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Isolation and Molecular Identification of Extreme an Halotolerant *Staphylococcus Sp.* 13cc from Brackish Water of Chilika Lake

P.D. Mahapatra and B.B. Mishra

Department of Microbiology, CPGS, OUAT, Bhubaneswar - 751 003, India.

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Brackish water is the most extraordinary reservoir of bacterial community with an adaptability of tolerance to saline condition. In this research work, the halotolerance of the bacterial isolates were screened for identification and characterization of halotolerant 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 halotolerance. A total of 25 bacterial isolates were obtained and almost all the isolates survived upto 1 M NaCl but only one bacteria tolerated above 2.5M NaCl and was extremely halotolerant .This strain was identified as *Staphylococcus sp.13CC.* and designated with accession number KF657328 . It is a potential urease producer which is an important industrial enzyme. Further assay of enzyme is yet under study.

Key words: Halotolerant bacteria, *Staphylococcus* sp., Urease.

Asia's largest brackish lake Chilika(19^0 28' - 19 ^{0}54' N latitude and $85^0$5' – 85^0 38'E longitude)(Fig.1) is an estuarine (Biswas *et al* .,1932) whose water is subjected to variation in salinity 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 halotolerance to this salinity. Halotolerance is the protoplasmic component of resistance to salt 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).These halotolerant bacteria produces large quantity of compatible solutes,gas vesicles, intracellular and extracellular enzymes, salt resistant proteins and high contents of acidic to basic amino acids which acts as adaptive mode to tolerate high salt concentrations (Saum and Muller, 2007). and some may have certain inherent ability of biotechnological application.The research on bacterial potency and their tolerance to variations in physico chemical parameters of this lake is still under veil. Keeping in view of the above facts the present investigation was under taken in order to study the halotolerance of the bacterial population and isolate the extreme halotolerant strains whose biotic potential is to be studied further.

* To whom all correspondence should be addressed.
Mob.: +91-8763530566;
E-mail :pratikshya.undefined@gmail.com

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 halotolerant 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 salt Agar for isolation of culturable bacteria . 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 Nutrient agar slant at low temperature (4^0C) for further characterization.

Screening for Halotolerance

The halotolerance of bacterial isolates was studied by incubating 100µl of culture in different tubes containing 5ml of NB with varying amount of NaCl at a difference of 0.5M concentration ranging from 0.5 M to 4 M of sodium chloride (NaCl). After incubating 18 to 24 hours at 37° C, a loopful of the culture was sub-cultured onto NA plates. All the plates along with a control NA plate without NaCl were incubated at 37°C for 18 to 24 hours. The highest concentration of NaCl on which growth occurred, was detected.

Biochemical Identification of the halotolerant bacteria

The halotolerant 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 halotolerant bacterial isolate

The growth of isolate was tested on Nutrient broth by incubating it at a difference of 5^0C temperatures ranging from 25°C to 55^0C for 37^0C/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'-AGAGTTTGATCC TGGCTCAG-3'

Reverse Primer: 5'-ACGGCTACCTT GTTACGACTT-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°C for 2 minutes followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1.5 minutes. Final extension was carried out at 72°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 is enumerated in Table 1.The alkalinity of the water is found to be high .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.

Screening for Halotolerance

Out of the the 25 bacterial isolates,all survived upto 1 M which gives a clear indication that the inherent bacterial flora is mildly halotolerant .However with increasing molarity very few survived above 2 M and only one was growing luxuriantly at 3M named 13cc .The halotolerance was detected according to (Kushner *et al* 1993). The details of tolerance to salinity is depicted in Fig 2.

Biochemical Identification of the Halotolerant bacteria 13CC

After gram staining it was found to be a gram positive cocci , It was positive for catalase , coagulase. It utilized almost all types of sugar while negative for Dulcitol and Inositol.It is hydrolysing lipid and producing little amount of DNAase, gelatinase, amylase but the urease 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 halotolerant bacteria are good source of enzymes. A parallel finding was scripted by (Daoud *et al*.,2013) where lipase enzyme is produced from halotolerant *Staphylococcus sp.*

Effect of temperature on bacterial isolate 13cc

The isolate could withstand a wide variation in temperature change. The optimum growth was observed at 35^{o}C but the cocci 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 Fig 3.

Molecular Identification

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

Phylogenetic tree 13ccc

The evolutionary history was inferred using the Neighbor-Joining method The optimal tree with the sum of branch length = 0.17552286 is shown. The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances

Table 1. Physicochemical parameters of the water samples

Name of the Parameters	Sample-1 (Barkul)	Sample-2 (Kalupada Ghat)	Sample-3 (Parikud)	Sample-4 (Rambha)
BOD	28.3 mg/L	32.5 mg/L	20.6 mg/L	30.0 mg/L
COD	41.8 mg/L	48.7 mg/L	39.6 mg/L	46.2 mg/L
pH	7.9	8.8	8.6	9.2
Alkalinity	60 mg/L	80 mg/L	80 mg/L	84 mg/L
Calcium Hardness	26.4 mg/L	25.7 mg/L	28.5 mg/L	29.6 mg/L
Dissolved Oxygen	8.6 mg/L	8.2 mg/L	7.2 mg/L	7.6 mg/L
Salinity	10.8 ppt	11.2 ppt	11.4 ppt	10.2 ppt
Iron	o.25 mg/L	0.22 mg/L	0.24 mg/L	0.19 mg/L

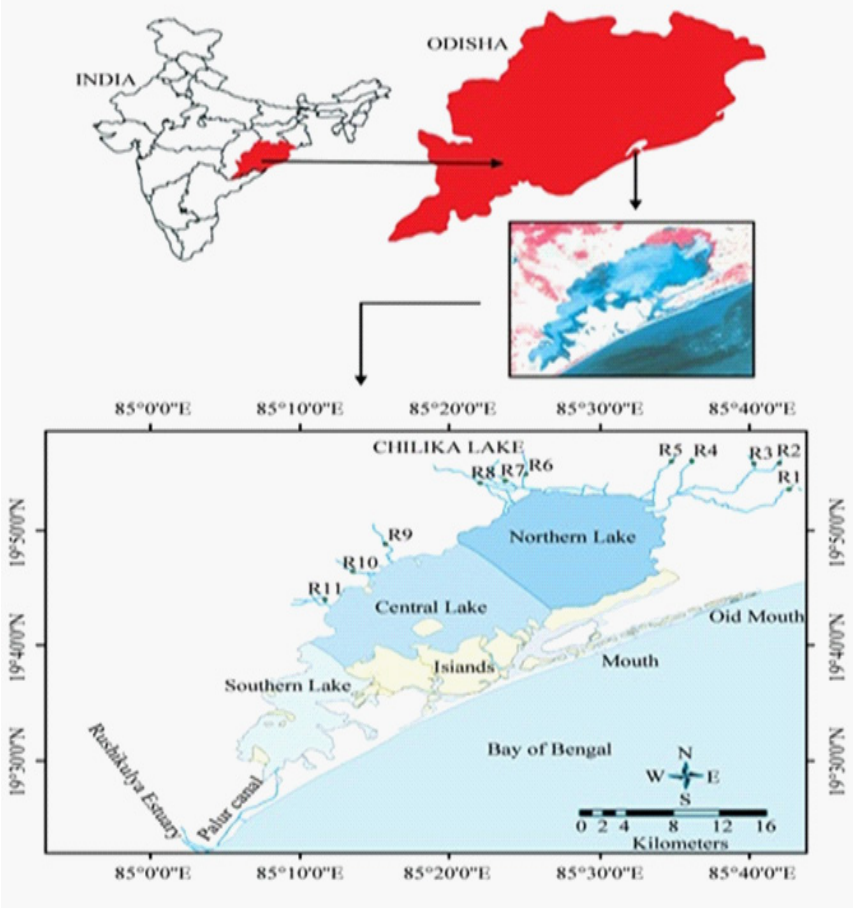
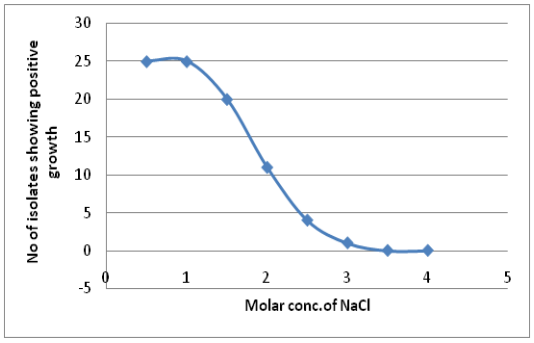
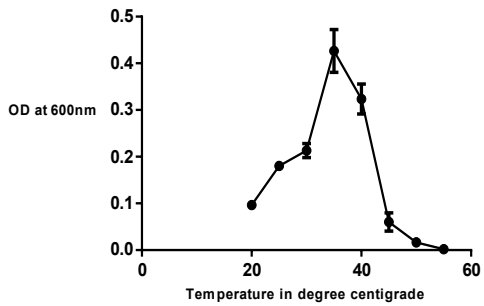


Fig. 1. Location and position of chilika



Statistical analysis	
r	-1
95% confidence interval	
P value	
P (one-tailed)	< 0.0001
Exact or approximate P value?	Exact
Significant (alpha = 0.05)	Yes
Number of XY Pairs	8

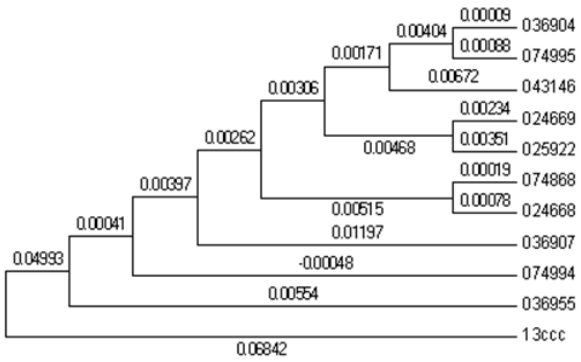
Fig. 2. Halotolerance of the bacterial isolates



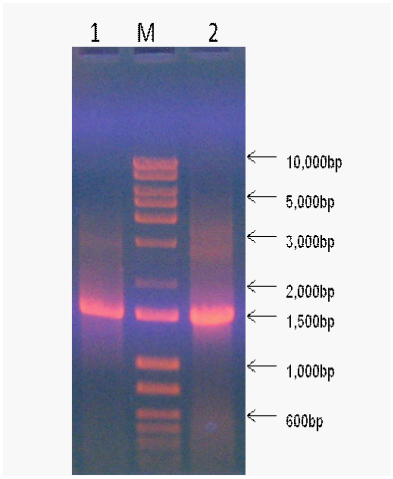
Statistical analysis	
Std. Deviation	0.1514
Std. Error of Mean	0.05353

Fig 3. Effect of temperature on the growth of bacterial isolate 13cc

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 1031 positions in the final dataset. Evolutionary analyses were conducted in MEGA5



ID	Description	% Similarity
13ccc	Analysed Sample	
074994	*Staphylococcus haemolyticus* JCSC1435 strain JCSC1435	99%
036955	*Staphylococcus haemolyticus* strain SM 131	99%
074868	*Staphylococcus lugdunensis* HKU09-01 strain HKU09-01	98%
024668	*Staphylococcus lugdunensis* strain ATCC 43809	98%
036907	*Staphylococcus xylosus* strain KL 162	98%
043146	*Staphylococcus simiae* CCM 7213 strain CCM 7213	98%
024669	*Staphylococcus pasteuri* strain ATCC51129	98%
036904	*Staphylococcus epidermidis* strain Fussel	98%
074995	*Staphylococcus epidermidis* RP62A strain RP62A	98%
025922	*Staphylococcus warneri* strain AW 25	98%



0.8% Agarose gel electrophoresis showed PCR product of 1.5 kb. Lane 1- 13CC M- 1kb DNA ladder (DNAmark™ Logic Marker) and Lane2-13CC

Fig. 4. PCR amplification of 16srRNA gene

Bioinformatic analysis

The bacteria is having 99% similarity with *Staphylococcus haemolyticus* and 98% similarity with *Staphylococcus xylosus, Staphylococcus pasteuri, Staphylococcus epidermidis, Staphylococcus simiae, Staphylococcus warneri.* The sample 13CC is having the closest identity with *Staphlococcus sp.*

CONCLUSION

The brackish water of chilika exhibit a variation in its salinity which may lead to the halotolerance of the bacterial flora.The sample 13CC was identified as *Staphylococcus sp.13CC* with accession number KF657328 designated by by Genbank. The *Staphylococcus sp.* usually tolerate 8% of NaCl(Ventosa *et al* 1998) however it is extremely halotolerant and growing at and surviving upto 45^0C. It is exhibiting a good urease

activity and the enzyme extraction is under study.

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