Archives of Microbiology (2023) 205:332
https://doi.org/10.1007/s00203-023-03661-2

ORIGINAL PAPER

Phylogenetically and physiologically diverse methanogenic archaea inhabit the Indian hot spring environments

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Received: 24 March 2023 / Revised: 10 August 2023 / Accepted: 15 August 2023 / Published online: 14 September 2023

Abstract

Mesophilic and thermophilic methanogens belonging to the hydrogenotrophic, methylotrophic, and acetotrophic groups were isolated from Indian hot spring environments using BY and BCYT growth media. Following initial *Hinf* I-based PCR–RFLP screening, 70 methanogens were sequenced to ascertain their identity. These methanogens were phylogenetically and physiologically diverse and represented different taxa distributed across three physiological groups, i.e., hydrogenotrophs (53), methylotrophs (14) and acetotrophs (3). Overall, methanogens representing three families, five genera, and ten species, including two putative novel species, were recognized. The highest number and diversity of methanogens was observed at 40 ℃, dominated by *Methanobacterium* (10; 3 species), *Methanosarcina* (9; 3 species), *Methanothermobacter* (7; 2 species), *Methanomethylovorans* (5; 1 species) and *Methanoculleus* (3; 1 species). Both putative novel methanogen species were isolated at 40 ℃ and belonged to the genera *Methanosarcina* and *Methanobacterium*. At 55 ℃, limited diversity was observed, and resulted in the isolation of only two genera of methanogens, i.e., *Methanothermobacter* (28; 2 species) and *Methanosarcina* (4; 1 species). At 70 ℃, only members of the genus *Methanothermobacter* (5; 2 species) were isolated, whereas no methanogen could be cultured at 85 ℃. Ours is the first study that documents the extensive range of cultivable methanogenic archaea inhabiting hot springs across various geothermal provinces of India.

Keywords Archaea · Diversity · Ecology · Phylogeny · Taxonomy

Introduction

Methane has been identified as one of the most important renewable biofuels and has multiple applications if produced and harvested in anaerobic digestion processes (Demirel and Scherer 2008; Pore et al. 2019). At the same time, methane is also a potent greenhouse gas (28 times CO_2), contributing to global warming for over 100 years if released directly into the atmosphere (Pachauri et al. 2014). The beneficial and harmful nature of methane, therefore, necessitates the study of methanogens, which produce most of the biogenic methane (Thauer et al. 2008). Methanogens are a group of strict anaerobic archaea that play a key role in the carbon cycle by facilitating interspecies hydrogen transfer (Ishii et al. 2005; Pak and Bartha 1998). These organisms thrive in diverse habitats like oil wells, the gut of herbivores, hydrothermal vents, subsurface coal mines, rice fields, hot springs, etc. (Joshi et al. 2018; Kumar et al. 2018). Methanogens are currently documented under four phyla, namely, *Euryarchaeota*, *Thermoproteota*, *Bathyarchaeota*, and *Verstraetearchaeota* (Berghuis et al. 2019; Evans et al. 2015; Vanwonterghem et al. 2016; Zhou et al. 2018), although the cultured isolates till date are only from phylum *Euroyarchaeota*, *Verstraetearchaeota* and *Thermoproteota* (Cheng et al. 2023; Hatzenpichler 2023). Phylogenetically, methanogens are divided into five families, comprising 33 genera and 153 species and can survive over a broad temperature range of 0–110 ℃ and pH of 4.1–10 (Franzmann et al. 1997; Michał et al. 2018). For growth, methanogens require a redox potential lesser than − 200 mV and can be either autotrophic or fastidious in nature. Various media, both generalized and specific, have been used to match the in situ culture conditions of different methanogens inhabiting diverse environments (Cheng et al. 2007; Joshi et al. 2018; Khelaifia et al.

Communicated by Kristina Beblo-Vranesevic.

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2013). Physiologically, methanogens are divided into three broad groups based on substrate requirements; hydrogenotrophic, methylotrophic, and acetotrophic or aceticlastic (Liu and Whitman 2008). Amongst, hydrogenotrophic methanogens utilize formate and H_2: CO_2 and are the most diverse, widely found, and fastest-growing (Zabranska and Pokorna 2018). The methylotrophic methanogens utilize methyl compounds like methylamines, and alcohols, including methanol, with a very few requiring a combination of hydrogen and methanol; while acetotrophic methanogens consume acetate (Demirel and Scherer 2008). Methanogens are integral to the microbial consortia in several industrially significant processes, like the degradation of organic wastes and xenobiotics and wastewater treatment (Enzmann et al. 2018). Hot springs are reported to harbour lignocellulolytic, hydrocarbon-degrading, and methane-producing microbes, serving as a promising source of thermophilic microbes and thermostable enzymes (Jardine et al. 2018; Kristjansson 1989; Mohammad et al. 2017; Tirawongsaroj et al. 2008; Yadav et al. 2018). Hot springs environments are also known to function as analogues for the hydrothermal systems of Mars, which are useful for habitability studies using methanogens (Hays et al. 2017). The Indian subcontinent is known to have more than 300 hot springs spread across seven geothermal provinces (Singh Bisht et al. 2011). Indian hot springs are majorly pristine niches and support diverse microbial communities (Narsing Rao et al. 2018; Poddar and Das 2018). The present study documents the diversity, distribution and phylogenetic relationships of culturable methanogens isolated from different Indian hot springs.

Materials and methods

Sampling, media, and enrichment

The water and sediment samples were collected from nine hot springs located across four geothermal provinces of India, namely Chumathang and Puga (Ladakh, Jammu and Kashmir); Unhavare, Tural, and Rajawadi (Western Maharashtra); two locations in Chopda, i.e., 1 and 2 (North Maharashtra); Unkeshwar (East Maharashtra) and Tattapani (Chhattisgarh). All hot springs were alkaline in nature, with pH ranging from 7.7 to 8.8, while the temperatures ranged from 40 to 80 ℃ (Table S1).

All the enrichments were set up in 60 ml glass serum bottles using two different media, i.e., BY (Joblin 2005) and BCYT (Touzel and Albagnac 1983) at four different temperatures of 40 ℃, 55 ℃, 70 ℃, and 85 ℃ (pH 6.8 ± 0.1). The BCYT medium comprised (per L) of 0.3 g KH_2PO_4, 0.6 g NaCl, 0.1 g $MgCl_2.6H_2O$, 0.08 g $CaCl_2.2H_2O$, 1 g NH_4Cl, 0.5 g yeast extract, 0.5 g trypticase, 1 ml resazurin (0.2%, w/v), 1.5 g $KHCO_3$, 10 ml each of trace element and vitamin solution, 1 g L-cysteine HCl, and 0.3 g $Na_2S.9H_2O$ (Table S2). On the other hand, the BY medium contained (per L) 170 ml each of solution A and B, 5 g $NaHCO_3$, 1 g yeast extract, 300 ml of centrifuged rumen fluid, 10 ml trace element solution, 1 ml resazurin (0.1%, w/v), 0.5 g L-cysteine HCl, and 10 ml vitamin solution (Table S2). The specific substrate requirements were provided for enriching each group of methanogens. For hydrogenotrophic methanogens, the medium was pressurized with H_2: CO_2 (80:20, v/v at 1 bar pressure) and re-pressurized weekly during incubation (Joshi et al. 2018). For the methylotrophic and acetotrophic groups, a methanol and trimethylamine mixture and sodium acetate were provided as substrates (final concentration, 50 mM), respectively. Streptomycin sulphate and benzylpenicillin were added to prevent bacterial contamination (final concentration, 200 µg/ml). The growth was monitored by checking the turbidity, fluorescence microscopy, and measurement of headspace methane using a gas chromatograph (Perkin Elmer, TCD, Argon carrier). The enrichments were checked for growth up to two months, after which they were scored positive or negative.

Isolation procedure

The positive enrichments were selected to isolate pure cultures of methanogens using serum bottle modification of the Hungate technique (Miller and Wolin 1974). For this, the dilutions were made up to 10^{-5}, and 1 ml of inoculum from each dilution was added to 125 ml glass bottles containing 10 ml of the respective medium, substrate, and gelling agent. The agar (2%) or Gelrite (0.8% mixed with 0.1% $MgCl_2{\cdot}6H_2O$) served as the gelling agents for mesophilic (40 ℃) or thermophilic (55–85 ℃) incubations, respectively. All the roll bottles were routinely checked for the growth of isolated colonies and methane production during incubation. After successful growth, the morphologically distinct colonies were picked under anaerobic conditions and grown under their respective growth conditions of media, substrate, and temperature.

Molecular characterization

The DNA of the pure cultures grown for 1 month was isolated using the cetyltrimethylammonium bromide (CTAB) method (Doyle 1991) with some modifications. Briefly, the cells were centrifuged at 10,510 rcf/10 min, and resultant pellets were suspended in 1 ml CTAB buffer and incubated at 50 ℃ with intermittent mixing until a clear solution was obtained. The solution was mixed with an equal volume of phenol: chloroform: isoamyl alcohol (P: C: I; 25:24:1), centrifuged, and the supernatant was mixed with an equal volume of C: I (24:1). Following re-centrifugation, an equal volume of isopropanol was added to the supernatant and

incubated at -20 ℃ for 12 h. The visible DNA threads were centrifuged twice at 17,760 rcf/15 min, and the pellet was washed with pre-warmed 80% ethanol. The washed pellet was re-centrifuged and dried before re-suspending in 50–100 µl MilliQ water and stored at − 20 ℃ until used.

The isolated DNA of all cultures was used for the PCR amplification of the 16S rRNA gene using the primer pair 86F and 1340R (Wright and Pimm 2003). Meanwhile, the 16S rRNA gene sequences of different genera and species of methanogens were downloaded from Nuccore, NCBI. Further, the in silico restriction digestion analysis was performed using the Cleaver software to identify the most appropriate restriction enzyme (Jarman 2006). The amplified PCR products were then subjected to the PCR-restriction fragment length polymorphism (RFLP) analysis using the identified restriction enzyme as per the manufacturer's protocol.

The digested PCR products were separated by electrophoresis (60 V for 5 h; Bio-Rad PowerPac™ Basic) on 3% agarose gel containing GelRed® Nucleic Acid Gel Stain (Biotium) in FAST buffer (Brody and Kern 2004). The isolates showing different restriction patterns or having the same patterns but differing in sampling locations and growth conditions were outsourced for sequencing at 1st BASE, Singapore. Using the BioEdit software, the acquired sequences were aligned and assembled (Hall 1999). The sequence similarity searches were carried out using NCBI GenBank BLASTn, limiting to sequence from type material.

For phylogenetic analyses, the 16S rRNA gene sequences of type strains of nearest matches were downloaded from the NCBI GenBank database. All the obtained sequences were aligned using the ClustalW programme (Thompson et al. 1994) with default settings in MEGAXI (Tamura et al. 2021). The aligned sequences were used to construct a phylogenetic tree in MEGAXI using the maximum-likelihood method based on the Tamura-Nei model (Tamura and Nei 1993) and tested by 500 bootstrap replications.

Following identification, the roll tubes were prepared again to document the colony characteristics like shape, size and colour. The cellular morphology was studied using a phase contrast and fluorescence microscope (Eclipse 80i, Nikon, Japan).

Ecological distribution

To correlate the niche diversity of the obtained methanogen strains recorded in this study to closely related taxa, 16S rRNA gene sequences were queried with the GenBank dataset. All the closely related cultured and uncultured sequences were downloaded, and their phylogenetic positions were inferred using a maximum-likelihood-based phylogenetic tree using MEGAXI. Sequence comparison was conducted using BLASTn with > 90% OTU coverage, > 98.0% identity, and an *e* value of $< 1e-25$. Additionally, the information on the habitat of related taxa was also recorded to understand the distribution across oxygen-restricted habitats.

Results and discussion

Enrichment and isolation

The abundance of archaea, in general, and methanogens, in particular, is usually less in a given niche than other microbes like bacteria (Aller and Kemp 2008; Flemming and Wuertz 2019). Hence, enrichment in the particular medium is desired to increase the population of targeted groups of methanogens. Methanogens have particular growth requirements that might differ for each genus or species (Joblin 2005; Whitman et al. 2014). The autotrophic methanogens require only the mineral medium to survive and grow, whereas the fastidious ones are known to have specific growth requirements in terms of amino acids, vitamins, trace elements, and co-enzymes (Joblin 2005; Saleem et al. 2013). These particular requirements are fulfilled by nutrient-rich components like rumen liquor, as in BY medium, which contains 28 different compound categories (Saleem et al. 2013). The rationale behind using two different media, viz. BCYT (basal mineral medium) and BY (with rumen liquor) was to facilitate the growth of both autotrophic and fastidious methanogens. Further, for the purpose of potential industrial applications, including anaerobic digestion, which generally function optimally at neutral pH; the similar pH range was selected for the enrichment process.

Of 216 total enrichments, 56 yielded positive results (Table S3). Samples from the western Maharashtra region yielded most of the positive enrichments, while the least were from the Ladakh region (Fig. 1). The maximum positive enrichments were obtained at 40 ℃ (28), followed by 55 ℃ (21), and 70 ℃ (7), whereas no enrichment was found positive at 85 ℃. It was observed that the enrichments across incubation temperatures (40–70 ℃) were obtained from the hot springs having high in situ temperature conditions (50–80℃), whereas positive enrichments at 40 ℃ were obtained majorly from lower temperature hot springs.

The substrate-wise analysis revealed the highest number of positive enrichments for hydrogenotrophic (total 26; 14 at 40 ℃, 9 at 55 ℃ and 3 at 70 ℃), followed by methylotrophic (total 18; 9 at 40 ℃, 7 at 55 ℃ and 2 at 70 ℃) and acetotrophic (total 12; 5 at 40 ℃, 5 at 55 ℃ and 2 at 70 ℃) methanogens (Table S3). In nature, the most abundant methanogens are hydrogenotrophic, as it is thermodynamically the most favourable reaction of all three (Garcia et al. 2000; Jabłoński et al. 2015). On the contrary, the process of acetotrophic methanogenesis is the least abundant in nature, as the reaction requires maximum free energy to proceed,

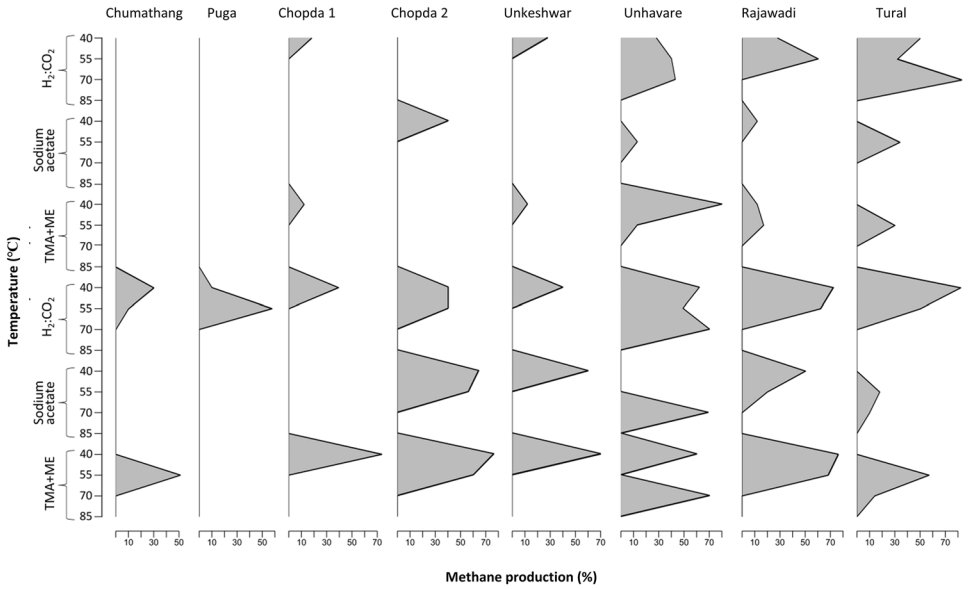


Fig. 1 Spatial graph depicting the positive enrichments established at different culturing conditions in an increasing number

resulting in less available energy (Deppenmeier and Müller 2008; Ferry 2011).

In the growth medium comparison, the BCYT medium supported the growth better, as indicated by 34 positive enrichments compared to 22 of the BY medium. No positive enrichment was obtained from the Tattapani sample, while Unhavare, Rajawadi and Tural hot springs yielded maximum positive enrichments across media, temperatures and substrates. The absence of growth in Tattapani enrichments can probably be attributed to the prevailing alkaline and high-temperature conditions, which might have limited the associated diversity.

The positive enrichments established at a mesophilic temperature of 40 ℃, particularly from the hot springs with a higher in situ temperature, indicate the presence of thermophilic and/ or thermotolerant methanogens capable of growing even at lower temperatures. Also, some of the hot springs are known to show seasonal and diurnal variations or the presence of gradients in the in situ temperature conditions that might also impact the population and diversity (Sigee 2005).

Thermophilic methanogens are known to have a faster growth rate and less doubling time than their mesophilic counterparts (Whitman et al. 2014); hence an earlier positive result was anticipated at higher temperatures. As expected, the enrichments set up at 55 ℃ and 70 ℃ were able to produce methane within 15 days of incubation, whereas the enrichments set up at 40 ℃ took three to four weeks to produce methane.

The isolation procedures from these enrichments led to the growth of morphologically diverse colonies of methanogens on roll bottles. Over 200 colonies were picked into their respective liquid medium and incubated at respective temperatures. Of the total colonies picked, the most diverse and most number of colonies were observed at 40 ℃, and a few at 55 ℃ but the roll tubes at 70 ℃ yielded only a single type and less number of colonies in all the samples.

Based on methane production, 120 isolates were noted positive, of which 78 were hydrogenotrophic (39 at 40 ℃, 26 at 55 ℃ and 13 at 70 ℃), 33 were methylotrophic (28 at 40 ℃ and 5 at 55 ℃) and 9 were acetotrophic (5 at 40 ℃ and 4 at 55 ℃).

Molecular characterization

The in silico analysis identified *Hinf* I as the most suitable restriction enzyme capable of generating different ribotypes (Table S4), thus differentiating methanogens at the genus level. The PCR amplification of isolated DNA generated PCR products of c.a. 1200 bp for all isolates. The actual restriction digestion of PCR products of one hundred and twenty isolates using *Hinf* I produced 6 types of restriction patterns (Fig. S1), which helped document the diversity and minimize the number of sequencing reactions. Seventy isolates were chosen for sequencing and identification based on differences in geographical locations and growing conditions.

The identification results established these isolates as members of ten different species under five genera; *Methanothermobacter* (*Mtb.*), *Methanoculleus* (*Mcu.*), *Methanosarcina (Msc.), Methanomethylovorans (Mmv.)* and *Methanobacterium* (*Mbt.*). All strains were members of families, *Methanobacteriaceae*, *Methanomicrobiaceae,* and *Methanosarcinaceae* of phylum *Euryarchaeota* (Table 1). Further, these cultures comprised all three groups, i.e., hydrogenotrophic (53), methylotrophic (14), and acetotrophic (3).

Table 1 Cultivation and identification details of pure isolates of methanogens obtained from different hot springs across India

Name of hot spring	Location	Substrate	Medium	Temperature in ℃	Number of identified strains	Representative strain		Closest phylogenetic affiliate and GenBank accession number	% sequence coverage	% similarity
						Strain name	GenBank accession number			
Chumathang	Ladakh	H_2:CO_2	BY	55	3	C55H7	ON107522	*Methanothermobacter thermautotrophicus* strain Delta H (CP064324)	100	100
				70	3	C70H5	ON107512	*Methanothermobater thermautotrophicus* strain Delta H (CP064324)	100	100
			BCYT	55	2	C55H9	ON107498	*Methanothermobacter marburgensis* strain Marburg (CP069376)	100	99.66
Puga	Ladakh	H_2:CO_2	BCYT	55	5	P55H1	ON107517	*Methanothermobacter thermautotrophicus* strain Delta H (CP064324)	100	100
Unhavare	West Maharashtra	H_2:CO_2	BY	55	1	U55H21	OQ600610	*Methanothermobacter thermautotrophicus* strain Delta H (CP064324)	100	100
					1	U55H7	ON107516	*Methanothermobacter marburgensis* strain Marburg (CP069376)	100	100
			BCYT	40	4	U40H2	ON107497	*Methanothermobacter marburgensis* strain Marburg (CP069376)	100	99.66
				55	6	U55H15	ON107511	*Methanothermobacter thermautotrophicus* strain Delta H (CP064324)	100	100
Tural	West Maharashtra	H_2:CO_2	BY	40	2	T40H1	ON107514	*Methanobacterium formicicum* strain MF (NR115168)	100	99.82
				55	2	T55H15	ON107507	*Methanothermobacter marburgensis* strain Marburg (CP069376)	100	99.74
			BCYT	40	2	T40H8	ON107499	*Methanothermobacter marburgensis* strain Marburg (CP069376)	100	99.74
				55	2	T55H21	ON107508	*Methanothermobacter marburgensis* strain Marburg (CP001710)	100	99.66
				70	2	T70H10	ON107513	*Methanothermobacter marburgensis* strain Marburg (CP001710)	100	99.74
		TMA + ME	BY	40	3	T40N3	ON107505	*Methanomethylovorans thermophila* strain L2FAW (NR043089)	100	99.73

Table 1 (continued)

Name of hot spring	Location	Substrate	Medium	Temperature in ℃	Number of identified strains	Representative strain		Closest phylogenetic affiliate and GenBank accession number	% sequence coverage	% similarity
						Strain name	GenBank accession number			
Rajawadi	West Maharashtra	H_2:CO_2	BY	40	1	R40H5	ON107523	*Methanobacterium bryantii* strain MOH (NR042781)	100	98.99
				55	3	R55H12	ON107509	*Methanothermobacter marburgensis* strain Marburg (CP001710)	100	99.74
			BCYT	40	2	R40H9	MK496647	*Methanobacterium aarhusense* strain H2 LR (NR042895)	100	97.05
					1	R40H16	ON107500	*Methanothermobacter marburgensis* strain Marburg (CP001710)	99	99.74
				55	3	R55H20	ON107510	*Methanothermobacter marburgensis* strain Marburg (CP069376)	99	99.74
		TMA + ME	BY	40	2	R40N10	ON107506	*Methanomethylovorans thermophila* strain L2FAW (NR043089)	98	99.40
Unkeshwar	East maharashtra	H_2:CO_2	BY	40	2	UP40H1	ON107519	*Methanobacterium formicicum* strain MF (NR115168)	100	100
Chopda 1	North Maharashtra	H_2:CO_2	BY	40	2	UC40H2	ON107518	*Methanobacterium formicicum* strain MF (NR115168)	100	99.91
			BCYT	40	1	H40UC1	ON107504	*Methanobacterium bryantii* strain MS1724 (MK680235)	100	100
		TMA + ME	BCYT	40	3	T40UC2	MG008506	*Methanosarcina horonobensis* strain HB-1 (CP009516)	100	98.50
Chopda 2	North Maharashtra	H_2:CO_2	BY	40	2	UCB40H6	OQ607805	*Methanoculleus hydrogenitrophicus* strain HC (NR116881)	100	100
			BCYT	40	1	UCB40H4	ON107520	*Methanoculleus hydrogenitrophicus* strain HC (NR116881)	100	99.51
		TMA + ME	BCYT	40	2	UCB40T9	ON107501	*Methanosarcina mazei* strain S-6 (CP009512)	99	100
					2	M40UCB1	OQ457565	*Methanosarcina thermophila* strain TM-1 (CP009501)	100	100
				55	2	UCB55M1	OQ457561	*Methanosarcina thermophila* strain TM-1 (CP009501)	100	100
		Sodium acetate	BCYT	40	1	UCB40N1	OQ457564	*Methanosarcina thermophila* strain TM-1 (CP009501)	100	100
				55	2	N55UCB5	ON107521	*Methanosarcina thermophila* strain TM-1 (CP009501)	100	100

From the hot springs of Ladakh, only the members of the thermophilic hydrogenotrophic genus *Methanothermobacter* were obtained; *Mtb. thermautotrophicus* (55 and 70 ℃, BY), and *Mtb. marburgensis* (55 ℃, BCYT) from Chumathang (Fig. 2a), and *Mtb. thermautotrophicus* (55 ℃, BCYT) from Puga (Fig. 2b). From the Unkeshwar hot spring of eastern Maharashtra, which had low in situ temperature, only hydrogenotrophic *Mbt. formicicum* was obtained on BY medium at 40 ℃ (Fig. 2c). From Rajawadi, Tural and Unhavare hot springs of western Maharashtra, which had in situ temperatures in the range of 50–65 ℃, three genera of methanogens viz., *Methanothermobacter*, *Methanobacterium*, and *Methanomethylovorans* were cultured. Amongst, maximum diversity was observed in Rajawadi (Fig. 2d), followed by Tural (Fig. 2e) and Unhavare (Fig. 2f) hot springs, from where four, three and two taxa of methanogens were isolated, respectively. At 40 ℃, members of the hydrogenotrophic genus *Methanobacterium* were isolated from Rajawadi (*Mbt. bryantii*) and Tural (*Mbt. formicicum*) hot springs on BY medium, and a putative novel species on BCYT medium from Rajawadi. In addition, a strict methylotrophic methanogen, *Mmv. thermophila* was also obtained at 40 ℃ from both these hot springs, but only on BY medium. At 55 ℃, *Mtb. marburgensis* was isolated from all three hot springs, whereas *Mtb. thermautotrophicus* was obtained only from Unhavare. Both *Methanothermobacter* spp. were obtained on both media. At 70 °C, only *Mtb. marburgensis* was isolated from the Tural hot spring, probably because of its higher in situ temperature than others. Methanogens belonging to all three groups, i.e., hydrogenotrophic, methylotrophic, and acetotrophic, were obtained from both the Chopda hot springs (northern Maharashtra) on BCYT medium; exclusively mesophilic from location 1 (Fig. 2g) but both mesophilic and thermophilic from location 2 (Fig. 2h). Two species of *Methanobacterium*, namely *Mbt. formicicum* and *Mbt. bryantii* and one putative novel species of methylotrophic *Methanosarcina* were cultured from location 1. From location 2, in addition to two species of *Methanosarcina,* i.e., *Msc. mazei* (40 °C), *Msc. thermophila* (40 °C, 55 ℃), and *Mcu. hydrogenitrophicus* (40 ℃) was also isolated. Some interesting observations were also made after analysing Venn diagrams to classify the methanogens according to the varying isolation conditions of medium, temperature, and substrate (Fig. 3). The media-wise analysis showed that three methanogens were obtained exclusively on BCYT medium, while two on BY medium (Fig. 3a). Fastidious methanogens such as *Mbt. formicicum*, which needs certain growth factors, amino acids, and co-enzymes, were

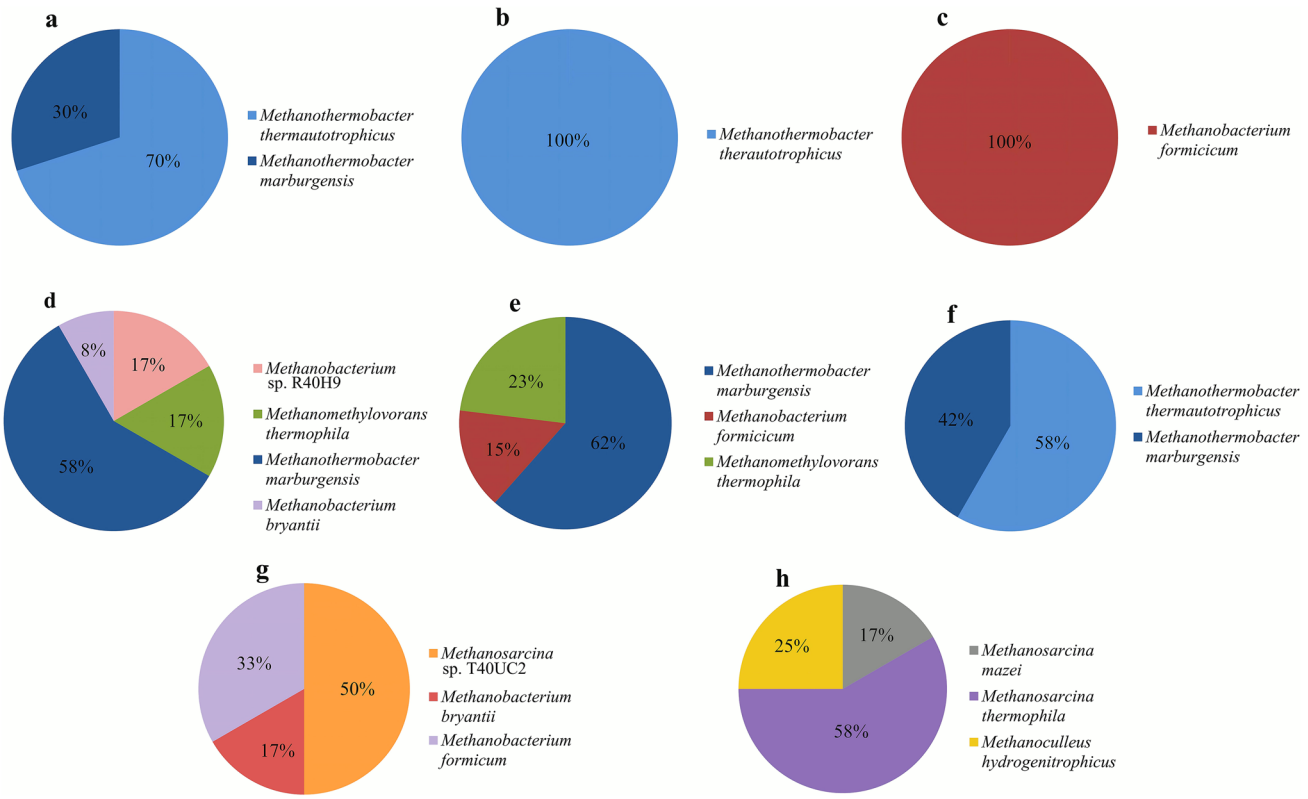


Fig. 2 Pie charts describing the diversity, distribution and abundance of various methanogenic species in Chumathang (**a**), Puga (**b**), Unkeshwar (**c**), Rajawadi (**d**), Tural (**e**), Unhavare (**f**), Chopda 1 (**g**) and Chopda 2 (**h**) hot springs

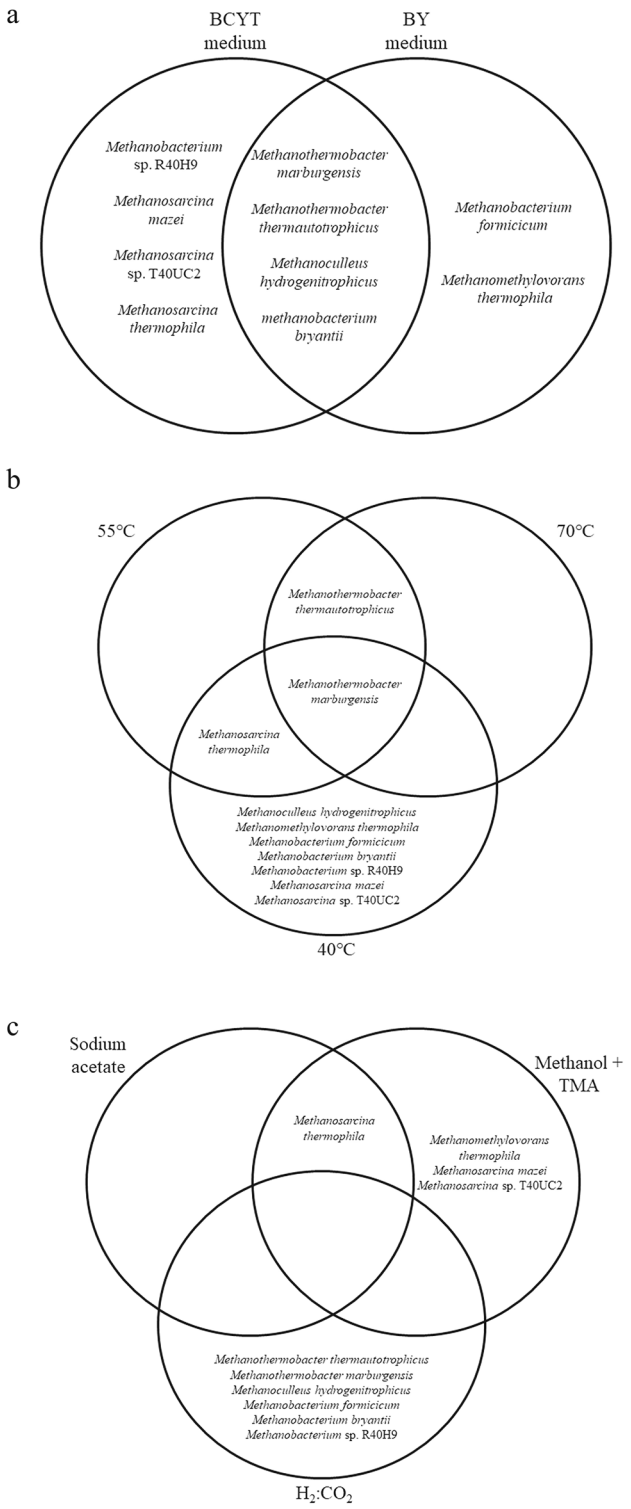


Fig. 3 Venn diagrams showing methanogenic distribution at the species level when cultured on different media (**a**), temperatures (**b**) and substrates (**c**)

isolated solely on BY medium (Khelaifia et al. 2013). In our earlier study, the BY medium was also found to support the growth of diverse hydrogenotrophic methanogens (Joshi et al. 2018). Also, *Mmv. thermophila* was cultured only on BY medium, probably owing to its specific requirement of cobalt, which is a constituent of BY medium (Jiang et al. 2005). In comparison, only the BCYT medium supported the growth of all the strains of *Methanosarcina*, which are known to be metabolically versatile. *Msc. mazei* requires trypticase and peptone for its growth and *Msc. thermophila* requires the growth factor *p*-amino benzoic acid. The presence of these components in the BCYT medium explains their successful cultivation. Interestingly, both the putative novel species were isolated only from the BCYT medium. *Methanothermobacter* and *Methanoculleus*, two genera recognized for their autotrophic nature, were cultured on both media (Whitman et al. 2014).

The temperature-based comparison revealed that except *Mtb. thermautotrophicus*, all other methanogen species were cultured at 40 °C (Fig. 3b). Barring, *Msc. thermophila*, only the members of the genus *Methanothermobacter, Mtb. thermautotrophicus* and *Mtb. marburgensis* were isolated at 55 °C and 70 ℃. Of these, *Mtb. marburgensis,* which optimally grows at 65 ℃, was found at all three temperatures (40, 55 and 70 ℃), signifying its broader temperature range (Wasserfallen et al. 2000). Various species of *Methanobacterium, Methanosarcina, Methanoculleus,* and *Methanomethylovorans*, including the two putative novel species, were cultured only at 40℃. Noticeably, *Mmv. thermophila* was obtained only at 40 °C, even though it has an optimal growth temperature in the thermophilic range (50 °C) (Jiang et al. 2005). A thermophilic acetotrophic methanogen, *Msc. thermophila,* which grows optimally at 50 ℃, was cultivated at 40 and 55 ℃ (Zinder et al. 1985). These results suggest that microbial diversity decreases with increasing temperature (Fig. S2). Such observations have been made in the past too, where various factors like the absence of vital enzymes at higher temperatures and specific requirements of thermophilic ecosystems to perform rare functions reportedly impact diversity (Ruhl et al. 2022; Sharp et al. 2014; Shu and Huang 2021). Also, the fact that thermophilic methanogens could be cultured even at low temperatures, but not vice versa, specifies their broader growth range. The broad temperature range of 40–75 °C for *Mtb. marburgensis* and *Mtb. thermautotrophicus* has been reported (Wasserfallen et al. 2000).

Substrate-wise, *Mmv. thermophila*, *Msc. mazei* and *Methanosarcina* sp. strain T40UC2 were isolated only on TMA + methanol, whereas *Msc. thermophila* was isolated on both, sodium acetate and TMA + methanol (Fig. 3c). The other six strains were strict hydrogenotrophs, comprising the members of the genus *Methanothermobacter*, *Methanobacterium* and *Methanoculleus*. Amongst, the genus

Methanosarcina has been reported as the most versatile methanogen, capable of utilizing multiple substrates, even though strain level variation does occur (Maestrojuan and Boone 1991). In comparison, *Mmv. thermophila* is reported to be a strict methylotroph, and all hydrogenotrophs are known to utilize H_2:CO_2 or formate (Demirel and Scherer 2008; Jiang et al. 2005).

The relationship between the methanogens isolated in this study using 16S rRNA gene-based phylogenetic analysis was also analyzed, which revealed the grouping of all the cultures in three clusters representing three different families of methanogens, namely *Methanobacteriaceae*, *Methanomicrobiaceae* and *Methanosarcinaceae* (Fig. 4). It was observed that all strains of the genus *Methanothermobacter* were highly related despite having different origins. Similarly, two identical strains each of *Mbt. bryantii* (Rajawadi and Chopda 1), *Mmv. thermophila* (Rajawadi and Tural), and three strains of *Mbt. formicicum* (Chopda 1, Unkeshwar and Tural) were obtained from different locations across the Maharashtra region. We also report two putative novel species belonging to the genera *Methanosarcina* and *Methanobacterium,* forming a separate clade with *Methanosarcina mazei* and *Methanobacterium bryantii,* respectively.

The colony characters varied from pinpoint or large circular (*Methanothermobacter*, *Methanoculleus*), and irregular (*Methanobacterium*) to large clump-forming colonies (*Methanosarcina* and *Methanomethylovorans*). The fluorescence microscopy highlighted different cell morphology for each genus, like large clumps (*Methanosarcina*), small cocci (*Methanoculleus*), rods (*Methanobacterium*), long rods (*Methanothermobacter*) and large irregular cocci (*Methanomethylovorans*) (Fig. 4).

Ecological distribution

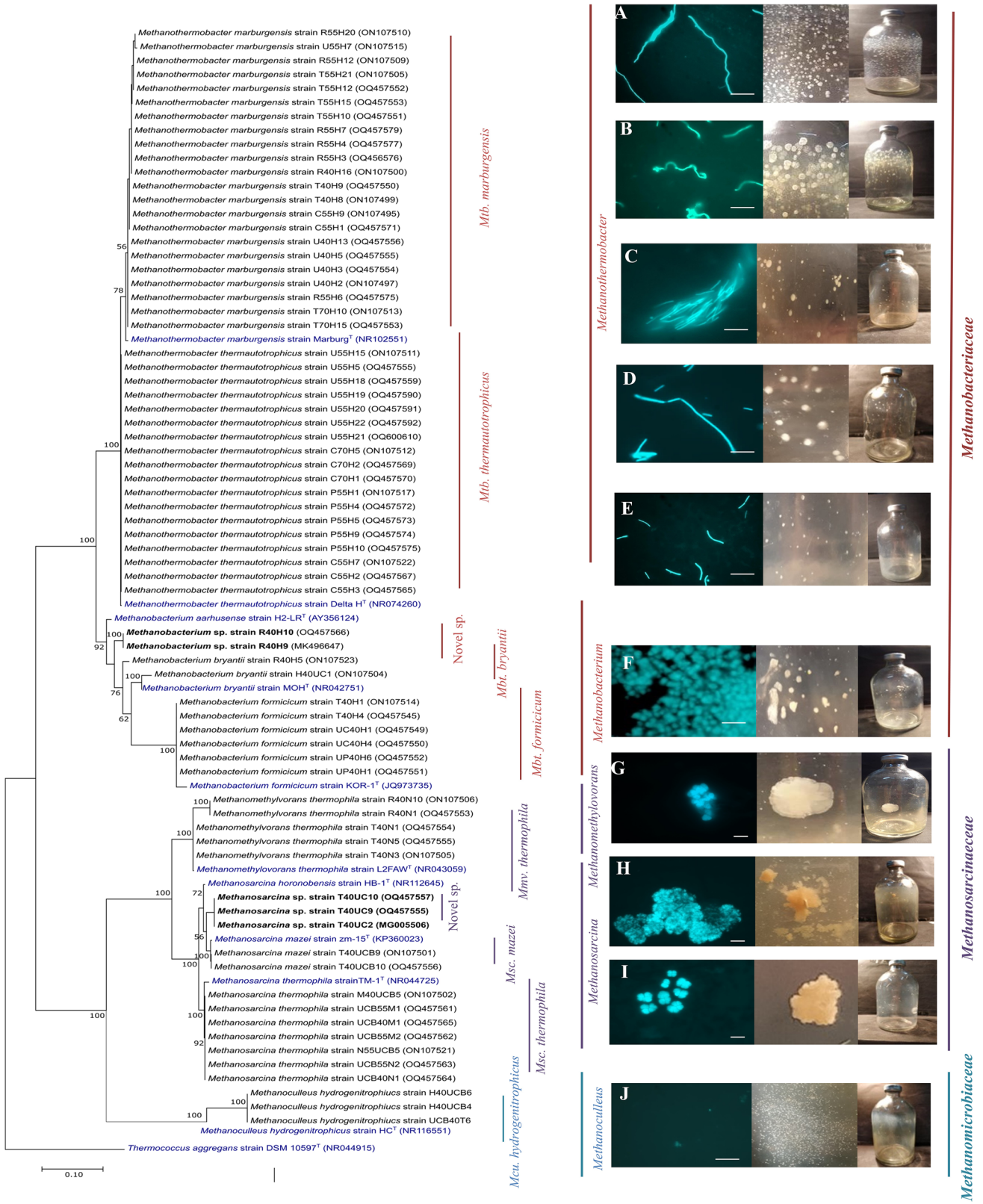
Methanogens have been found in various oxygen-restricted habitats (Liu and Whitman 2008). Though they prefer pH levels around neutral for their survival and proliferation, methanogens can grow across a broad range of temperature conditions, from psychrophilic to hyperthermophilic (Hoehler et al. 2010). Their substrate requirements also vary, but the most common and diverse methanogens represent the hydrogenotrophic group across broad pH and temperature conditions (Zabranska and Pokorna 2018). In this study, we were able to culture different strains of methanogens from Indian hot spring sediments, representing both mesophilic and thermophilic, and hydrogenotrophic, acetotrophic, and methylotrophic groups.

The majorly hydrogenotrophic genus *Methanobacterium* comprises 25 valid species (https://lpsn.dsmz.de/search?word=methanobacterium). Several of them are autotrophic and are found in habitats like anaerobic digesters, sewage sludge, aquatic sediments, marshy soils, gastrointestinal tracts of animals, geothermal areas, etc. (Boone 2015a; Whitman et al. 2014). In the present study, three species of this genus, including one putative novel species, were cultured from hot springs of Tural, Rajawadi, Unkeshwar and Chopda 1, showing widespread distribution in addition to previously known environments (Fig. S3). The phylogenetic analysis showed that all the isolated *Mbt. formicicum* strains (Unkeshwar, Chopda 1 and Tural hot springs) were related to the ones obtained from a pig slurry digester (Korea), biogas reactor (Germany), farm animals (New Zealand), sheep rumen and cow dung (India) and gas storage facility (Russia). The related uncultured clones were found in an anaerobic digester (Netherlands) and wastewater reactor (Japan). The *Mbt. bryantii* strains cultured from Rajawadi and Chopda hot springs were related to the strains isolated from a sewage digester (Germany), methane hydrate (India), rice field soils (France), and a goat faecal sample (India), and uncultured clones from rice paddy fields (Japan), coal bed water (China), and a faecal sample (UK). The putative novel *Methanobacterium* sp. strain R40H9 isolated from Rajawadi hot spring showed no similarity to cultured or uncultured sequences.

The genus *Methanothermobacter* comprises eight valid species (https://lpsn.dsmz.de/search?word=Methanothermobacter), all of which are thermophilic, hydrogenotrophic and autotrophic and are mainly found in oil fields and anaerobic digesters (Boone 2015b; Boone et al. 2015a). Except for Unkeshwar and Chopda, two *Methanothermobacter* species, *Mtb. thermautotrophicus* and *Mtb. marburgensis* were isolated from every hot spring (Fig. S4). The strains of *Mtb. thermautotrophicus* obtained were related to isolates from a power gas reactor (Germany), bay sediments (China), oil fields (Russia and India), and uncultured clones from oil fields (Japan and Singapore). While an uncultured affiliate was from a saline lake in Spain, the cultured affiliates of *Mtb. marburgensis* strains were related to isolates from sewage digesters/thermophilic biogas plants (Germany) and hot springs (China and India).

Methanoculleus is one of the most physiologically diverse genera and contains 11 valid species (https://lpsn.dsmz.de/search?word=Methanoculleus), which inhabit a range of environments with varying temperatures, substrates, and salinities, including marine, freshwater, and volcano sediments, bioreactors, etc. (Boone et al. 2015b; Lai 2019) A single species, *Mcu. hydrogenitrophicus* strains were obtained from only one hot spring (Chopda 2) in this study, which was related to the isolates from wetland soil (USA and India), and uncultured clones from rumen fluid (China); and cattle manure compost, rice paddy soil and groundwater from sedimentary rock (Japan) (Fig. S5).

The genus *Methanomethylovorans* comprises three valid species (https://lpsn.dsmz.de/search?word=Methanomethylovorans), all of which are strictly methylotrophic



and reported from freshwater/ wetland sediments and anaerobic digesters (Boone et al. 2015c; Kim and Rhee 2019). In this work, only the *Mmv. thermophila* strains were isolated from Tural and Rajawadi hot springs and were phylogenetically identical to *Mmv. thermophila* type strain (thermophilic anaerobic digester, USA). Our strains

◂**Fig. 4** The phylogenetic tree and corresponding macroscopic and microscopic images of different strains of methanogens. The 16S rRNA gene-based maximum-likelihood tree constructed using Tamura 3 parameter model with uniform rates showing the phylogenetic position of different strains, including putative novel species (in bold) with their respective type strains (in blue). Bootstrap values (≥50%) based on 500 replicates are indicated at branching points. The GenBank accession number of each strain is listed in parentheses. Scale bar, 0.1 substitutions per site. The images showing the fluorescence microscopic characteristics (left) and colony morphologies (right) of *Methanothermobacter thermautotrophicus* strain U55H20 (**a**), *Methanothermobacter marburgensis* strain R55H7 (**b**), *Methanobacterium bryantii* strain H40UC1 (**c**), *Methanobacterium* sp. strain R40H9 (**d**), *Methanobacterium formicicum* strain UP40H6 (**e**), *Methanomethylovorans thermophila* strain R40N1 (**f**), *Methanosarcina thermophila* strain UCB55M1 (**g**), *Methanosarcina* sp. strain T40UC2 (**h**), *Methanosarcina mazei* strain T40UCB10 (**i**) and *Methanoculleus hydrogenitrophicus* strain H40UCB6 (**j**), respectively. Scale bar, 10 µM (colour figure online)

also grouped alongside uncultured clones reported from hot springs (Taiwan and Armenia), oil reservoirs (USA) and anaerobic digesters (Netherlands) (Fig. S6).

Methanosarcina is metabolically the most versatile of methanogens and comprises 14 valid species (https://lpsn.dsmz.de/search?word=Methanosarcina), which can be distinctly identified because of their aggregate or clump-forming quality. The members of this genus are present across all three physiological groups and reported from diverse environments like anaerobic digesters and various sediments (Boone et al. 2015c; Wagner 2020). *Methanosarcina* members are known to be fast-growing and robust and were isolated only from Chopda hot springs in this study. We cultivated three species, including a putative novel one from Chopda 1 and, *Msc. thermophila* and *Msc. mazei* from Chopda 2 hot springs. The strains of *Msc. thermophila* were closely related to the cultured strains isolated from an anaerobic digester (USA) and a termite gut (India). The related uncultured clones were obtained from cattle manure compost (Japan). The strains of *Msc. mazei* were closely related to the isolates from the estuarine environment and marine sediment (Taiwan), while uncultured clones were obtained from cattle manure compost (Japan). As expected, *Methanosarcina* sp. strain T40UC2, isolated from Chopda 1 location, did not have any related cultured affiliates, but similar uncultured clones were found from a thermal vent of Yellowstone (USA). (Fig. S7).

In contrast to the diverse methanogens cultured in the current study, most of the comparable strains have been reported majorly from artificial environments like digesters, wastewater treatment plants, etc., which highlights the relevance of these hot springs as holding a unique collection of diverse methanogens.

Conclusion

The hot springs harbour diverse methanogenic archaeal communities, making them a unique environmental niche for studying microbial processes and potential industrial applications. We successfully cultivated five genera and ten mesophilic and thermophilic methanogen species representing hydrogenotrophic, methylotrophic and acetotrophic physiological groups. The most diverse sets of taxa were obtained at 40 ℃ on both BY and BCYT media, whereas higher incubation conditions majorly supported the growth of hydrogenotrophic methanogens. While methanogens with specific nutrient requirements were cultivated on either BY or BCYT medium, both media supported the growth of autotrophic methanogens. Some methanogen genera, like *Methanoculleus*, *Methanosarcina*, and *Methanomethylovorans*, were restricted to particular hot springs, whereas the methanogens belonging to *Methanobacterium* and *Methanothermobacter* were widespread over several hot springs across geothermal regions. This study also documents two putative novel species in the *Methanobacterium* and *Methanosarcina* genera, unravelling their taxonomy, diversity and distribution. To the best of our knowledge, our study represents the first report of the isolation of *Mmv. thermophila*, *Msc. thermophila*, *Msc. mazei* and *Mbt. bryantii* from hot spring habitats. This study also suggests that *Hinf* I can be used to differentiate different genera of methanogens using PCR–RFLP. Changing media and temperature conditions were shown to isolate distinct methanogens from the same environmental niches selectively. Further work needs to be conducted to compare the growth rate of different methanogens, following which efficient strains can accelerate rates of various microbial processes.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00203-023-03661-2.

Acknowledgements The authors are thankful to Dr Karthick Balasubramanian, Agharkar Research Institute, Pune and Dr. Raymond A Duraiswami, Savitribai Phule Pune University, for sampling support. We duly acknowledge the help Mr. Sai Hivarkar and Mr. Radhakrishnan Cheran extended during the study. We also appreciate the technical assistance from Dr. Vikram Lanjekar. We are grateful to the Director, Agharkar Research Institute for providing the necessary infrastructure support.

Author contributions SSD and PKD conceived the project and designed the experiments. KSD conducted the experiments, analysed the data and wrote the first draft of the manuscript. PKD and SSD revised the manuscript. All authors have read and approved the final version of the manuscript.

Funding The authors acknowledge the support of the Science and Engineering Research Board (SERB), Department of Science and Technology (DST) for financial support (Project no. YSS/2015/000718). Kasturi Deore thankfully acknowledges University Grants Commission,

Delhi (India) for the junior research fellowship (Ref. No. 23/06/2013(i) EU-V).

Declarations

Conflict of interest The authors declare that they do not have any conflicts of interest.

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