An alkalophilic *Methanosarcina* isolated from Lonar crater

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Craters formed by hypervelocity meteoritic impact offer unique ecological environment. The Lonar crater situated on the outskirts of Lonar town in District Buldhana, Maharashtra is the only known crater formed by hypervelocity meteoritic impact in basaltic rock. The lake water is very salty and alkaline (pH 9.5–10.0). A methanogen isolated from this unique environment grows well with acetate, methanol, methylamine (mono-, di- and tri-), pyruvate and 1° propanol. It shows poor growth on H$_2$:CO$_2$ (80:20 v/v). The isolate is a typical acetoclastic methanogen. The novelty of the isolate is its ability to grow at alkaline pH of 9.5. The isolate requires 0.5% (w/v) NaCl along with pH 9.0 for its optimum growth. Morphological and biochemical characteristics suggest that the isolate belongs to the genus *Methanosarcina*.

The methanogenic archaea are one of the earliest divergences of extant life forms. Several lines of evidence suggest that these microorganisms represent the third kingdom, namely archaea. Methanogens are widely distributed in nature, but confined to strictly anaerobic environments. A variety of methanogens have been isolated and characterized from different ecological niches such as sea sediment, salt pans, thermal vents, anaerobic digester and lake sediment. The sediment system of crater has been studied less for the methanogen, although environmental conditions are favourable for methanogenesis.

The alkaline lake of Lonar crater is situated in Maharashtra (lat. 19°58', long. 76°34'). Based on geological studies, it is postulated that the lake originated as a meteorite impact crater around 50–60 thousand years ago. The Lonar crater, the third largest in the world, is the only crater in basaltic rock. The lake has a circular periphery and is situated in a hollow, 0.14 km below the ground level with an amphitheatre of vertical cliffs (Figure 1). The diameter of the lake around the top of the banks is about 2 km, while at the bottom is 1.2 km. Water enters the lake through rain, ground water seepage and the springs situated in the cliffs at the edge of the lake. It does not receive any industrial discharges. The lake water is alkaline, having an average pH of 9.5–10.0. Alkalinity of the lake is attributed to the high content of sodium carbonate and hence was used previously as a source of washing soda. The microbial ecosystem prevailing in this lake has not been studied in detail so far. Therefore, it was thought to undertake studies on anaerobic bacteria present in Lonar crater.

Amongst different kinds of methanogens, the *Methanosarcina* sp. having acetoclastic activity play a pivotal role in the methanogenic food chain. The family Methanosarciniaceae contains certain halophilic and alkalophilic methylotrophic methanogens. However, none of the species of *Methanosarcina* show alkalophilic character along with the halotolerant or halophilic nature. In this paper, we describe the isolation of an alkalophilic and halotolerant methanogenic archaea belonging to the genus *Methanosarcina*, from Lonar crater.

Four samples of water and sediment were collected from four different sites of Lonar crater. The in situ temperature and pH of the lake water at these sites were 35°C and 9.5–10.0, respectively. Water and sediment samples were collected with the help of Auger Driller and Scooper in sterile N$_2$-flushed bottles. During the collection and transportation period, care was taken to maintain the anoxic conditions. On bringing the samples to the laboratory, microscopic observations were made for all four samples using a fluorescence microscope equipped with excitation filter UV 300–380 (Nikon Optiphot with epifluorescence attachment). Microscopic observations revealed the presence of single morphological type of cells showing blue-green fluorescence, a typical characteristic of methanogens, under UV light. Based on these observations, it was decided to mix all the sediment samples together to obtain a composite sample for further studies.

Chemical analysis of the water sample was done for the determination of total solids, volatile solids, conductivity, chloride as NaCl, alkalinity as CaCO$_3$, phosphate (PO$_4^{3-}$), according to Greenberg *et al.* The results of chemical analysis are as follows (g l$^{-1}$): total solids 15.5, volatile solids 2.7, alkalinity as CaCO$_3$ 3.6, chloride 3.0, phosphate (PO$_4^{3-}$) 0.2 and conductivity.

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**Figure 1.** Alkaline lake of Lonar crater, Dist. Buldhana, Maharashtra.
9.3 milli mho cm\textsuperscript{-1}. Chemical analysis showed that lake water is highly alkaline (pH 9.5–10.0) and rich in various salts, carbonate and bicarbonate.

By considering the chemical characteristics of the lake water and nutritional requirements of methanogens, a nutrient medium was devised for the enrichment and further studies. The medium, henceforth referred to as LNHP (Lunar Lake High pH), having the following composition (in g l\textsuperscript{-1}) was used: yeast extract 2.0, tryptone 2.0, NH\textsubscript{4}Cl 1.0, NaCl 30.0, KH\textsubscript{2}PO\textsubscript{4} 3.0H\textsubscript{2}O 0.4, KCl 1.5, trace element solution\textsuperscript{12} 1 ml, resazurine solution (0.1% w/v) 1 ml and Na\textsubscript{2}S\textsubscript{5}H\textsubscript{8}O\textsubscript{5} 0.5. The medium was boiled and cooled to room temperature under a stream of O\textsubscript{2}-free N\textsubscript{2}. The pH of the medium was adjusted to 9.5 with Na\textsubscript{2}CO\textsubscript{3}. It was then distributed anaerobically as 18 ml aliquots in 65 ml capacity serum vials, which were sealed with butyl rubber stopper and crimped with aluminium seals. Sterilization was carried out by autoclaving at 121°C for 20 min.

All experiments using the solid media were performed in an anaerobic glove box (Forma Scientific, USA). The solid media were prepared using agar (Difco, Detroit) as the solidifying agent at a concentration of 30 g l\textsuperscript{-1} and poured into glass Petri plates inside the anaerobic glove box. The solid and liquid media were prepared according to the anaerobic techniques described by Hungate\textsuperscript{12}, subsequently modified by Bryant\textsuperscript{13}, Miller and Wolin\textsuperscript{14}, and further described by Ranade and Gadre\textsuperscript{15}.

Enrichments were set up for methanogens in LNHP medium, with acetate as the growth substrate. We inoculated 2 g of aliquot from the composite sample of sediment into 65 ml serum bottle containing 18 ml of medium. After three successive transfers, vancomycin hydrochloride (100 μg ml\textsuperscript{-1}) was used in further enrichments to inhibit nonmethanogenic contaminants. All bottles were incubated at 35°C in static condition. In the first transfer of enrichment, vials containing LNHP medium with acetate as the substrate produced methane after two weeks of incubation at 35°C. Microscopic observation of the acetate enrichment shows the presence of one morphological type of methanogens. In subsequent transfers there was better growth of methanogens, as indicated by increase in methane content in identical periods of incubation. Vancomycin was shown to reduce the proportion of nonmethanogenic contaminant during enrichment studies.

Isolation of methanogens from successful enrichment was made by the roll tube method\textsuperscript{12} using solidified LNHP medium and acetate as the substrate, after suitable dilution of the eighth enrichment. After two weeks of incubation at 35°C, roll tubes that showed the presence of methane were taken for further studies. Single morphological type of colonies with irregular margin, opaque and lightish brown in colour were observed in roll tubes, which showed the presence of methane. A well-isolated colony was picked up and aseptically transferred into liquid LNHP medium in anaerobic glove box. The resulting culture was reincultured into the roll tube to check its purity. The isolated culture was found to be pure on the basis of microscopic observations and the presence of single morphological type of colonies in the roll tube. The purity of the resulting culture was also confirmed by checking the absence of aerobic growth on nutrient agar plate and anaerobic contaminant in PYG (Peptone–Yeast extract–Glucose) agar medium\textsuperscript{16}. Thus, from sediment samples single morphological type of methanogen was obtained, which was designated as LN 1.

Microscopic observation revealed that cells of LN 1 isolate were nonmotile, pseudosarcina and occurred as large aggregates. LN 1 isolate showed blue-green fluorescence under UV light (Figure 2).

LN 1 isolate was maintained by serial transfer in liquid LNHP medium after every three weeks. The culture was routinely observed to check the purity during maintenance.

The amount of methane was determined by gas chromatograph (Chemito 3800) equipped with Porapak Q column (80/100, 3.2 mm × 2 m, SS), thermal conductivity detector\textsuperscript{4,17}. Hydrogen (40 ml min\textsuperscript{-1}) was used as the carrier gas. Oven, injector and detector temperatures were 60°C, 60°C and 80°C, respectively. Acetate was measured by gas chromatography of free acids\textsuperscript{18}. Pure methane (Span Gas, India) was used as the external standard. Data integration and analysis were done using Winchrom EX software.

For further characterization of isolate LN 1, its ability to use different carbon sources, growth at different pH, temperature and salt tolerance were tested\textsuperscript{19}. For testing its catabolic substrates range, anoxic stocks of filter-sterilized substrates, viz. acetate, methanol, formate, pyruvate, methylamine (mono-, di- and tri-), propanol, butanol, ethanol and dimethylsulphide (DMS) were pre-

![Image](image_url)

Figure 2. Cells of isolate LN 1 showing blue-green fluorescence under UV-light (magnification, 75 ×; scale bar 100 μm).
pared (stock 2M). These substrates were added separately at 50 mM concentration. The vials with H2 : CO2 (80 : 20 v/v) as the substrate were pressurized every alternate day at 1 kg cm\(^{-2}\), to maintain adequate supply of the substrate. Freshly-grown culture was inoculated at 10\% v/v. All bottles were incubated for maximum period of 30 days and monitored on every alternate day for methanogenesis by gas chromatography. Methane formation in test bottles was compared with the methane formation in control lacking catabolic substrates. These results showed that isolate LN 1 has the ability to use acetate, methanol, pyruvate, methylamines and 1\° propanol. The isolated culture did not use formate, ethanol, butanol and DMS. Compared to these growth substrates, it gave less methane and scanty growth with H2 : CO2 as the substrate. LN 1 produced more amount of methane with acetate (50 mM) as the substrate.

To determine the optimum pH for the growth of LN 1 isolate, LNHP medium adjusted to different pH values from 6.0 to 10.5 was used. The pH of the medium was adjusted using 1 N HCl or Na\(_2\)CO\(_3\). Acetate was used as growth substrate. All the vials were incubated at 35\°C in static condition and analysed for methanogenesis, as described earlier. The cells of isolate LN 1 grew well in the pH range of 6.5–9.5, with optimum growth at pH 9.0 (Figure 3).

Optimum temperature for the growth of the isolate was determined in LNHP medium. Acetate was used as growth substrate. All bottles were incubated at 15\°C to 55\°C, at a temperature interval of 5\°C. All bottles were inoculated and analysed for growth as described previously, for a maximum period of 30 days. Isolate LN 1 showed growth in the temperature range of 30–45\°C, with optimum growth at 35–37\°C.

Requirement of NaCl was examined in LNHP medium with different salt concentrations ranging from 0.06 to 10\% (w/v). All bottles were inoculated, incubated and analysed for growth as described earlier. The isolate LN 1 grew in the presence of 3\% NaCl, with optimum growth at 0.5\% (w/v) NaCl. This isolate produced methane at low salt (0.06\%) concentration. Methanogenesis by this culture, however, was completely inhibited at NaCl concentration higher than 3\% (w/v).

The data on optimum pH, optimum temperature and requirement of NaCl by isolate LN 1 are the average values of results obtained from three independent experiments.

The physiological and morphological characteristics clearly show that isolate LN 1 belongs to the genus *Methanosarcina*. A further study using molecular techniques is necessary for a more defined taxonomic and phylogenetic classification of the LN 1 isolate.

*Methanosarcina* is an important genus in the group of methanogens. This is mainly due to universal occurrence of these methanogens in aquatic sediments of sea, river, lake and anaerobic digesters\(^1\). Within the genus, five different species have been reported so far\(^2\). All species of *Methanosarcina* have pH optima around 6.0–7.0. Only few species can grow in broad pH range of 4.0–8.5 (ref. 2). It is worthwhile to mention here that three cultures of *Methanosarcina* which were obtained from MACS collection of microorganisms, ARl, Pune, namely *Methanosarcina mazei* S-6 (MCM 709), *Methanosarcina barkeri* MS (MCM 711) and *M. barkeri* 227 (MCM 710) were unable to grow in LNHP medium. Compared to these species, isolate LN 1 has pH optima at 9.0 and pH range of 6.5–9.5 for growth. Certain species of *Methanosarcina* are halophilic or halotolerant in nature, such as *Methanosarcina thermophila*, which can grow up to 7.0\% (w/v) NaCl, with its optimum growth at 3.5\% (w/v) NaCl (ref. 2) and *M. barkeri* A-12 and A-13, which can grow up to 3\% NaCl with its optimum growth at 0.06\% NaCl (ref. 5). Compared to these species, the isolate LN 1 has low salt (0.5\% w/v) requirement and salt tolerance of 3\% (w/v) NaCl. Although isolate LN 1 shared characteristics with different known species of *Methanosarcina*, it stands apart from these reported species with respect to its optimum growth at alkaline pH of 9.0. Thus, isolation of methanogen LN 1 in the present report can be considered as unique.

Degradation of organic matter under anaerobic condition or in absence of oxygen is a complex microbial phenomenon\(^3\). It involves activities of a number of anaerobic bacteria. In the end of the process, methane is produced by the methanogens. Thus production of methane or presence of methanogens in the sediment sample of Lonar lake confirms the anaerobic degradation of organic matter in the sediment. This also means that not only methanogens are present in the sediment sample, but various other anaerobic bacteria also inhabit the sediment system of Lonar. All these bacteria play an

**Figure 3.** Effect of pH on methane formation and acetate utilization by isolate LN 1. Each data point represents the average value of three independent experiments.
important role in the degradation of organic matter, thus making the Lunar ecosystem a live one. Isolation of aerobic bacteria from Lunar lake sediment has already been reported by Kanekar et al. This signifies the biodiversity of Lunar lake with respect to microbial life.

In conclusion, strain LN 1 isolated from the sediment of Lunar crater is an alkalophilic, halotolerant methanogenic archaean that can grow at pH 9.0, which differs from the known species of the Methanosarcina.


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Microfauna and age of the Sangcha Malla Formation of Garhwal Tethys Himalaya, India

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Biostratigraphic investigations of the Sangcha Malla Formation in the type area of the Garhwal Tethys Himalaya were carried out during an expedition. Systematic investigations from this unit led to the recovery of several well-preserved species of Archaeoglobigerina, Rosita, Globotruncanita, Heterohelix and Pseudotextularia. The faunal assemblage is indicative of deposition under a deep marine condition. The foraminiferal taxa are recorded from the upper part of the Sangcha Malla Formation, which is the youngest marine litho-unit deposited in the Garhwal Tethys Himalaya. Stratigraphic distribution of the taxa indicates that this part of the Sangcha Malla Formation was deposited during the Campanian times. The fauna recovered herein from the Garhwal Tethys Himalaya shows a close affinity with that of the Zanskar region of Ladakh Himalaya and the Siplt region of Himachal Pradesh, suggesting thereby that during the Late Cretaceous times there were marine connections in these regions and the Upper Cretaceous sediments were deposited under similar (deep marine) palaeoenvironment.

The fossiliferous sedimentary succession of the remote Malla Jolar area in the Kiogad sector of the Garhwal Tethys Himalaya has been studied since early times. Heim and Gansser1 gave a detailed geological account of the area and differentiated various litho-units. Several other geologists contributed to the geology of the area2–4. The present contribution is based on the fieldwork carried out by two of the authors (K.P.J. and S.K.P.) in an expedition in the Garhwal Tethys Himalaya organized by the Wadia Institute of Himalayan Geology, Dehra Dun in 1998.

A perusal of the literature reveals that age of the Sangcha Malla Formation of the Garhwal Tethys Himalaya was established mainly on the basis of planktonic foraminifera1,2,3 studied in thin sections of rocks. Planktonic foraminiferal studies based on thin sections without their morphotypes have been questioned by several workers5, as the latter provide additional characteristics such as peripheral keels, ventral and dorsal suture lines, etc. for specific determination. For precise identifica-

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