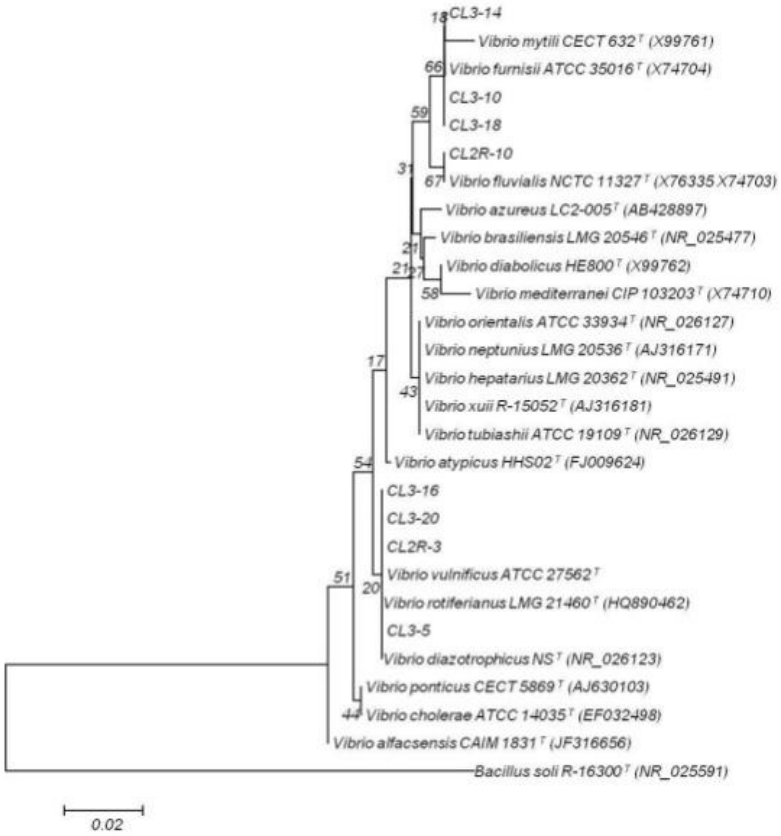
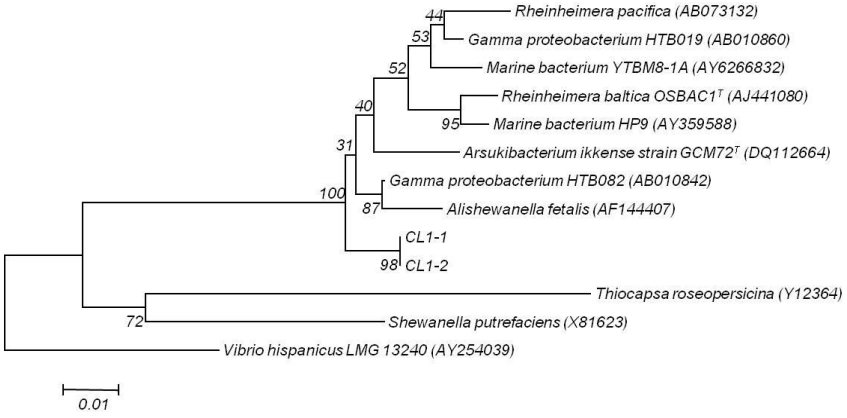


b)

Figure 22: Phylogenetic analysis using neighbour-joining of MEGA 5, based on 16S rRNA gene sequences of bacterial isolates from Chilka Lake, Odisha with their respective genera a) *Pseudoalteromonas* and b) *Alteromonas.* Percentage bootstrap values are indicated at node.

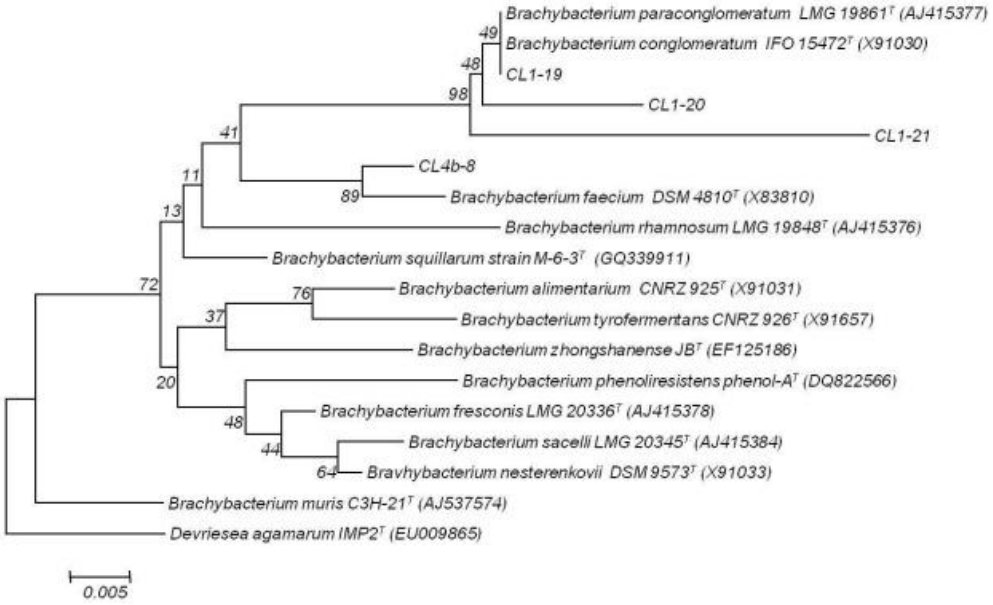


a)

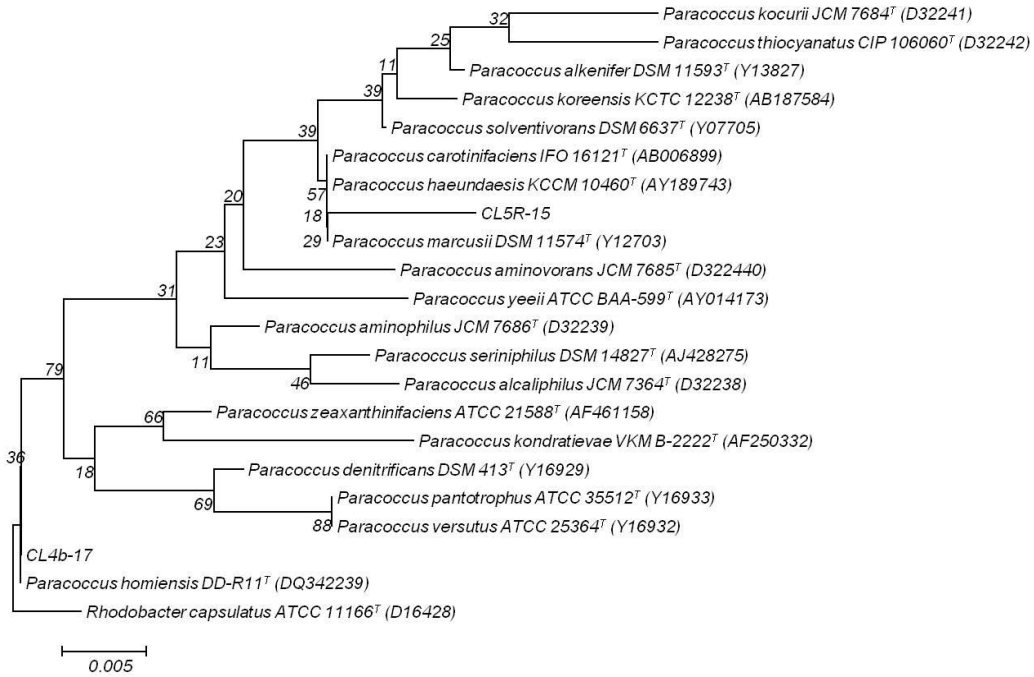


b)

Figure 23: Phylogenetic analysis using neighbour-joining of MEGA 5, based on 16S rRNA gene sequences of bacterial isolates from Chilka Lake, Odisha with their respective genera a) *Vibrio* and b) *Arsukibacterium*. Percentage bootstrap values are indicated at node.

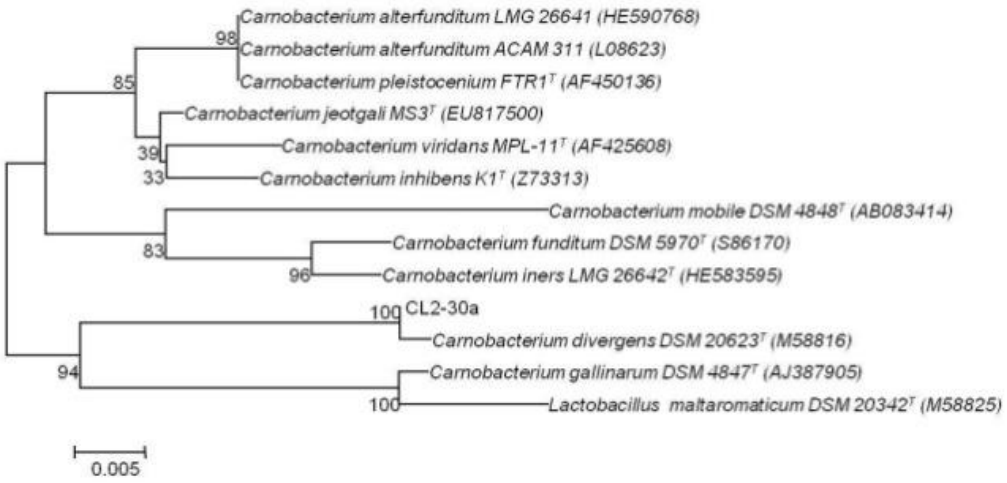


a)

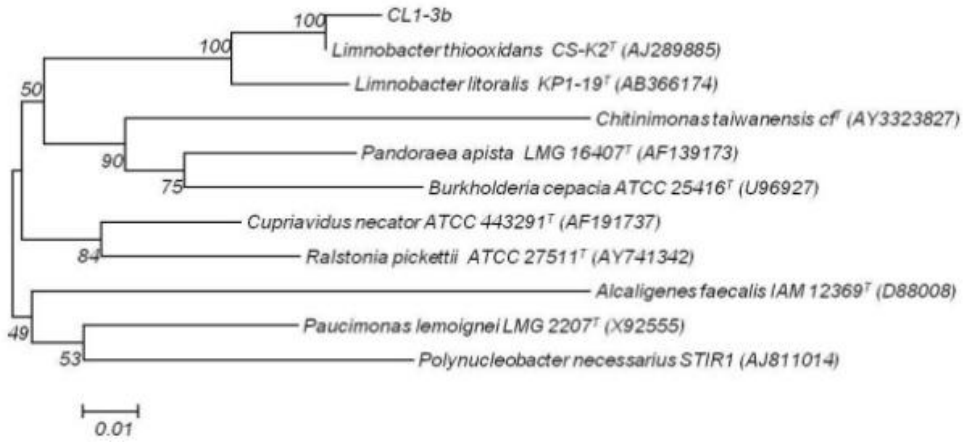


b)

Figure 24: Phylogenetic analysis using neighbour-joining of MEGA 5, based on 16S rRNA gene sequences of bacterial isolates from Chilka Lake, Odisha with their respective genera a) *Brachybacterium* and b) *Paracoccus.* Percentage bootstrap values are indicated at node.

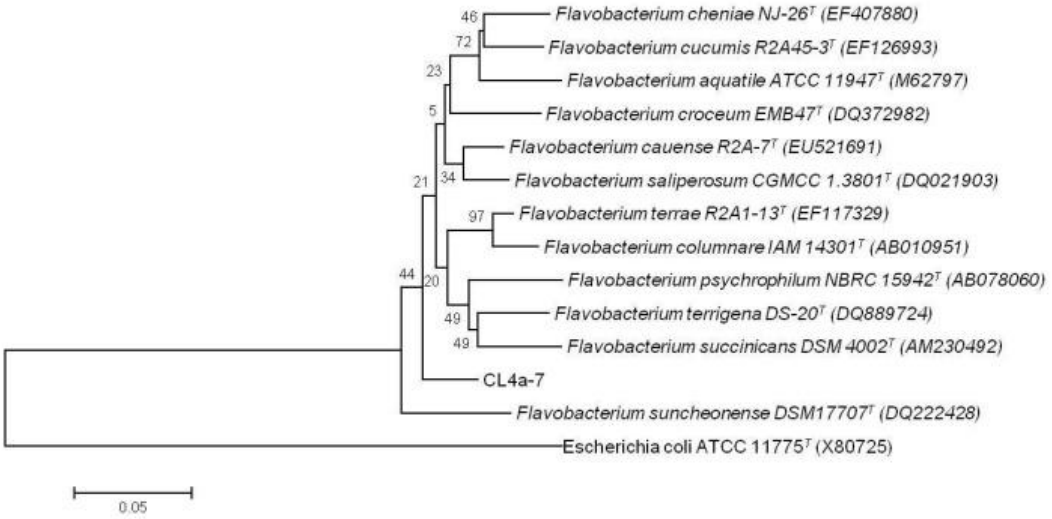


a)

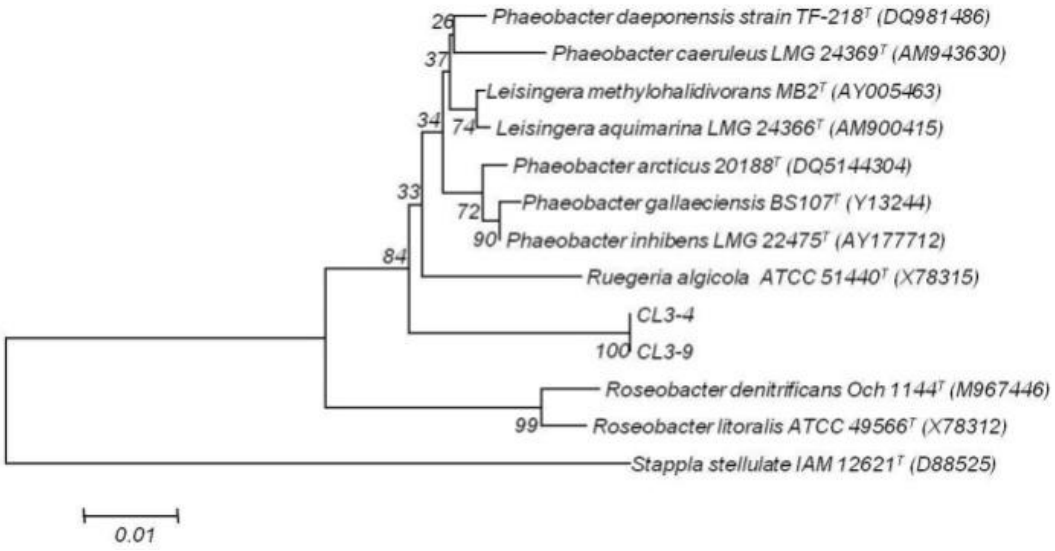


b)

Figure 25: Phylogenetic analysis using neighbour-joining of MEGA 5, based on 16S rRNA gene sequences of bacterial isolates from Chilka Lake, Odisha with their respective genera a) *Carnobacterium* and b) *Limnobacterium*. Percentage bootstrap values are indicated at node.

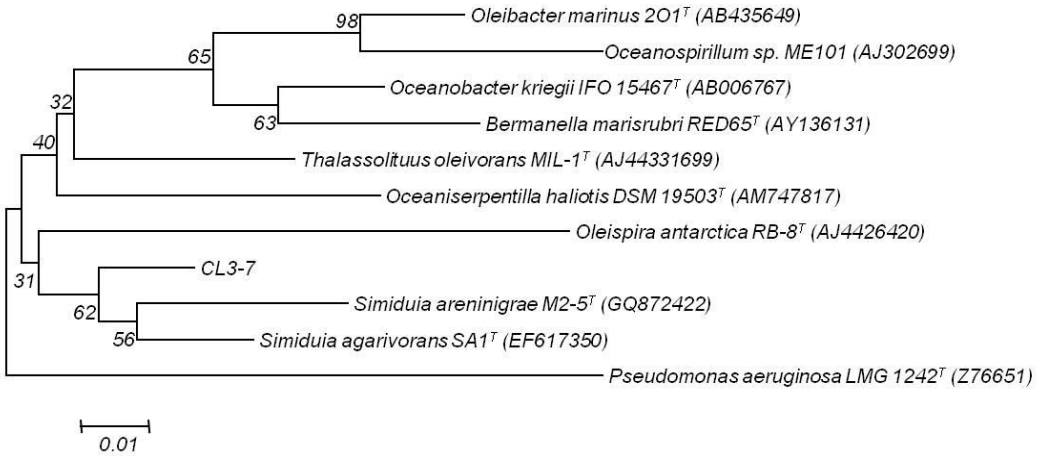


a)

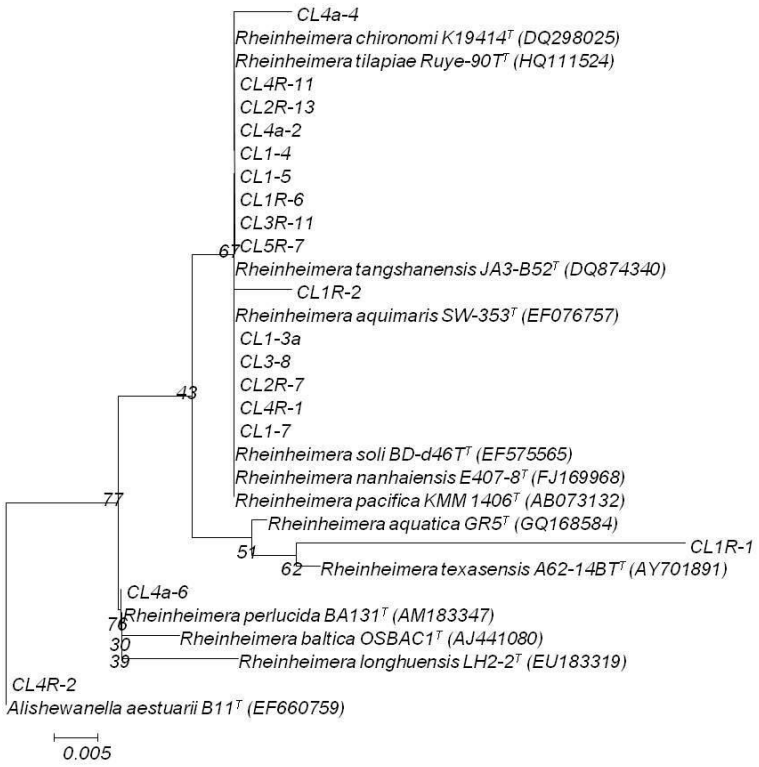


b)

Figure 26: Phylogenetic analysis using neighbour-joining of MEGA 5, based on 16S rRNA gene sequences of bacterial isolates from Chilka Lake, Odisha with their respective genera a) *Flavobacterium* and b) *Phaeobacter.* Percentage bootstrap values are indicated at node.

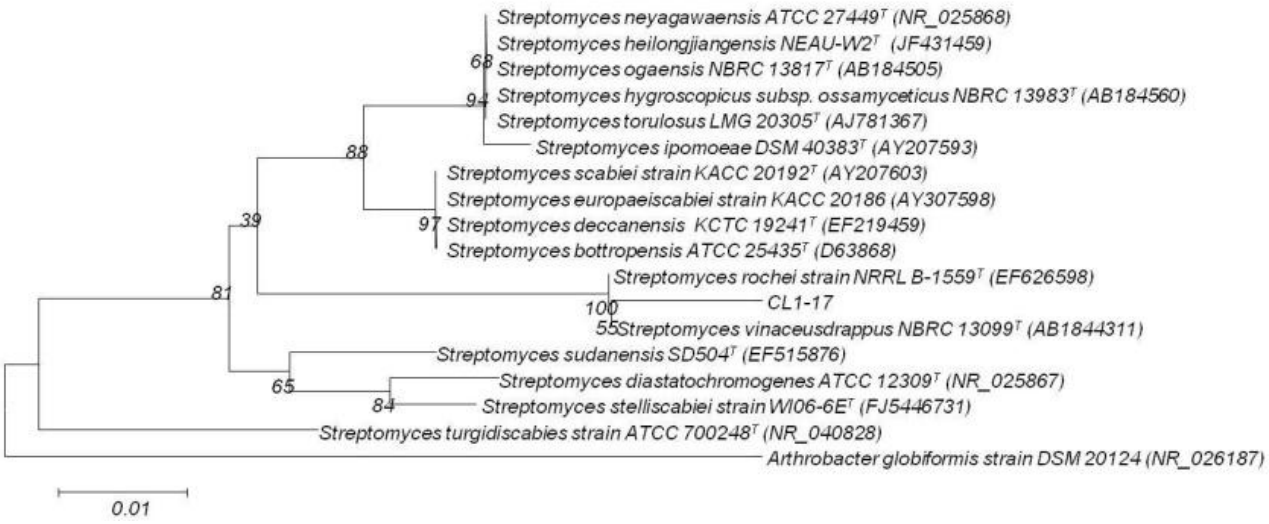


a)

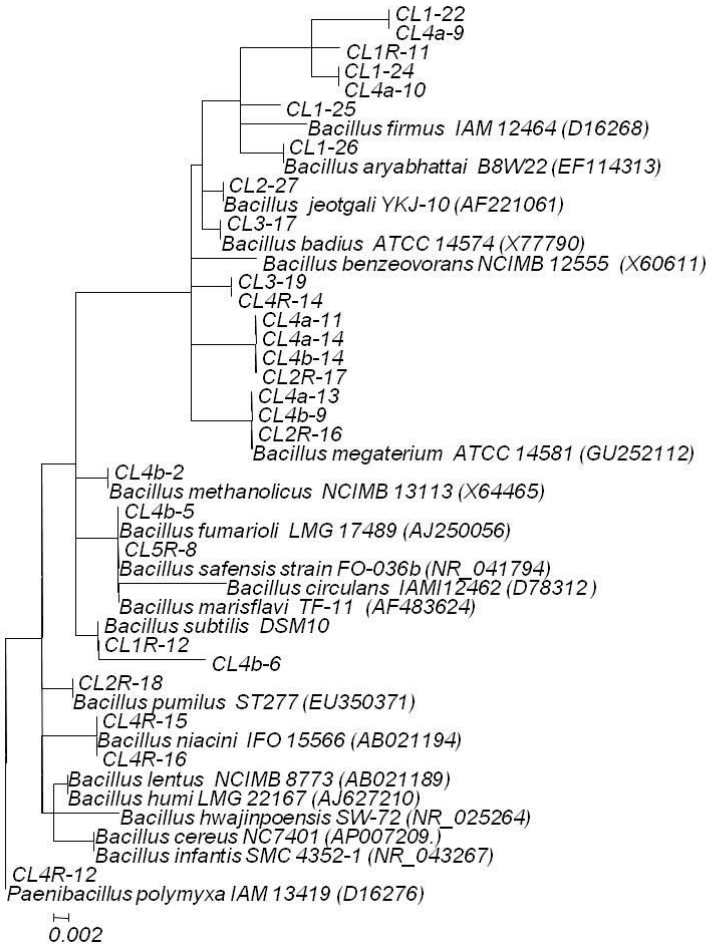


b)

Figure 27: Phylogenetic analysis using neighbour-joining of MEGA 5, based on 16S rRNA gene sequences of bacterial isolates from Chilka Lake, Odisha with their respective genera a) *Simiduia* and b) *Rheinheimera.* Percentage bootstrap values are indicated at node.

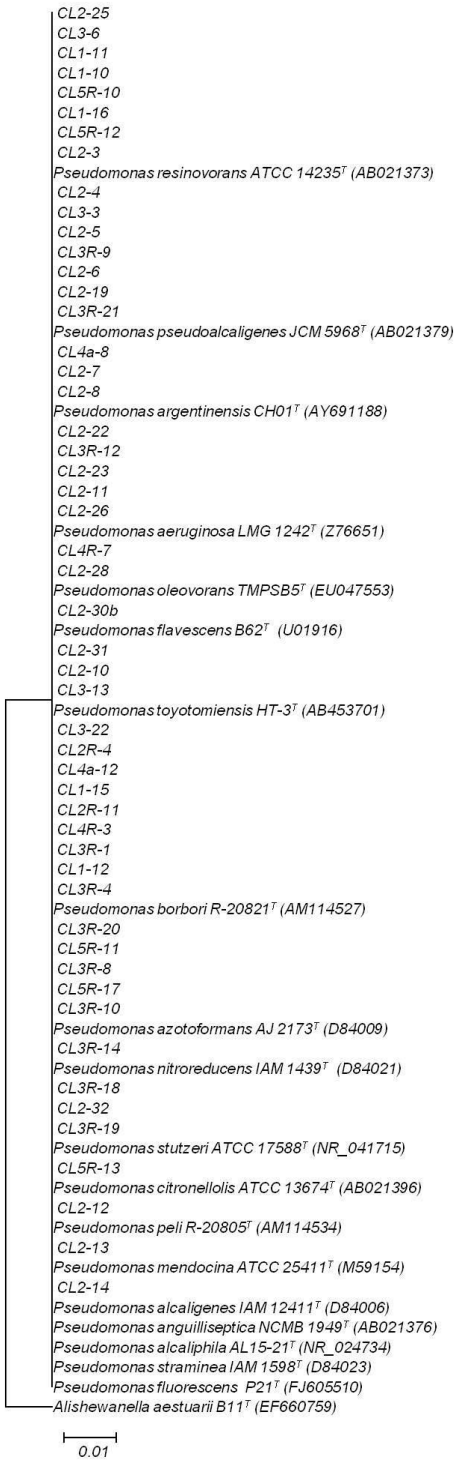


a)



b)

Figure 28: Phylogenetic analysis using neighbour-joining of MEGA 5, based on 16S rRNA gene sequences of bacterial isolates from Chilka Lake, Odisha with their respective genera a) *Streptomyces* and b) *Bacillus.* Percentage bootstrap values are indicated at node.



y)

Figure 29: Phylogenetic analysis using neighbour-joining of MEGA 5, based on 16S rRNA gene sequences of bacterial isolates from Chilka Lake, Odisha with their respective genus *Pseudomonas.* Percentage bootstrap values are indicated at node.

3.5. Characterization of unique bacterial isolates: Since, most of the bacterial isolates belong to same species or similar strains, only one representative of each species or strain was selected. Out of 151 successfully sequenced isolates, 52 were used for further characterization.

3.5.1. Physiological characterization:

3.5.1.1. pH tolerance of bacteria from Chilka Lake, Odisha: The tolerance of isolates to different pH was tested from pH 5.0 to pH 12.0 and all the bacteria grew at pH 7.0-8.0 suggesting that the optimum pH is around 7. None of the bacteria grew at pH 5.0 and only one (CL2-30a) grew at pH 6.0. Most of the isolates grew at pH 9.0 (CL1-9, CL1-16, CL2-4, CL2-6, CL2-23, CL2-27, CL2-28, CL2-32, CL3-6, CL3-19, CL4a-3b, CL4b-2, CL4b-5, CL4b-6, CL4b-7, CL4b-8, CL4b-12, CL1R-2, CL2R-3, CL2R-4, CL2R-6, CL2R-7, CL4R-12, CL4R-15, CL5R-3, CL5R-8 and CL5R-13), pH 10.0 (CL1-9, CL1-16, CL2-4, CL2-6, CL2-27, CL2-28, CL2-32, CL3-6, CL3-19, CL4b-2, CL4b-5, CL4b-6, CL4b-7, CL4b-8, CL4b-12, CL1R-2, CL2R-3, CL2R-4, CL2R-6, CL2R-7, CL5R-3 and CL5R-13), pH 11.0 and 12.0 (CL1-9, CL1-16, CL2-4, CL2-6, CL2-27, CL2-28, CL2-32, CL3-6, CL3-19, CL4b-2, CL4b-5, CL4b-7, CL4b-8, CL4b-12, CL1R-2, CL2R-3, CL2R-4, CL2R-6, CL2R-7, CL5R-3 and CL5R-13).

3.5.1.2. Temperature tolerance of bacteria from Chilka Lake, Odisha: The tolerance of bacterial strains was tested from 4^{o}C to 45^{o}C and the bacteria isolated showed a wide range of temperature tolerance. All the isolates grew at 25°C. Many bacteria were able to grow at temperatures as low as 4°C and as high as 45°C. Out of the 52 unique isolates, 19 bacteria (CL1-17, CL1-19, CL1-24, CL1-25, CL2-4, CL2-27, CL3-7, CL3-19, CL4a-3b, CL4a-11, CL4a-12, CL4a-14, CL4b-2, CL4b-5, CL4b-12, CL4b-14, CL4R-12 and CL4R-15) could tolerate 45°C temperature. Most of the isolates grew even at 4°C (CL1-9, CL1-16,CL1-17, CL1-18, CL1-19, CL2-4, CL2-6, CL2-17, CL2-23, CL2-27, CL2-28, CL2-30a, CL2-32, CL3-6, CL3-7, CL3-9, CL3-13, CL3-19, CL4a-3b, CL4a-11, CL4a-12, CL4a-14, CL4b-2, CL4b-5, CL4b-6, CL4b-7,

CL4b-8, CL4b-12, CL4b-13, CL4b-17, CL1R-2, CL1R-5, CL2R-3, CL2R-4, CL2R-6, CL2R-7, CL2R-8, CL2R-11, CL3R-6, CL4R-15, CL5R-3, CL5R-8, CL5R-13 and CL5R-15). Very few isolate could not tolerate 15°C (CL1-3b, CL1-9 and CL4R-10), 30°C (CL1-9, CL2-30a, CL2R-6, CL2R-7 and CL4R-10) and 37°C (CL1-9, CL2-30a, CL2R-6, CL2R-7, and CL4R-10).

3.5.1.3. Salt tolerance of bacteria from Chilka Lake, Odisha: Majority of the bacterial isolates showed extreme salt tolerance growing even at 10.0% salt concentration. Eleven isolates (CL1-9, CL3-4, CL3-9, CL1R-2, CL1R-5, CL2R-3, CL2R-6, CL2R-7, CL2R-8, CL4R-10, and CL5R-15) could not grow without salt indicating that these are obligatory halophilic. CL1-9, CL2R-6and CL2R-7 could only grow at 2.0% salt.

3.5.2. Biochemical characterization of bacterial isolates from Chilka lake, Odisha: Various biochemical tests were conducted to characterize bacteria according to production of enzymes such as urease, lipase, caseinase, β-galatosidase, phosphatase etc. (Table 7). Most of the isolates were positive for β-galatosidase whereas very few bacteria could produce DNase. These bacteria produced many commercially important enzymes, proving their economic importance. The isolates were categorized in to different physiological groups based on the combinations of enzymes they produced (Table 8). Among the 52 isolates, 11 strains (CL2-27, CL4b-8, CL1-24, CL3-19, CL4a-14, CL4b-7, CL4b-13, CL4b-14, CL1R-2, CL5R-8 and CL4b-2) produced a combination of 4 enzymes and 4 isolates (CL4b-5, CL4b-12, CL1R-5 and CL3R-6) exhibited more than 5 enzymes in combination (Table 8), indicating that these isolates play an important role in Chilka Lake ecosystem and further they can be explored for the purpose different commercially viable enzymes.

Table 8. Biochemical characterization of bacterial isolates from Chilka Lake, Odisha

S. No.	Sample	Gelati-nase	Lipase	Amylase	DNAse	Urease	Caseinase	Beta-galactosidase
1	CL1-3b	-	+	-	-	-	-	-
2	CL1-9	-	+	-	-	-	-	-
3	CL1-16	-	-	-	-	-	-	-
4	CL1-17	-	+	+	-	+	-	+
5	CL1-18	-	-	-	-	-	-	+
6	CL1-19	+	-	+	-	-	+	+
7	CL1-24	+	+	+	-	-	+	+
8	CL1-25	+	+	+	-	-	+	-
9	CL2-4	-	-	+	-	-	-	-
10	CL2-6	-	-	+	-	-	-	+
11	CL2-17	-	-	+	-	+	-	+
12	CL2-23	-	-	+	-	-	-	+
13	CL2-27	+	-	+	+	+	+	-
14	CL2-28	-	-	-	-	+	-	-
15	CL2-30a	-	-	-	-	+	-	-
16	CL2-32	-	+	-	-	+	-	+
17	CL3-4	-	-	-	-	-	-	+
18	CL3-6	-	-	-	+	+	-	+
19	CL3-7	-	-	-	-	+	-	+
20	CL3-9	+	-	-	-	+	-	+
21	CL3-13	+	-	-	-	-	-	+
22	CL3-19	+	+	+	-	-	+	+
23	CL3-21	+	+	+	-	-	-	+
24	CL4a-3b	-	+	-	-	+	-	+
25	CL4a-11	+	+	+	-	-	-	+
26	CL4a-12	-	+	+	-	-	-	+
27	CL4a-14	+	+	+	-	-	+	+
28	CL4b-2	+	+	-	+	+	-	+
29	CL4b-5	+	+	+	+	-	+	+
30	CL4b-6	-	+	+	-	-	-	+
31	CL4b-7	+	+	+	-	-	+	+
32	CL4b-8	+	-	+	-	+	+	+
33	CL4b-12	+	+	+	+	-	+	+
34	CL4b-13	+	+	+	-	-	+	+
35	CL4b-14	+	+	+	-	-	+	+
36	CL4b-17	-	-	-	-	-	-	+
37	CL1R-2	+	+	+	-	-	+	+
38	CL1R-5	+	+	+	+	-	+	+
39	CL2R-3	-	+	+	-	+	-	+
40	CL2R-4	-	+	+	-	+	-	+
41	CL2R-6	-	+	-	-	-	-	+
42	CL2R-7	-	-	-	-	-	-	+
43	CL2R-8	-	+	-	-	-	+	+
44	CL2R-11	-	+	+	-	-	-	+
45	CL3R-6	+	+	+	+	+	-	+
46	CL4R-10	-	+	-	-	-	+	+
47	CL4R-12	+	+	-	-	-	+	+
48	CL4R-15	+	+	-	-	-	+	+
49	CL5R-3	-	+	-	-	-	-	+
50	CL5R-8	+	+	-	+	-	+	+
51	CL5R-13	-	+	+	-	-	+	+
52	CL5R-15	-	+	-	-	-	-	+

* + (positive), - (negative)

Table 9. Number of bacterial isolates from Chilka Lake, Odisha producing combinationof enzymes*

Enzymes	No. of isolates	Name of the isolate
A+B	2	CL2-6, CL2-23
G+B	1	CL3-13
L+B	4	CL2-32, CL2R-6, CL5R-3, CL5R-15
U+B	1	CL3-7
A+U+B	1	CL2-17
D+U+B	1	CL3-6
G+U+B	1	CL3-9
L+A+B	3	CL4a-12, CL4b-6, CL2R-11
L+C+B	2	CL2R-8, CL4R-10
L+U+B	1	CL4a-3b
G+A+C+B	1	CL1-19
G+L+A+B	2	CL3-21, CL4a-11
G+L+A+C	1	CL1-25,
G+L+C+B	2	CL4R-12, CL4R-15
L+A+C+B	1	CL5R-13
L+A+U+B	3	CL1-17, CL2R-3, CL2R-4
G+A+D+U+C	1	CL2-27
G+A+U+C+B	1	CL4b-8
G+L+A+C+B	7	CL1-24, CL3-19, CL4a-14, CL4b-7, CL4b-13, CL4b-14, CL1R-2
G+L+D+C+B	1	CL5R-8
G+L+D+U+B	1	CL4b-2,
G+L+A+D+C+B	3	CL4b-5, CL4b-12, CL1R-5
G+L+A+D+U+B	1	CL3R-6

* A-amylase, B-β galactosidase, C-Caseinase, D-DNase, G-Gelatinase, L-Lipase and U-Urease

3.6. Characterization of 10 potential novel isolates from Chilka Lake, Odisha: Ten bacteria (CL1-16, CL1-17, CL3-4, CL3-9, CL4a-14, CL4b-2, CL4b-8, CL1R-2, CL2R-4 and CL3R-6) showing ≤98.0% similarity, based on 16S rRNA gene, compared to subject sequences in NCBI database, were predicted as potential novel species. These 10 strains were subjected to additional biochemical tests to find out their enzymatic activity, substrate consumption and their species status. Genera *Pseudomonas*, *Bacillus*, *Rheinheimera* and *Phaeobacter* were represented by two species each, amongst these 10 bacteria. Rest belonged to the genera *Brachybacterium* and *Streptomyces.* Amongst these 10 isolates, two strains-CL1-17 and CL3R-6-showed ≤97.0% similarity to their

respective phylogenetic neighbours, the *Streptomyces rochei* and *Rheinheimera aquimaris* According to the current species definition, they represent novel species of the genera *Streptomyces* and *Rheinheimera* respectively.

3.6.1. Morphological and Physiological characterization of bacteria isolated from Chilka Lake, Odisha: Ten potentially novel isolates were grouped based on Gram-staining and 5 strains were as Gram-positive and rest five were Gram-negative. All Gram-positive isolates were motile and amongst Gram-negatives, except two (CL1-16 and CL3R-6) all were negative, further their colony characteristics are listed Table 9, besides listing their ability to grow at different temperature, pH and salt tolerance in section 3.5.1.

Table 10. Morphological and physiological characteristics of the isolates showing ≤98.0%similarity with their phylogenetic neighbours*

S.No.	Sample	Colony morphology	Motility test	KOH string test
1	CL1-17	Mustard coloured, irregular, crateriform, rough, convex, diameter: 1mm	+	Gram +ve
2	CL3-4	Cream coloured, round, convex, shiny, smooth, diameter: 1mm	+	Gram +ve
3	CL3-9	White coloured, Opaque, round, convex, shiny, smooth, diameter: 1mm	+	Gram +ve
4	CL4b-2	Cream coloured, opaque, round, convex, shiny , smooth, diameter: 1mm	+	Gram +ve
5	CL4b-8	Lemon yellow coloured, round, convex, shiny, smooth, diameter: 1mm	+	Gram +ve
6	CL1-16	Transparent, round, convex, shiny, smooth,4diameter:0.5mm	+	Gram -ve
7	CL4a-14	Mustard coloured, round, convex, rough, diameter: 1-2mm	-	Gram -ve
8	CL1R-2	Cream coloured, round, convex, shiny, smooth, diameter: 1-2mm	-	Gram -ve
9	CL2R-4	Yellow coloured, transparent, round, shiny, smooth, diameter: 1mm	-	Gram -ve
10	CL3R-6	Yellow coloured, translucent, irregular, convex, shiny, smooth, diameter: 1-2mm	+	Gram -ve

* + (positive), - (negative)

3.6.2. Biochemical characterization:

3.6.2.1. Biochemical characterization of potentially novel species from Chilka Lake:

Production of oxidase, catalase and cellulase, arginine dihydrolase, arginine and glutamic acid decarboxylase were tested for the 10 potentially novel species. Most of these were positive or weakly-positive for enzymes oxidase and catalase, while the production of arginine dihydrolase was variable amongst the strains. All the potential novel isolates were capable of producing arginine and glutamic acid decarboxylase. None of the isolates could hydrolyse aesculin and cellulase or ferment lactose, glucose, sucrose with or without production of H_2S (Table 10).

Table 11. Biochemical characterization of the important bacteria from Chilka lake, Odisha*

S. No.	Sample	Oxidase	Catalase	Arginine dihydrolase
1	CL1-16	+	+	+
2	CL1-17	+/-	+	-
3	CL3-4	+	+/-	-
4	CL3-9	+/-	+	+
5	CL4a-14	+	+	+
6	CL4b-2	+/-	+/-	+
7	CL4b-8	-	+	-
8	CL1R-2	+/-	-	-
9	CL2R-4	+	-	-
10	CL3R-6	+	-	+

* + (positive), +/- (weakly positive), - (negative)

3.6.2.2. Biochemical characterization of potentially novel species from Chilka Lake, Odisha using HiMedia kits:

Potentially novel bacteria were further characterized using HiMedia kits (KB009 HiCarbohydrate™ kit and KB003 Hi25™ Enterobacteriaceae identification kit) to check the enzyme production and substrate utilization. The results are indicated in Tables 11 and 12.

Table 12. Substrate utilization by important bacterial isolates from Chilka Lake, Odisha based on using HiMedia KB009 HiCarbohydrate™ kits*

S.No.	Test	Samples									
		CL1-16	CL1-17	CL3-4	CL3-9	CL4a-14	CL4b-2	CL4b-8	CL1R-2	CL2R-4	CL3R-6
Kit No.: KB009											
Part A											
1	Lactose	-	-	-	-	-	+	-	-	-	-
2	Xylose	-	-	-	+	-	+	+	-	-	+
3	Maltose	-	-	-	-	-	-	-	-	+	+
4	Fructose	-	-	-	-	-	-	+	-	-	+
5	Dextrose	-	-	-	+	-	+	+	-	-	+
6	Galactose	-	-	-	-	-	+	-	-	-	+
7	Raffinose	-	-	-	-	-	-	-	-	-	-
8	Trehalose	-	-	-	-	-	-	-	-	-	+
9	Melibiose	-	-	-	-	-	+	+	-	-	+
10	Sucrose	-	-	-	-	-	-	-	-	-	+
11	L-Arabinose	-	-	-	+	-	+	-	-	-	+
12	Mannose	-	-	-	+	-	+	+	-	-	+
Part B											
13	Inulin	-	-	-	-	-	-	-	-	-	-
14	Sodium gluconate	-	-	-	-	-	-	-	+	-	-
15	Glycerol	-	+	-	-	-	-	-	+	-	-
16	Salicin	-	-	-	-	-	-	-	-	-	-
17	Dulcitol	-	-	-	-	-	-	-	-	-	-
18	Inositol	-	-	-	-	-	-	-	-	-	-
19	sorbitol	-	-	-	-	-	-	-	-	-	-
20	Mannitol	-	-	-	-	-	-	-	-	-	-
21	Adonitol	-	-	-	-	-	-	-	-	-	-
22	Arabitol	-	-	-	-	-	-	-	-	-	-
23	Erythritol	-	-	-	-	-	-	-	-	-	-
24	α-Methyl-D-glucoside	-	+	-	+	+	+	+	-	-	-
Part C											
25	Rhamnose	-	-	-	-	-	-	-	-	-	-
26	Cellubiose	-	-	-	-	-	-	-	-	-	-
27	Melezitoise	-	-	-	-	-	-	-	-	-	-
28	α-Methyl-D-mannoside	-	-	-	-	-	-	-	-	-	-
29	Xylitol	-	-	-	-	-	-	-	-	-	-
30	ONPG	-	+	+	+	+	+	+	+	-	+
31	Esculin hydrolysis	-	-	-	-	+	+	+	+	-	+
32	D-Arabinose	-	-	-	-	-	-	-	-	-	-
33	Citrate utilization	+	+	+	+	+	+	+	+	+	+
34	Malonate utilization	+	+	+	+	+	+	+	+	+	+
35	Sorbose	-	-	-	-	-	-	-	-	-	-

* + (positive), - (negative)

Table 13. Substrate utilization and biochemical characterization of important bacterial isolates from Chilka lake, Odisha (using HiMedia KB003 Hi25™ Enterobacteriaceae Identification kit)*

S. No.	Test	Samples									
		CL1-16	**CL1-17**	**CL3-4**	**CL3-9**	**CL4a-14**	**CL4b-2**	**CL4b-8**	**CL1R-2**	**CL2R-4**	**CL3R-6**
Kit No.: KB003											
Strip 1											
1	ONPG	-	+	+	-	+	+	+	+	-	+
2	Lysine utilization	-	-	-	-	+	+	+	+	-	-
3	Ornithine utilization	-	+	-	-	+	+	+	+	-	-
4	Urease	-	+	-	-	-	+	+	+	-	-
5	Phenylalanine deamination	-	-	-	-	-	-	-	-	-	-
6	Nitrate reduction	-	-	-	+	-	-	-	+	+	-
7	H_2S production	-	-	+	-	-	-	+	-	-	-
8	Citrate utilization	+	+	+	+	+	+	+	+	+	+
9	Vogues Proskauer's	-	-	-	-	-	-	-	-	-	-
10	Methyl Red	-	-	-	-	-	-	-	-	-	-
11	Indole	-	-	-	-	-	-	-	-	-	-
12	Malonate utilization	+	+	+	+	+	+	+	+	+	+
Strip 2											
13	Esculin hydrolysis	-	+	-	-	+	+	+	+	+	+
14	arabinose	-	-	-	-	-	-	-	-	-	-
15	Xylose	-	-	-	-	-	+	-	-	-	-
16	Adonitol	-	-	-	-	-	-	-	-	-	-
17	Rhamnose	-	-	-	-	-	-	-	-	-	-
18	Cellubiose	-	-	-	-	-	-	-	-	-	-
19	Melibiose	-	-	-	-	-	+	-	-	-	-
20	Saccharose	-	-	-	-	-	-	-	-	-	-
21	Raffinose	-	-	-	-	-	-	-	-	-	-
22	Trehalose	-	-	-	-	-	-	-	-	-	-
23	Glucose	-	-	-	+	-	+	-	+	-	-
24	Lactose	-	-	-	-	-	-	-	-	-	-

* + (positive), - (negative)

3.6.2.3. Characterization of potentially novel species from Chilka Lake, Odisha for sensitivity to antibiotics: All 10 bacterial isolates were tested for their susceptibility to 23 different antibiotics (Table 13). All the isolates showed sensitivity to amikacin, chloramphenicol, kanamycin, streptomycin and tetracycline. The susceptibility of all ten isolates was variable as indicated in Table 13.

Table 14. Antibiotic susceptibility of the isolates showing ≤98.0% similarity with their phylogenetic neighbours*

S.No.	1	2	3	4	5	6	7	8	9	10
Sample Name	CL1 R16	CL1 R17	CL3 R4	CL3 R9	CL4a R14	CL4b R2	CL4b R8	CL1R R2	CL2R R4	CL3R R6
Antibiotic										
Amikacin	S	S	R	S	S	S	S	S	S	S
Ampicillin	R	R	R	S	S	R	S	S	S	S
Cefazolin	R	R	R	R	S	R	R	R	R	S
Cefoperazone	S	S	R	S	S	R	S	S	S	S
Cefuroxime	R	R	R	R	S	R	R	R	R	R
Cephotaxime	S	S	R	S	S	R	S	S	S	S
Chloramphenicol	S	S	R	S	S	S	S	S	S	S
Ciprofloxacin	S	S	R	S	S	S	R	S	S	S
Colistin	R	R	S	R	R	R	R	S	S	S
CoRTrimoxazole	R	S	S	S	S	S	S	S	S	S
Doxycycline hydrochloride	R	R	S	S	S	R	S	S	S	S
Erythromycin	S	R	R	S	S	S	S	S	S	S
Kanamycin	S	S	S	S	S	S	S	S	S	S
Lincomycin	R	R	R	R	S	R	R	R	R	S
Lomefloxacin	S	S	S	S	S	S	R	S	S	S
Nalidixic acid	S	R	S	S	R	S	R	S	S	S
Nitrofurantoin	S	S	R	S	S	R	R	S	S	S
Norfloxacin	S	S	R	S	R	S	R	S	S	S
Penicillin G	R	R	R	R	S	R	R	R	R	R
Streptomycin	S	S	R	S	S	S	S	S	S	S
Tetracyclin	S	S	S	S	S	S	S	S	S	S
Tobramycin	S	S	S	S	S	S	S	S	S	S
Vancomycin	R	R	S	R	S	R	S	S	R	R

*S-susceptibility and R-resistance

4. DISCUSSION

Bacterial diversity studies have been carried out for a long time and have helped in understanding their diversity, ecology and identifying the novel species found in a particular ecosystem. Bacterial populations differ in air, water and soil but they are certain of being present everywhere. Microbes are responsible for maintaining several biogeochemical cycles, playing an important role in sustenance of life on earth.

The advent of culture-independent technique has helped in identifying non-cultivable species but very few cultivable bacterial species (11564 species) are known till date. The present study was carried out to increase the knowledge about bacterial diversity of brackish water lakes. The total counts of the brackish water have been found to be 4.9-7.3 X 10^6 and 5.6-9.2 X 10^6 per ml in lakes St. Lawrence estuary, Canada (Monfort *et al.,* 1992) and Ria de Aveiro, Portugal (Almeida *et al.,* 1992) respectively. The bacterial number for Chilka Lake samples was less than the above mentioned lakes, indicating that the nutrients play an important role. As the sample were collected at an interval of 1km, it was seen that there was not much change in the total count of bacteria but the species found in each sample varied to a great extent. In the present investigation, an estimated amount of 9.3% of bacteria could be cultured using Zobell marine agar medium when compared to the total cell count. Our study has lead to the cultivation of reasonably high bacterial number compared to previous studies (Amann *et al.,* 1990a).

16S rRNA gene analysis showed majority of the bacteria isolated from Chilka Lake were *Gammaproteobacteria* and *Firmicutes*. This data corroborated with studies on other brackish water lakes such as Pulicat Lake, Tamil Nadu, India (Sahay *et al.,* 2011) and Pangong Lake, Jammu & Kashmir (Sahay *et al.,* 2012). There was a huge difference in the genera found in all these lakes. *Bacilli, Halobacilli* and *Halomonas* were the major genera found in Pulicat Lake (Sahay *et al.,* 2011), *Tsukamurella, Alishewanella, Sphingomonas,*

Ochrobactrum and *Brevundimonas sp.* were found in Pangong Lake (Sahay *et al.,* 2012), while the present study showed that Chilka Lake is mostly inhabited by *Bacilli, Pseudomonas and Rheinheimera* with other 22 genera and most of them were not reported from other brackish water lakes indicating that Chilka lake harbours a different bacterioplankton.

The isolates from Chilka Lake belonged to various species. Genera *Bacillus, Pseudomonas* and *Pseudoalteromonas* were represented by 11, 10 and 5 species respectively, showing the rich bacterial diversity of this lake. But certain isolates belonged to less common genera like *Arsukibacterium, Limnobacter* and *Simiduia* for which only one or two species have been identified till date. These bacteria have not been reported in other brackish water lakes. Thus, the isolates showing similarity with such rare genera have immense possibility of being new species.

Previously, studies on these lakes have shown that majority of the bacteria are halotolerant or halophilic. The bacterial populations of Shira Lake, Russia have also been shown to be halotolerant, surviving at both low and high salt concentrations (Lobova *et al.,* 2010). Similar results were obtained in this study. The 52 isolates characterized for salt tolerance can be placed in the category of obligatory halophilic or halotolerant. Strains CL1R-5 and CL2R-8 are halophilic as they did not grow on media without salt while strain CL1-3b did not survive in salt concentrations beyond 0.5% suggesting that it is mildly halotolerant. Halophilic microorganisms are potential sources of enzymes uniquely adapted to activity at high salt concentrations with application in beverages, pharmaceutical and detergent industries (Kamekura *et al.,* 1992; Kobayashi *et al*., 1994; Adams & Kelly, 1995; Li *et al.,* 2002), leather industry (Birbir *et al.,* 1996) and food preservative industries (Ventosa *et al.,* 1995; Mellado *et al,*. 2003).

Due to change in water temperature in different seasons, the bacteria have adapted to survive at extremes of temperature and were expected to survive in a wide range of temperature. The study proved that these bacteria can grow at 4-45°C but flourish at temperature range of 15-30°C, being mesophilic in nature. The bacteria of Lake Shira and

Pangong Lake were found to be psychrotolerant (Lobova *et al.*, 2010; Sahay *et al.*, 2012). The bacteria isolated can survive at pH 7.0-12.0, citing their significance in food and pharmaceutical industry. But, the optimum pH for their growth was between pH 7.0-8.0.

Bacterial diversity studies also aimed at finding bacteria of industrial use. The bacteria obtained in the present study produced enzymes such as gelatinase, urease, DNase, β-galactosidase, caseinase etc. depicting that survival in extreme conditions is mediated by such enzymes and these bacteria can be used for various industrial processes. Variations in the production of industrial enzymes were reported among the isolates from Howz Soltan Lake, Iran (Rohban et al., 2009). The study theorized that extremely halophilic/halotolerant and Gram positive halophilic bacteria produced more extracellular enzymes in comparison to moderate halophilic and Gram-negative isolates (Rohban et al., 2009). Majority of the bacterial isolates produced important enzymes or hydrolyzed a variety of substrates. Hence, these bacteria can be a source of enzymes for commercial use and benefit the biotechnology industry.

Using 16S rRNA sequences for BLAST similarity search and phylogenetic analysis, and Biochemical characterization, potentially novel species were found. As required per the bacterial species definition, two of the novel bacteria- CL1-17 and CL3R-6 showed ≤97.0% 16S rRNA gene sequence similarity with their respective nearest phylogenetic neighbours. The comparison of enzyme/substrate consumption profiles of the two potential novel strains with their nearest neighbours is shown in Table 14. These isolates show differences in enzyme production from their neighbours confirming that these are new species. Besides the biochemical characterization, phylogenetic trees constructed on the basis of 16S rRNA sequences served as the most efficient method in identifying a bacterium (Figure 18). The results are corresponded with BLAST similarity search results.

Table 15. Comparison of biochemical characters of CL1-17 and CL3R-6 with their nearest phylogenetic neighbours*

Characteristic	**Srain no.**	**Closest speciest**	**Characteristic**	**Srain no.**	**Closest speciest**
Enzyme	CL1-17	*Streptomyces rochei*	Enzyme/substrate	CL3R-6	*Rheinheimera aquimaris*
Gelatinase	-	+	Aesulin	-	+
Lipase	+	-	Nitrate reduction	-	+
Amylase	+	+	Amylase	+	+
DNase	-	-	DNase	+	+
Urease	+	+	Glucose assimilation	+	+
Cellulase	-	+	Arabinose assimilation	+	-
β-galactosidase	+	-	Citrate utilization	+	-

*+ (positive), - (negative)

Based on polyphasic taxonomic characterization, the two novel species (CL1-17 and CL3R-6) were found to possess the following characters. CL1-17 is Gram positive, motile, halophilic, can grow in a wide range of temperature and produces enzymes such as lipase, amylase, urease, oxidase, catalase, decarboxylases and β-galactosidase and exhibited >97.0% similarity with *S. rochei* of the genus *Streptomyces*. The strain is tentatively named as *Streptomyces indica* sp. nov. CL3R-6 is Gram negative, motile, halophilic and can grow in a wide range of temperature but it also produces gelatinase, arginine dihydrolase and DNase apart from enzymes produced by CL1-17 and exhibited 96.0% similarity with *R. aquimaris* of the genus *Rheinheimera*. The strain is tentatively named as *Rheinheimera chilkensis* sp. nov. Besides these two, rest 8 strains could represent novel species provided the DNA-DNA hybridization is performed with their respective phylogenetic neighbour (Stackebrant & Geobel, 1994).

Prior studies on the bacterial diversity of Chilka Lake have resulted in identification of two novel species- *Shewanella chilikensis* and *Thiorhodococcus modestalkaliphilus*

(Sucharita *et al.*, 2009; Sucharita *et al.*, 2010). These bacteria grew at a temperature of 25-42°C, salt concentration of 0.5-4.0% and pH 6.5-9.5. Interestingly, our study also confirmed the presence of the genus *Shewanella* and in addition, 24 other genera were also detected.

CONCLUSION: The present study lead to the identification of 52 species belonging to 25 genera and all the strains are mesophilic had an optimum pH of 7.0 to 8.0; major isolates are halotolerant and had a wide range of growth temperature with an optimum of 25 to 30 ^{o}C. Based on polyphasic taxonomic characteristics, close to 10 isolates were identified as representing novel species and based on less than 97.0% 16S rRNA gene sequence similarity and differences exhibited with nearest phylogentiic neighbors, two isolated were identified as belonging to novel species and named them as *Streptomyces indica* sp. nov (Strain no. CL1-17) and *Rheinheimera chilkensis* sp. nov (Strain no.CL3R-6).

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