Cibtech Journal of Bio-Protocols ISSN: 2319–3840 (Online)
An Open Access, Online International Journal Available at http://www.cibtech.org/cjbp.htm
2014 Vol. 3 (1) January-April, pp.11-18/Mahapatra and Mishra
Research Article

ISOLATION AND MOLECULAR IDENTIFICATION OF AN EXTREME ALKALI-TOLERANT *PSEUDOMONAS MENDOCINA 2E* FROM BRACKISH WATER OF CHILIKA LAKE

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ABSTRACT

Brackish Lakes are the most extraordinary natural reservoir of fluctuating salinity and alkalinity in its water quality. This variation leads to adaptability of tolerance for alkaline condition of the residential bacterial community in the lake. In this research work, the alkali-tolerance of the bacterial isolates were screened for identification and characterization of alkali-tolerant bacteria from the brackish water of the Chilika lake, Odisha. Representative samples of water was collected aseptically from four important sites of the lake *viz* Barkul, Kalupada Ghat, Parikuda and Rambha during the period from January to June 2012. Maximum possible number of culturable bacteria were obtained and screened for alkali-tolerance. A total of 25 bacterial isolates were obtained and almost all the isolates survived upto pH 8 but only one bacteria tolerated upto pH 13 and was extremely alkali-tolerant. This strain was identified as *Pseudomonas mendocina 2E* and designated with accession number KF657327. It is a potential lipase producer which is an important industrial enzyme. Further assay of enzyme is yet under study.

Keywords: *Alkali-Tolerant Bacteria, Pseudomonas Mendocina, Lipase*

INTRODUCTION

Asia's largest brackish lake Chilika (19^0 28' –19 ^{0}54' N latitude and $85^0$5' –85^0 38'E longitude) (Figure 1) is an estuarine (Biswas et al., 1932) whose water is subjected to variation in alkalinity due to the confluence of sea water from Bay of Bengal and fresh water from Daya and Bhargabi rivers and its tributaries (Nayak et al., 2004). This precisely affect the residential bacterial community which have developed alkali-tolerance to this alkaline condition. Tolerance is the protoplasmic component of resistance to stress. It involves the degree to which the protoplasm, can tolerate the ionic imbalance associated with salt stress, and the osmotic and toxic effects of increased ion concentrations (Larcher et al., 2001). Microorganisms, particularly bacteria, broadly distributed in nature and the majority of them grow and reproduce optimally at just about neutral pH (close to pH 7). Alkaliphiles microorganisms have been reported to grow in extreme alkaline environments and these can be categorized into two main categories; alkaliphiles and alkali-tolerant microorganisms (Krulwich and Guffanti, 1989; Yumoto, 2002). Alkaliphiles (alkali from the Arabic for soda ash and phile- lovingand haloalkaliphiles. Alkaliphiles require an alkaline pH of 9 and pH 10 for optimal growth (i.e. obligate requirement for alkaline growth conditions). The growth requirements of haloalkaliphilic microorganisms are for both an alkaline pH of 9 and high salinity up to 4M NaCl (which is 8 times the salt content of normal sea water) (Horikoshi, 1999). Bacillus alcalophilus is an example of an alkaliphilic bacterium which retains its intracellular pH between 8.5 and 9.0 (Jones and Grant, 2000). Alkali-tolerant microorganisms, on the other hand, can survive at pH 10 and sometimes above, but optimum growth takes place at pH of near neutral medium (in some published works they are called facultative alkaliphiles) (Krulwich and Guffanti, 1989). The research on bacterial potency and their tolerance to variations in physico chemical parameters of this lake Chilika is still under veil. Keeping in view of the above facts the present investigation was under taken in order to study the alkali-tolerance of the bacterial population and isolate the extreme alkali-tolerant strains whose biotic potential is to be studied further.

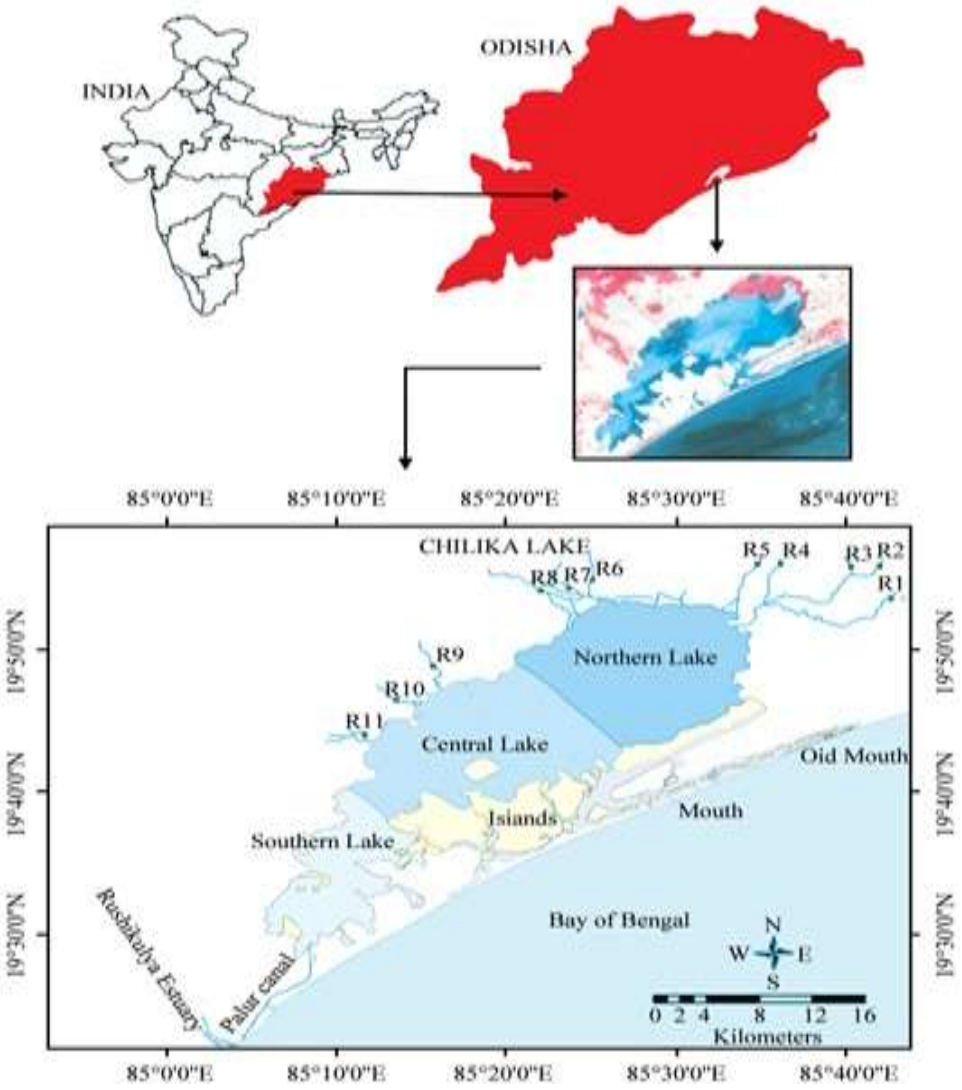


Figure 1: Location and position of Chilika

MATERIALS AND METHODS

Collection of Sample

Water samples were collected aseptically from four sites of Chilika including Barkul, Kalupada Ghat, Parikuda and Rambha from January to June 2012 for investigation. The physico chemical parameters of the samples were analysed and processed immediately in the laboratory to isolate inhabiting bacterial flora, study their morphology and other characteristics features required for their identification and isolate the alkali-tolerant bacteria.

Bacteriological Analysis of Water

Water samples were inoculated aseptically in the liquid medium i.e. Nutrient broth at 37ºC/24 hours. Hundred microlitres of inoculums was taken and spread plated on two different media viz., Nutrient Agar (NA) and Marine Agar for isolation of culturable bacteria.The bacterial load was calculated of all the four sites using serial dilution method. From these bacterial isolates, colonies showing different morphology were selected, subcultured once or twice on Nutrient agar plates to obtain pure culture and were preserved in respective Marine agar slant at low temperature (4^0C) for further characterization.

Screening for Alkali-Tolerance

Alkaline selective medium (Horikoshi medium) and marine agar medium were used to isolate alkali-tolerant bacteria. These media were prepared and different pH values were used (6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0, 13.0, 14.0). The pH value of liquid media was rechecked after autoclaving and shown not alter by more than ±0.05 pH units. The pH value of the suspension was measured using a bench top pH meter which had been standardised using pH 10.0 and pH 4.0 buffers. The alkali-tolerance of bacterial isolates was studied by incubating 100µl of culture in different tubes containing the liquid medium with varying pH. After incubating 18 to 24 hours at 37^{o}C, a loopful of the culture was sub-cultured onto NA plates. All the plates were incubated at 37^{o}C for 18 to 24 hours. The highest pH on which growth occurred, was detected.

Biochemical Identification of the Alkali-Tolerant Bacterial Isolate

The alkali-tolerant isolate from the screening was identified by studying colony characteristics on different pseudo selective media, Gram reaction and identification through a series of biochemical tests and also other features required for their characterization following standard microbiological techniques by (Collins and Lyne, 1970) with further study on their sugar utilization and enzymatic activities.

Effect of temperatures on growth of alkali-tolerant bacterial isolate

The growth of isolate was tested on Nutrient broth by incubating it at a difference of 5^{0}C temperatures ranging from 25^{o}C to 60^{0}C for 37^{0}C/24 hours, respectively. Then OD was taken at 600nm and its temperature resistance was observed from its turbidity.

Molecular Identification of the Isolate

*DNA extraction and PCR Amplification***:**

The genomic DNA was isolated from the given organism using genomic DNA extraction Kit (Bhat Biotech).

PCR Amplification of the 16s rRNA gene was performed using the universal primers.

Forward primer: 5'-AGAGTTTGATCCTGGCTCAG-3'

Reverse Primer: 5'-ACGGCTACCTTGTTACGACTT-3'

PCR was performed as follows in a total volume of 50 µl in a 0.2 ml thin walled PCR tube.

Components	**Volume**
Nuclease free water	37 µl
Genomic DNA (0.1µg/µl)	2.0 µl
Forward Primer (10µM)	2.0 µl
Reverse Primer (10µM)	2.0 µl
10X Reaction Buffer	5.0 µl
dNTP Mix (10mM)	1.5 µl
Taq DNA polymerase (5 U/µl)	0.5 µl
Total volume	50 µl

The amplification was carried out in a Master cycler® Thermocycler (Eppendorf, Germany) using the following program.

Initial denaturation of 94^{o}C for 2 minutes followed by 40 cycles of denaturation at 94^{o}C for 1 min, annealing at 55^{o}C for 1 min and extension at 72^{o}C for 1.5 minutes. Final extension was carried out at 72^{o}C for 10 min.The ~1500 bp PCR product was purified to remove unincorporated dNTPS and Primers before sequencing using PCR purification kit (Norgen Biotek, Canada).

*Sequencing***:**

The strand of the rDNA region amplified by PCR were sequenced by automated DNA sequencer -3037*xl* DNA analyzer from Applied Biosystems using BigDye® Terminator v3.1 cycle sequencing Kit (Applied Biosystems). Sequence data were aligned and dendrogram was generated using Sequence analysis

software version 5.2 from Applied biosystems. The sequences obtained for plus and minus strands were aligned using DNA baser software before performing the bioinformatics analysis.

Bioinformatics analysis:

Sequences were compared to the non-redundant NCBI database by using BLASTN, with the default settings used to find the most similar sequence and were sorted by the E score. A representative sequence of 10 most similar neighbours was aligned using CLUSTAL W2 for multiple alignment with the default settings. The multiple-alignment file was then used to create phylogram using MEGA5 software.The data is submitted to Genbank for accession number.

RESULTS AND DISCUSSION

Physico-Chemical Parameters of Water Sample:

The analysed result of various parameters of water samples is enumerated in Table 1.The alkalinity of the water is found to be highest at site 4(Rambha).There is a variation in the salinity of the water. Highest salinity is found from parikud site of the lake. The BOD values indicates the presence of aquatic life in the lake and variation of these parameters in different sites of the same lake gives an indication of tolerance of microbes to this shifting habitat.

Table 1: Spatial variations of physico-chemical variables in Lake Chilika (mean ± SD) from January to June, 2012

Name of the Parameters	BOD in mg/L	COD in mg/L	pH	Alkalinity in mg/L	Calcium Hardness in mg/L	Dissolved Oxygen in mg/L	Salinity in ppt	Iron in mg/L
Site-1 (Barkul)	28.3 ±1.7	41.8 ±7.1	7.9±0.1	60.2±0.6	25.4 ±0.2	8.6 ±1.2	10.8 ±2.2	o.25 ±0.1
Site-2 (Kalupada Ghat)	28.5±2.0	48.7 ±8.4	8.8±0.1	80.6±0.8	25.7 ±0.1	8.2 ±0.9	11.2 ±1.6	0.22 ±0.3
Site-3 (Parikud)	20.6 ±3.3	39.6 ±8.2	8.6±0.2	80.2±0.4	26.1±0.3	7.2 ±0.5	12.4 ±0.8	0.24 ±0.2
Site-4 (Rambha)	30.2±1.2	46.2 ±7.1	9.2±0.3	84 ±0.2	25.6 ±0.5	7.6 ±0.5	10.2±1.2	0.19 ±0.6

Bacterial Load of the Water Sample

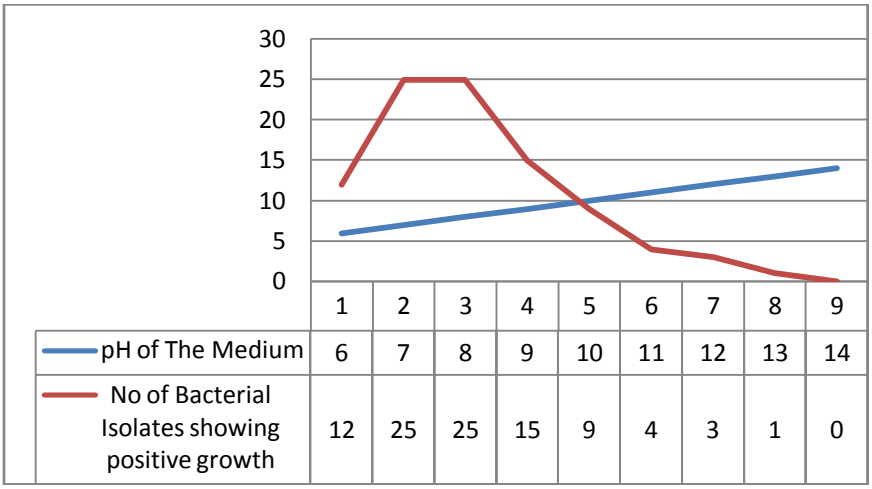
There was a slight spatial variation noted in the bacterial load in the water samples collected from four sites as depicted in table 2**.**Generally, the Northern part of the lake had the highest number of total count of bacteria as indicated by its mean value of 1.8×108± 7.3×107/ml. The bacterial load between Rambha (site 4) and Parikud (site 3) were not significantly different (Table 2). There was a significant difference in the total counts of bacteria between the 4 sites (One way ANOVA, $F(2, 99)=3.926$, $P<0.05$).Analysis indicated significant difference in terms of Total count between the Parikud and Barkul and Rambha and Barkul, Kalupada Ghat and Barkul sampling sites ($P<0.05$). The Rambha (site 4) and Parikud (site 3) were not significantly different ($P>0.05$) and they recorded lower numbers compared to the site1 sampling point.

Table 2: Bacterial load of Water samples from sampling sites of Chilika lake:

Sampling Sites	**Mean no. of bacteria in CFU/ml**
Site-1 (Barkul)	$1.8\times10^{8}\pm 7.3\times10^{7}$
Site-2 (Kalupada Ghat)	$1.4\times10^{8}\pm 3.5\times10^{7}$
Site-3 (Parikud)	$1.4\times10^{8}\pm 4.5\times10^{7}$
Site-4 (Rambha)	$1.4\times10^{8}\pm 6.9\times10^{7}$

Screening for Alkali-Tolerance

All the 25 isolates were growing at pH 8 which gives a clear indication that the inherent bacterial flora is mildly alkalitolerant, however there was a fall in the number when the pH increased to 10 but one isolate 2E was able to grow at pH 13.This extreme alkali-tolerant bacteria was isolated from the water sample at site 4 in the southern sector of the lake at Rambha. The details of tolerance to alkalinity of the isolates is depicted in Figure 2.However there was no growth in Horikoshi medium and hence it was conferred that no alkaliphiles were there in the isolates. Alkali-tolerant bacteria are usually found in the brackish lakes recently a group of alkali-tolerant bacteria are isolated from Hungarian soda lake (Borsodi, 2008).



	1	2	3	4	5	6	7	8	9
pH of The Medium	6	7	8	9	10	11	12	13	14
No of Bacterial Isolates showing positive growth	12	25	25	15	9	4	3	1	0

Figure 2: Alkali-tolerance of The Bacterial Isolates

Biochemical Identification of the Alkali-Tolerant Bacteria 2E

After gram staining it was found to be a gram negative rod, It was positive for catalase, coagulase. It utilized almost all types of sugar while negative for Rhamnose and Inositol. It is producing little amount of DNAase, gelatinase, amylase but the lipase activity is more as compared to other enzymes. This enzyme can be extracted and purified for further characterization.It gives a clear indication that these alkali-tolerant bacteria are good source of enzymes. A parallel finding was scripted by (Qamsari *et al*, 2011) where lipase enzyme is produced from alkailtolerant *Pseudomonas sp.*

Effect of Temperature on Bacterial Isolate 2E

The isolate could withstand a wide variation in temperature change. The optimum growth was observed at 40^{o}C but the rod could grow at a temperature of 50^{0}C.However It was unable to survive above 55^{0}C.The details of growth at various temperature is represented in Figure 3.

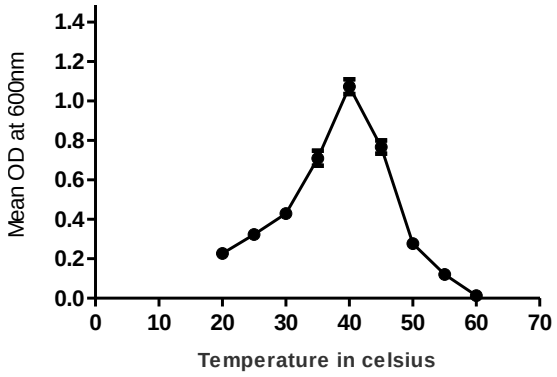


Figure 3: Effect of temperature on the growth of bacterial isolate 2E

Molecular Identification

After DNA extraction, the PCR amplification is carried and DNA was sequenced. The PCR amplification on agarose gel is given in Figure 4.After sequence analysis the phylogenetic tree was constructed.

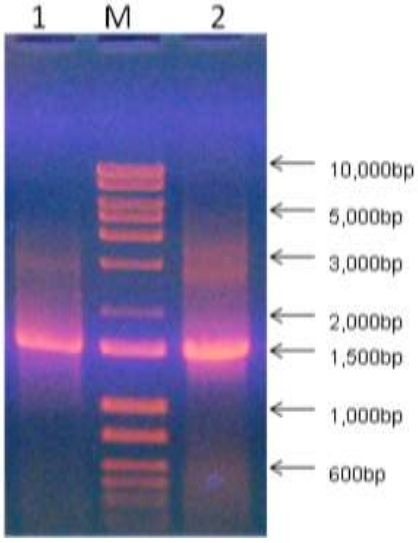
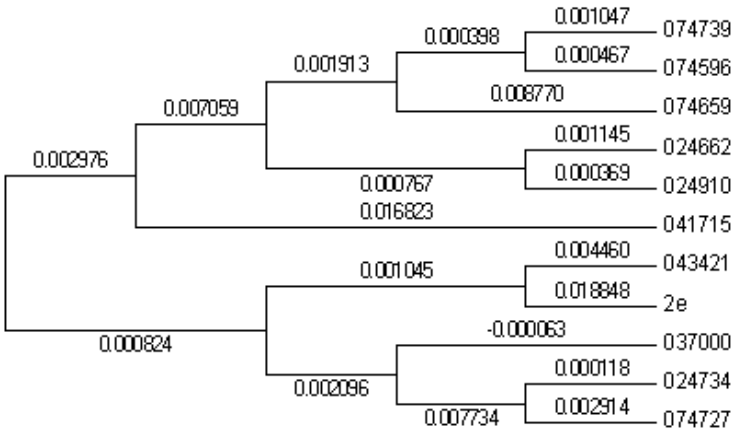


Figure 4: PCR amplification of 16srRNA gene

0.8% Agarose gel electrophoresis showed PCR product of 1.5 kb. Lane 1- 2E M- 1kb DNA ladder (DNAmark™ Logic Marker) and Lane2-2E

Phylogenetic Tree 2E

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.07970913 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 661 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.



ID	Description	% Similarity
2e	Analysed Sample	
037000	Pseudomonas pseudoalcaligenes strain Stanier 63	99%
024734	Pseudomonas alcaliphila strain AL15-21	98%
043421	Pseudomonas mendocina strain NCIB 10541	98%

074727	Pseudomonas mendocina ymp strain ymp	98%
024910	Pseudomonas monteilii strain CIP 104883	97%
024662	Pseudomonas plecoglossicida strain FPC951	97%
074739	Pseudomonas putida F1 strain F1	97%
074596	Pseudomonas putida KT2440 strain KT2440	97%
041715	Pseudomonas stutzeri ATCC 17588 = LMG 11199 strain ATCC 17588	97%
074659	Pseudomonas fulva 12-X strain 12-X	97%

Bioinformatic Analysis

The bacteria is having 99% similarity with *Pseudomonas pseudoalcaligenes strain Stanier 63*and 98% similarity with *Pseudomonas alcaliphila strain AL15-21, Pseudomonas mendocina strain NCIB 10541, Pseudomonas mendocina ymp strain ymp.* The sample 2E is having the closest identity with *Pseudomonas sp.*

DISCUSSION

As a simplified, discrete and productive system, Lake Chilika offers a suitable object for research on bacterial ecology. Lake Chilika shows such fluctuations causing spatial variations in their physico-chemical characteristics as shown by this study. Similar pattern both in the lake and the catchment area was believed to be instrumental in driving these temporal and spatial fluctuations, causing major changes especially in the chemical environment of the soda alkaline lakes (Oduor and Schargel, 2007).The brackish water quality of chilika exhibit a variation in its alkalinity which may lead to the alkali-tolerance of the bacterial flora. The sample 2E was identified as Pseudomonas mendocina 2E with accession number KF657327**.** designated by by Genbank. 16S rRNA gene analysis showed the bacteria isolated from Chilka Lake belonged *Gammaproteobacteria* group. This data corroborated with studies on other brackish water lakes such as Pulicat Lake, Tamil Nadu, India (Sahay, 2011) and Pangong Lake, Jammu & Kashmir (Sahay, 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, 2011), *Tsukamurella, Alishewanella, Sphingomonas*, The *Pseudomonas sp.* usually tolerate upto pH 10 (Qu, 2011) however the isolated strain is extremely alkali-tolerant and growing at and surviving upto 50^0 C. It is exhibiting a good lipase activity and the enzyme extraction is under study.

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