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Molecular Biology and Biochemistry for Enhanced Biomethanation

Sanjay Kumar Ojha • Snehasish Mishra* • Sarat Kumar Nayak • Mrutyunjay Suar

School of Biotechnology, KIIT University, Bhubaneswar-751024 (ODISHA), India

Corresponding author: * **snehasish.mishra@gmail.com**

ABSTRACT

Methanogenic bacteria are one amongst the three classes of Archaebacteria representing the most primitive dwellers of the Earth, reportedly since some 3.5 billion years ago. While their activity is inhibited by oxygen, these bacteria are robust enough to appear in a wide variety of ecological niches, such as, the intestinal tracts of ruminants, sewage digesters, groundwater and deep soil/water. Biomethanation by these is an interesting biotechnology that converts almost all types of organic polymers including the recalcitrant lignocelluloses, to methane and carbon dioxide. This process can be enhanced by manipulating various physical, chemical and molecular factors, though molecular level manipulation needs deeper understandings. Research in genetics, gene regulation and expression of methanogens is rapidly progressing. Relatively proficient genetic manipulation system, including cloning, expression and identification of new species in the last few years is definitely going to provide direction and leads to future investigations. Methyl CoM reductase (MCR), the enzyme responsible for biomethanation, constitutes approximately 10% of the total protein in methanogenic cultures. The significance and abundance of MCR inevitably focused initial attention on elucidating its structure and the mechanisms directing its synthesis and regulation. MCR-coding genes have been cloned and sequenced from various methanogens, though biomethanation process as a whole needs to be further understood and standardised. A plausible solution to biomethanation enhancement at the molecular level seems to lie in metagenomics. The biochemistry and microbiology of anaerobiosis of organic polymers to methane and the roles of the participating microbes are discussed here, along with their molecular biology, application and suggestions for enhanced biogas production.

Keywords: Archaea, biogas, methanogen, methyl Coenzyme-M reductase, metagenome
Abbreviations: **ATP**, adenosine tri-phosphate; **CoB**, coenzyme B; **CoM**, coenzyme M; **MB**, methanogenic bacteria; **MCR**, methyl coenzyme-M reductase; **PCR**, polymerase chain reaction; **rRNA**, ribosomal ribonucleic acid

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INTRODUCTION

Renewable energy is the energy that comes from natural resources such as sunlight, wind, tides, geothermal heat, as also biological sources. Energy production from fossil fuels becomes more and more problematic since this resource is non-renewable. Further, burning of coal, oil and natural gas is connected with emissions of the green-house gases including carbon dioxide. Biomethane also emits carbon dioxide at an intensity of 11 compared to 67.9, 95.8 and 96.7 for natural gas, diesel and gasoline respectively (Anon 2005). For these reasons, use of renewable energies is promoted by national programs in many countries. Long-term objectives of this policy are to ensure future energy supply and to reduce green-house gas emissions. The dependency on non-renewable sources is slowly but steadily decreasing. In

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Invited Review

2008, renewable sources contributed approximately 19% of the global final energy consumption (Anon 2011). Biogas is a combination of gases (CH_4, CO_2) in different proportions and is produced during anaerobic fermentation of organic substrates by specific microbial communities (Ohmiya *et al*. 2005). The main source of biogas is a class of bacteria known as methanogens. This sort of bacteria decomposes the large macromolecules to smaller form and finally degrading into its components. This context of biogas production from renewable resources or organic wastes is of socio-economic importance (Weiland 2003; Yadvika *et al*. 2004).

METHANOGENS

Regarded as among the oldest earthly creatures, the methanogens are extremophiles and adapt to thrive in such harsh habitats. Their discovery established the kingdom *Archaeobacteria* that includes some of the other extremophiles such as halophiles, thermophiles, psychrophiles, radophiles, barophiles, etc. and also the sulphur-dependent organisms. Woese *et al*. (1990) proposed a separate kingdom for methanogens and other Archaeobacteria as Archaea. Currently, there is a superkingdom Archaea that contains the two most prominent phyla Euryarchaeota and Crenarchaeota and the methanogens being under Euryarchaeota which again is subdivided in six classes. Methanobacteria, Mathanomicrobia and Metanococcoi are the classes which comprises all the methanogens. *Methanobacterium formicicum* is the representative organism of the phylum Euryarchaeota.

History of Archaea and methanogens

Molecular fossils are found by looking for the membranes formed from isoprene chains unique to Archaea. These do not decompose at high temperatures and make good markers for the presence of ancient Archaea. These ancient life forms have also been found in the oldest known sediment (3.8 billion years old) on earth, in the Isua district of Greenland, which indicates that they appeared within one billion years of the earth's formation, in an atmosphere that was rich in ammonia and methane. They have also been found in Mesozoic, Paleozoic and Precambrian sediments and it is thought that these initial habitants of earth were most likely methanogens.

The activity of phylum Euryarchaeota in methanogenesis can be well studied on the basis of an essential enzyme for methanogenesis, *i.e.*, Methyl CoM Reductase. The Database when searched and filtered it shows the following groups with their respective MCR-active organism. Methanobacteriales (244), Methanococcales (101), methanomicrobia (337), *Methanopyrus kandleri* (10) and environmental samples (2094). Environmental sample represents the total number of organisms (metagenome) reported so far but yet to be cultured and classified at organismal level (Uniport Organization 2011).

Habitat of methanogens

Methanogens inhabit in some of the most extreme environments on earth, including the rumen of ruminants living on hydrogen and carbon dioxide produced by other microbes, helping digest cellulose, as well as being necessary for protein synthesis. They can be found in places like muck of swamps and marshes, hydrothermal vents, porous rock, sewage sludge, termite-gut and oil-contaminated groundwater at underground oil storage facilities (Watanabe *et al*. 2002). Based on their natural habitat, some are thermophiles, the methanogens found in volcanic hot springs and solfataras, where temperatures span from 40-100°C and in marine environments in undersea hydrothermal vents where the temperatures can reach up to 350°C due to high pressure. Psychrophily is rare among methanogens with only a few species being identified till now (Nozhevnikova *et al*. 2003). Also, there are halophiles (salt-loving), (Riffat and Krongthamchat 2006) acidophiles (acid-loving), (Zhou and Ren 2007) alkaliphiles (base-loving) (Thakker and Ranade 2002), radophiles (radiation-loving) and so on. Malakahmed *et al*. (2009) reported a 93% bacterial, 5% protozoan and 2% fungal population in a 50-l anaerobic bioreactor, using 75% kitchen waste and 25% sewage sludge as substrate respectively. They showed that, fast-growing bacteria which are robust enough to grow on high substrate concentration and reduced (acidic) pH were dominant in the acidification zone of the ABR, *i.e.*, the front compartment of reactor. The terminal part of this ABR exhibited slower scavenging bacteria that grow excellently at high (alkaline) pH.

Morphology of methanogens

Archaea exhibit a wide variety of shapes, sizes, and ultrastructural variations, not unlike bacterial cells. Two shapes, *i.e.*, rods and coccoid, seem to dominate though. Examples of rods are *Methanobacterium* spp. and *Methanopyrus kandkandleri*, and coccoids include *Methanococcus* and *Methanosphaera*. *Methanoculleus* and *Methanogenium* exhibit coccoid to irregular shapes, possibly due to the loosely bound S-layers on the walls. Methanogens are not just limited to these shapes, but also include a plate (*Methanoplanus*), long thin spiral (*Methanospirillum*), and cluster of round (*Methanosarcina*) cells (Sirohi *et al*. 2010). Methanogens are known to lack murein, though some may contain pseudomurein, which can only be distinguished from its bacterial counterpart through chemical analysis (Sprott and Beveridge 1994; König 1988). Methanogens that do not possess pseudomurein have at least one paracrystalline array (S-layer), the proteins that fit together in an array like jigsaw pieces that do not covalently bind to one another, in contrast to a cell wall that is one giant covalent bond. The S-layer proteins of some methanogens (e.g., *Methanococcus* spp.) are glycosylated thereby facilitating stability (Beveridge and Schultze-Lam 1996; Shlimon *et al*. 2004).

IDENTIFICATION OF METHANOGENS

There are a number of methods available for identification of methanogens. Some have also been popular amongst the researchers, and some others are not so. All these methods can be broadly classified into two categories, culture-dependent, and culture-independent. The former one is losing its relevance at the cost of the later one, owing mainly to the cost, time, and reliability factors. Both these techniques are discussed below.

CULTURE-DEPENDENT TECHNIQUES

Ecologists studying microbial life in the environment have recognized the enormous complexity of microbial diversity for more than a decade (Whitman *et al*. 1998). Methanogens, which require very low redox potential for the growth, are perhaps the strictest anaerobes. Many workers have defined different ways for its growth but a modified Hungate culture technique has been the most appropriate one. Use of Freter type anaerobic glove box with an inner ultra low oxygen chamber has been described by Edwards and McBride (1975) to isolate and grow methanogens. The inner chamber is specially modified to maintain the redox potential and pressure necessary to grow methanogens. For this, the chamber is periodically flushed with H_2 and CO_2 (80:20). Cultures are plated in the outer anaerobic glove box and immediately placed in the inner chamber. Though this method is relatively expensive than Hungate procedures, it offers unique advantages like low skill, manual dexterity, and allows routine genetic procedures.

Various special designed media are available now days for growth and culture of anaerobes such as methanogens. The main constituents include a nutrient source (such as casein enzymic hydrolysate), oxygen-devouring compounds (such as sodium thioglycollate and sodium formaldehyde sulphoxylate) to facilitate anerobiosis and an indicator

against oxygen, such as methylene blue and resazurin (Brewer 1942).

Some of the approved and commercially available culture media are:

1. Anaerobic agar: it is recommended for the cultivation of anaerobic bacteria especially *Clostridium* species and other anerobic microorganism. In this casein and dextrose act as nutirnent source, sodium thioglycollate and sodium formaldehyde sulphoxylate as to provide anaerobiosis and methyllene blue as indicator. This is suitable for cultivation of facultative and obligate anaerobes and for the study of colonial morphology as colonies can be readily seen on the light colored agar and are easily accessible.
2. Anaerobic agar (brewer): Brewer designed this media for use with Brewer anaerobic cover to permit the surface growth of anaerobes and microaerophiles on agar without the use of anaerobic jar. Used for, cultivation of both facultative and obligate anaerobes and to study the colony morphology. The indicator used in this is resazurin.
3. Anaerobic agar without dextrose: Anaerobic agar without dextrose is used for carbohydrate fermentation studies and for studies of hemolytic activity of *Clostridia*, *Streptococci* and other organisms.

CULTURE-INDEPENDENT TECHNIQUES

It is long recognized that standard culture methods fail to adequately represent the enormous microbial diversity that exists in nature. To avoid reliance on cultivation, many culture-independent methods are employed to search for novel bacterial species such as analysis of their antigenic relationship, polyamine content, molecular weight of methyl reductase subunit and molecular weight of polar lipids, and many more. Conway de Macario *et al.* (1981) described a novel way to identify methanogens at genus and species level using cross-reactivity of immunoglobulins.

ELISA

Other than the culture method ELISA, an antigenic and antibody-based technique was used for identification of methanogens. In this a polyclonal antisera was developed against different strains of methanogens. The specificity is increased when cross reacted with cells. Sørensen and Ahring (1997) used this technique for identifying the microconstria of an anerobic digestor and reported unique pattern of different methanogenic strains.

The development of a variety of culture-independent methods, many of them coupled with high-throughput DNA sequencing, has allowed microbial diversity to be explored in ever greater detail (Moreira and Lopez-Garcia 2002; Rappe and Giovannoni 2003; Handelsman 2004; Harris *et al*. 2004). These include screening of expression libraries with immune serum, nucleic acid subtractive methods, small molecule detection with mass spectroscopy and many more (Relman 2002). Sequence-based methods are more in application now-a-days because of their general applicability and the continued expansion of high-throughput, low cost, sequencing capacity.

Molecular techniques

The basis of culture-independent identification of Archaeal species is sequence analysis of the sufficiently well-conserved (across species) rRNA genes that can be readily amplified using random PCR primers based on highly conserved sequences, yet are sufficiently diverse to differentiate archaeal species (Kušar and Avguštin 2010). Woese and Fox (1977) and Woese (1982) initially used small subunit (16S) rRNA gene sequences for construction of phylogenetic trees of cultivated organisms, but this method was subsequently applied to libraries of rRNA genes which are PCR-amplified from the unculturable environmental DNA samples (Stahl *et al*. 1984, 1985; Ward *et al*. 1990; Schmidt *et al*. 1991; Bergmann *et al*. 2010). A striking collective result from the application of this technique to numerous environmental samples was the realisation that cultivated organisms represent a tiny fraction of species present in most environmental samples. In fact, a very few currently recognised bacterial phyla contain cultivated members and thus the utility of culture-independent technique (Hugenholtz *et al*. 1998; Rappe and Giovannoni 2003).

To maximise the utility of 16S rRNA gene analysis for species determination, the entire 16S rRNA gene is amplified and sequenced in its entirety through bi-directional sequencing of cloned 16S amplicons (Hugenholtz 2002). After sequencing, 16S sequences are clustered into groups and a threshold of sequence similarity is established (usually 98 or 99%) to distinguish genus and species. This approach has been applied to biogas-producing microbial communities as well (Huang *et al.* 2002; McHugh *et al.* 2003; Mladenovska *et al.* 2003; Huang *et al.* 2005; Shigematsu *et al.* 2006; Klocke *et al.* 2007; Tang *et al.* 2007; Klocke *et al.* 2008). Using 16S rRNA and RFLP, Joulion *et al.* (1998) phylogenitically characterised the four major groups of methanogens from rice field soil.

While PCR amplification of 16S rRNA sequences has been of enormous value, there are some loopholes to this approach. In most of cases organisms that carry sequence differences within the highly conserved regions used for primer design may not amplify at all or do so less efficiently that the representation in cloned libraries may be a mismatch or incorrect, especially if the number of 16S rRNA sequences sampled is small (Kroes *et al.* 1999). Such errors may be recognized and corrected by hybridisation-based methods such as *in situ* hybridisation with species or strain-specific 16S oligonucleotides applied to the original (or similar) sample (Amann *et al*. 1995; Bosshard *et al*. 2000).

Another drawback of 16S rRNA sequencing is the need for high-throughput sequencing capacity that, except in high-throughput sequencing centers, remains relatively slow compared to hybridisation-based methods. As an alternative, several strategies employing 16S rRNA gene microarrays have been presented and offer speed compared to sequencing of many samples for comparison (Rudi *et al*. 2000; Small *et al*. 2001; Loy *et al*. 2002, 2005). For the most part, these studies employed oligonucleotide probes designed for detection of specific organisms such as sulphate-reducing bacteria or *β*-proteobacteria and have offered acceptable sensitivity. Application to highly complex environmental samples has been limited by sensitivity and difficulties in differentiating related species, but it seems reasonable to expect further improvement in this technology and eventual application of marker genes.

In addition to the 16S-rDNA target, other marker genes such as *mcrA* encoding the α-subunit of methyl coenzyme-M reductase have been used to elucidate the composition of methanogenic consortia (Lueders *et al*. 2001; Luton *et al*. 2002; Friedrich 2005; Juottonen *et al*. 2006; Rastogi *et al*. 2008). To eliminate potential problems with non-specific amplification, some researchers have developed primers for the gene sequence of the α-subunit of the methyl coenzyme M reductase (*mcrA*) (Springer *et al.* 1995; Hales *et al.* 1996; Luton *et al.* 2002). Mcr catalyses the last step of methanogenesis and is conserved among all methanogens. Phylogenetic inference with *mcrA* sequences is similar to that obtained with 16S rRNA gene sequences, suggesting no lateral transfer (Bapteste *et al.* 2005). Moreover, Mcr is absent in all nonmethanogens, with the exception of the anaerobic methane-oxidising *Archaea*, which are closely related to the methanogens (Hallam *et al.* 2003). Due to the fact that methanogens may be examined exclusively from other bacteria present in an environment, *mcrA* has been increasingly used for phylogenetic analysis coupled with, or independent of, 16S rRNA genes.

Table 1 A list of all methanogens whose gene sequences have been reported so far (November, 2011) to the NCBI.

Organism / Strain	Family / Class	*Size	GC	Ref Seq
Methanoculleus marisnigri JR1	Methanomicrobiaceae / Methanomicrobia	2.4781	62.1	NC_009051.1
Methanopyrus kandleri AV19	Methanopyraceae / Methanopyri	1.69497	61.2	NC_003551.1
Methanosphaerula palustris E1-9c	Unclassified / Methanomicrobiales	2.92292	55.4	NC_011832.1
Methanocella paludicola SANAE	Methanocellaceae / Methanomicrobia	*3	54.9	NC_013665.1
Methanoregula boonei 6A8	Methanomicrobiaceae / Methanomicrobia	2.54294	54.5	NC_009712.1
Methanosaeta thermophila PT	Methanosaetaceae / Methanomicrobia	1.87947	53.5	NC_008553.1
Methanocorpusculum labreanum Z	Methanocorpusculaceae / Methanomicrobia	1.8	50.0	NC_008942.1
Methanoplanus petrolearius DSM 11571	Methanomicrobiaceae / Methanomicrobia	*2.8	50	NC_014507.1
Methanothermobacter thermautotrophicus δ H	Methanobacteriaceae / Methanobacteria	1.75138	49.5	NC_000916.1
Methanospirillum hungatei JF-1	Methanospirillaceae / Methanobacteria	3.54474	45.1	NC_007796.1
Methanosarcina acetivorans C2A	Methanosarcinaceae / Methanomicrobia	5.75149	42.7	NC_003552.1
Methanohalophilus mahii DSM 5219	Methanosarcinaceae / Methanomicrobia	2	42.6	NC_014002.1
Methanosarcina mazei Go1	Methanosarcinaceae / Methanomicrobia	4.1	41.5	NC_003901.1
Methanococcoides burtonii DSM 6242	Methanosarcinaceae / Methanomicrobia	2.57503	40.8	NC_007955.1
Methanosarcina barkeri str. Fusaro	Methanosarcinaceae / Methanomicrobia	4.87341	39.2	NC_007355.1
Methanosalsum zhilinae DSM 4017	Methanosarcinaceae/ Methanomicrobia	* **2.1**	**38**	NC_015676.1
Methanocaldococcus infernus ME	Methanocaldococcaceae / Methanococci	*1.3	33.5	NC_014122.1
Methanococcus maripaludis C6	Methanococcaceae / Methanococci	1.74419	33.4	NC_009975.1
Methanococcus maripaludis C7	Methanococcaceae / Methanococci	1.77269	33.3	NC_009637.1
Methanococcus maripaludis S2	Methanococcaceae / Methanococci	1.66114	33.1	NC_005791.1
Methanococcus maripaludis C5	Methanococcaceae / Methanococci	1.8083	33.0	NC_009135.1
Methanobrevibacter ruminantium M1	Curculionoidea / Methanobacteria	*2.9	32.6	NC_013790.1
Methanocaldococcus fervens AG86	Methanocaldococcaceae / Methanococci	1.522	32.2	NC_013156.1
Methanocaldococcus sp. FS406-22	Methanocaldococcaceae / Methanococci	*1.812	32.0	NC_013887.1
Methanocaldococcus vulcanius M7	Methanocaldococcaceae / Methanococci	*1.7157	31.6	NC_013407.1
Methanocaldococcus jannaschii DSM 2661	Methanocaldococcaceae / Methanococci	1.73997	31.3	NC_000909.1
Methanococcus vannielii SB	Methanococcaceae / Methanococci	1.72005	31.3	NC_009634.1
Methanobrevibacter smithii ATCC 35061	Methanobacteriaceae / Methanobateria	1.85316	31.0	NC_009515.1
Methanococcus aeolicus Nankai-3	Methanococcaceae / Methanococci	1.5695	30.0	NC_009635.1
Methanococcus voltae A3	Methanococcaceae / Methanococci	*1.9	28.6	NC_014222.1
Methanosphaera stadtmanae DSM 3091	Methanobacteriaceae / Methanobacteria	1.7674	27.6	NC_007681.1
Methanobacterium sp. AL-21	Methanosarcinaceae/ Methanobacteria	* **2.6**	NA	NC_015216.1
Methanobacterium sp. SWAN-1	Methanosarcinaceae/ Methanobacteria	* **2.5**	NA	NC_015574.1
Methanococcus maripaludis X1	Methanococcaceae / Methanococci	**1.75**	NA	NC_015847.1
Methanohalobium evestigatum Z-7303	Methanosarcinaceae / Methanomicrobia	*2.36	NA	NC_014253.1
Methanosaeta concilii GP6	Methanosaetaceae / Methanomicrobia	**3.02**	NA	NC_015416.1
Methanothermobacter marburgensis Marburg	Curculionoidea / Methanobacteria	*1.6044	NA	NC_014408.1
Methanothermococcus okinawensis IH1	Methanococcaceae / Methanococci	* **1.72**	NA	NC_015636.1
Methanothermus fervidus DSM 2088	Methanothermaceae / Methanobacteria	*1.2	NA	NC_014658.1

*Size is estimated, otherwise genome size is calculated based on existing sequences listed at http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi

Metagenomics

Since library construction by classical cloning of fragmented DNA is not necessary for 454-pyrosequencing, biases should be relatively negligible when this technique is used for whole genome shotgun sequencing of microbial community metagenomes. The metagenome of a biogas-producing microbial community from a production-scale biogas plant fed with renewable primary products has been analysed by applying the ultrafast 454-pyrosequencing technology. Community structure analysis of the fermentation sample revealed that *Clostridia* from the phylum *Firmicutes* is the most prevalent taxonomic class, whereas, species of the order *Methanomicrobiales* are dominant among methanogenic *Archaea* (Krause *et al.* 2008a). Many sequence reads could be allocated to the genome sequence of the Archaeal methanogen *Methanoculleus marisnigri* JR1. This result indicated that species related to those of the genus *Methanoculleus* play a dominant role in hydrogenotrophic methanogenesis in the analysed fermentation sample (Schlüter *et al.* 2008).

Short-read-length libraries are generally not preferred for metagenomic characterisation of microbial communities (Wommack *et al*. 2008). On the other hand, other authors describe the phylogenetic classification of short environmental DNA fragments obtained by high-throughput sequencing technologies (Krause *et al.* 2008b; Manichanh *et al.* 2008). Ingrid *et al.* (2009) developed Anaerochip (a molecular tool), oligonucleotide probes targeting the 16S rRNA gene of methanogens. It allows screening for the presence or absence of most lineages of mesophilic and thermophilic methanogens within complex anaerobic samples in a single test. Application of this microarray to complex samples should result in a greater knowledge of the methanogenic communities. The study showed the dominance of *Methanoculleus* in a sub-optimally operating acidified anaerobic biowaste digester.

As per NCBI, sequencing of 62 organisms of phylum Euryarchaeota has been completed, and other 69 are in progress (**Table 1**). Various researchers suggest that higher is the GC content, higher is the stability of the genome. It includes the debatably alleged thermo-stability to the DNA, as also its conservatism through generations. The table has thus been arranged as per the total GC contents including all chromosome and plasmids, in a descending order. The total genome including all chromosome and plasmid has been accounted for, and the ones with an asterisk mention the estimated size.

METHANOGENESIS

Methane or biogas is produced from agricultural, municipal, and industrial waste biomass with the help of methanogens. These are physiologically united as methane producers in anaerobic digestion (Mshandete and Parawira 2009; Yu and Schanbacher 2010). Though the main substrates are acetate, H_2 and CO_2, methylamines, CO, formate and methanol are also converted to CH_4. Methanogen metabolism is unusual as H_2, CO_2, formate, methylated C1 compounds and acetate are used as energy and carbon sources. Methane is a big contributor to global warming, which necessitates understanding methanogenesis to use methane human good and

Table 2 The classification of methanogenic bacteria (MB) based on metabolic distinction.

Group	Substrate	Examples	Reaction equation
MB I	Acetate	*Methanosaeta* spp.	$CH_3COOH \rightarrow CO_2 + CH_4$
MB II	H_2 and formate	*Methanobrevibater* spp.	$CO_2+H_2O \rightarrow CH_4 + 2H_2O$
		Methanogenium spp.	
MB III	Methylated compounds	*Methanolobus* spp.	$CH_3OH \rightarrow CH_4 + CO_2 + 2H_2O$
		Methanococcus	$4CH_3NH_2 + 2H_2O \rightarrow 3CH_4 + CO_2 + 4NH_4^+$
MB IV	Acetate, H_2 and methylated compounds	*Metahanosarcina* spp.	Combinations of all

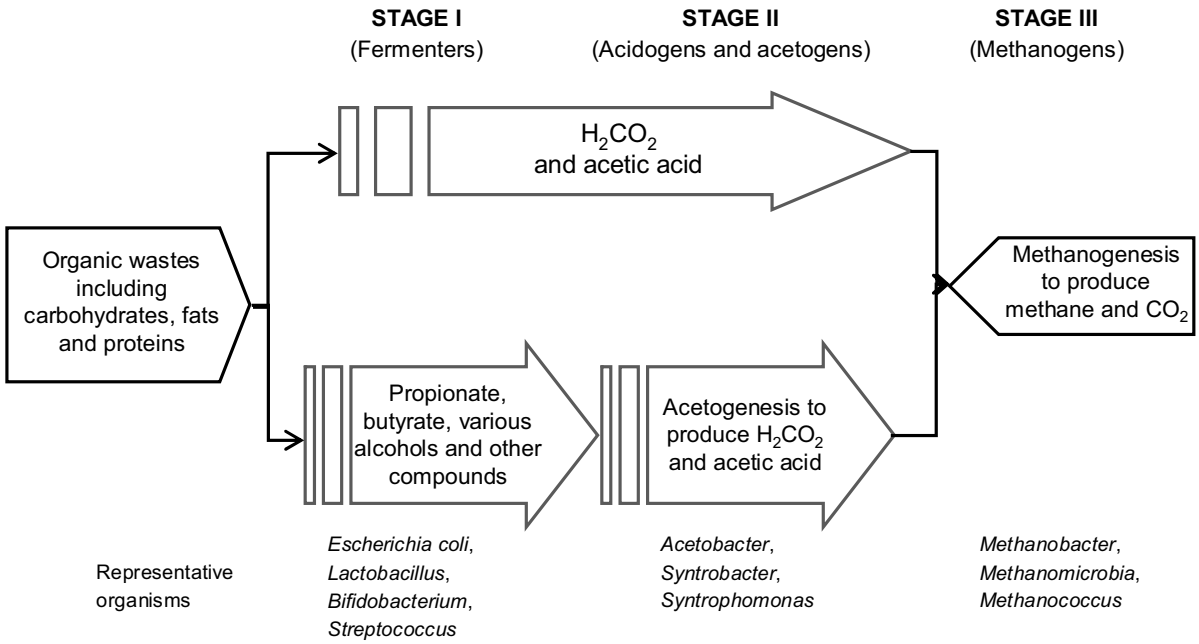

Fig. 1 Schematic presentation representing the stages involved in biotransformation of organic material to methane.

limit its greenhouse gas effect (Simpson *et al*. 2006; Aluwong *et al*. 2011).

Substrates for methanogenesis

In contrast to their huge phylogenetic diversity, methanogens can only use a few simple substrates, most of them being C1 compounds, like CO_2, formate, methanol and methylamines (Liu and Whitman 2008). In fact, the metabolism is restricted to only one or two of above substrates, the exceptions being *Methanosarcina* and *Methanolacina*.

Carbon-di-oxide reduction by molecular hydrogen, followed by formate utilisation is the common energy-yielding reaction in methanogens (Ferry 2010). Acetate is a substrate for *Methanosarcina* and *Methanosaeta*, while methylotrophic genera (e.g., most members of the *Methanosarcinaceae*) utilise methanol, several methylamines or methylsulphide. Furthermore, some species grow on primary and secondary short-chain alcohols. Many species are dependent on special growth factors like vitamins, amino acids or acetate. All methanogens can use ammonium as a nitrogen source. A few species (e.g., *Methanosarcina barkeri* (Scherer 1989) and *Methanococcus thermolithotrophicus*) fix molecular nitrogen too. Methanogenic bacteria (MB) can be categorised into four groups based on the substrate use (**Table 2**). MB I, II and III are the groups which exclusively use acetate, formate and methylated compounds, respectively. MB IV, a comprehensive group, can use a variety of compounds as substrate.

All catabolic processes finally lead to the formation of a mixed disulphide from coenzyme M and coenzyme B that functions as an electron acceptor of certain anaerobic respiratory chains. Molecular hydrogen, reduced coenzyme F_{420} or reduced ferredoxin is used as electron donors (Deppenmeier 2002). The redox reactions are coupled to proton translocation across the cytoplasmic membrane. The resulting electrochemical proton gradient is the driving force for ATP synthesis as catalysed by an A_1A_0-type ATP synthase. Other energy-transducing enzymes involved are the membrane-integral methyltransferase and the formylmethanofuran dehydrogenase complex.The former enzyme is a unique, reversible sodium ion pump that couples methyl-group transfer with the Na^+ transport across the membrane. The formylmethanofuran dehydrogenase is a reversible ion-pump that catalyses formylation and deformylation of methanofuran (Breitung and Thauer 1990; DiMarco *et al.* 1990).

Stages of methanogenesis

The processes in methanogenesis can be studied under 3 stages. Stage I holds the class of microbes which acts as the initiators. Here the fermentative bacteria hydrolyse and ferment complex insoluble organics to simple compounds such as acids, alcohol and others. In stage II, the intermediate products are transformed into acetic acids and H_2CO_2 through acetogenesis. Methanogens come into action in stage III of the whole process. They utilise the products thus formed in stages I and II (**Fig. 1**) thus producing methane.

Biochemistry of methanogenesis

Methanogenesis is an anaerobic respiration, and oxygen inhibits methanogens. Terminal electron acceptor here is the carbon of low molecular weight compounds CO_2 and acetic acid (Lessner 2009):

$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$ and $CH_3COOH \rightarrow CH_4 + CO_2$

The methanogenic pathway, which utilises CO_2 and H_2, involves methanogenic-specific enzymes that catalyses unique reactions using novel coenzymes (**Fig. 2**). Methanofuran, the first C1 carrier found only in methanogenic and sulphur-reducing Archaea, is reduced to Formylmethano-

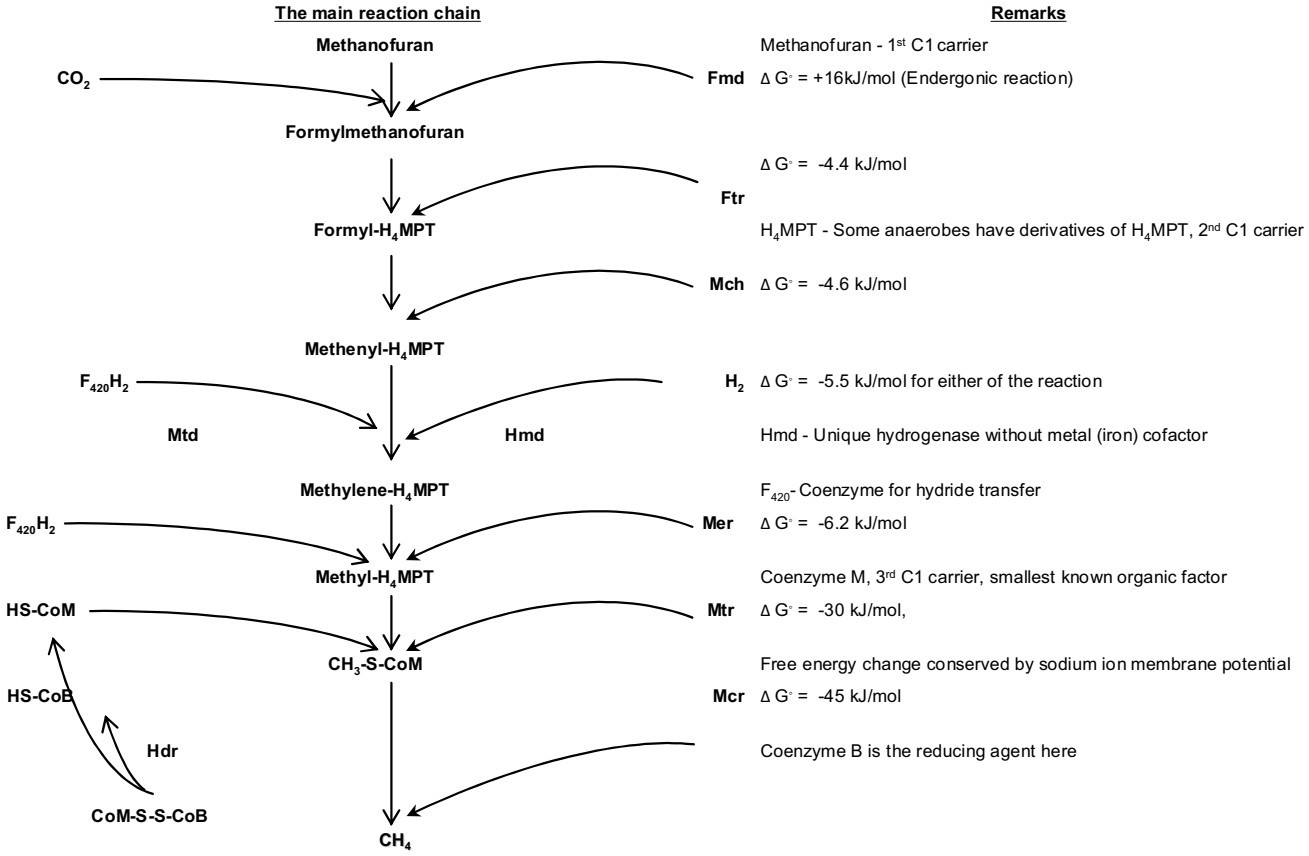

Fig. 2 The methanogenesis pathway showing the reaction steps and major catalysis. All Δ G◦ data are referenced from Thauer 1998. Abbreviations/ acronyms used: Formylmethanofuran dehydrogenase (Fmd); Formylmethanofuran-H4MPT formyltransferase (Ftr); Methenyl-H4MPT cyclohydrolase (Mch); F420 dependent methylene-H4MPT dehydrogenase (Mtd); H2-forming methylene- H4MPT dehydrogenase (Hmd); Methylene-H4MPT reductase (Mer); Methyl-H4MPT coenzyme M methyltransferase (Mtr); Methyl-coenzyme M reductase (Mcr); Heterodisulphide reductase/hydrogenase (Hdr); F420-reducing hydrogenase (Frh); Tetrahydromethanopterin (H4MPT); Coenzyme B (HS-CoB); Coenzyme M (HS-CoM); Heterodisulfide of coenzyme M and coenzyme B (CoM-S-S-CoB).

furan as CO_2 binds to it. The reaction is catalysed by Formylmethanofuran dehydrogenase (Fmd) (Thauer *et al.* 1993). This reaction is the only endergonic ($\Delta G^{\circ} = +16$kJ/mol) reaction in the whole process. The required energy is sourced from sodium ion membrane potential (Kaesler and Schonheit 1989a, 1989b). The second C1 carrier is tetrahydromethanopterin (H_4MPT) and the formyl group is now transferred to H_4MPT, catalysed by formylmethanofuran-H_4MPT formyltransferase (Ftr) (Donnelly and Wolfe 1986; Breitung and Thauer 1990). The formyl H_4MPT is then changed to N^5N^{10}-methenyl-H_4MPT and the reaction is catalysed by methenyl-H_4MPT cyclohydrolase (Mch) (Breitung *et al.* 1991) (ΔG = -4.6 kJ/mol). The N^5N^{10}-methenyl-H_4MPT is now reduced to methylene-H_4MPT in two ways, i.e., either the reduction is F_{420}-dependent or independent. The independent reduction is catalysed by H_2-forming methylene-H_4MPT dehydrogenase (Hmd) (Thauer *et al.* 1996) and the dependent pathway which also requires F_{420}-reducing hydrogenase (Frh) (not shown in the figure) for F_{420} reduction is catalysed by F_{420}-dependent methylene-H_4MPT dehydrogenase (Mtd) (Thauer *et al.* 1993). Reduction of Methylene-H_4MPT to Methyl-H_4MPT is now F_{420}-dependent and is catalysed by methylene-H_4MPT cyclohydrolase (Mer). Now, the methyl group from methyl-H_4MPT is transferred to a third C1 carrier, i.e., Coenzyme M. The reaction is catalysed by methyl-H_4MPT-coenzyme M methyltransferase (Mtr), (DiMarco *et al.* 1990; Gottschalk and Thauer 2001).

Mtr is an integral membrane protein complex of 670 kDa. The negative free energy change of this reaction (-30 kJ/mol) is conserved by sodium ion membrane potential. This is a typical methyltransferase that is coupled with ion transport and energy conservation. The sodium ion membrane potential that is formed by Mtr reaction is mainly used as a driving force for the first reaction (Gottschalk and Thauer 2001). Methyl-coenzyme M is finally reduced to methane by Methyl-coenzyme M reductase (Mcr) (Thauer 1998). The reductant here is Coenzyme B, which is the archaeal methanogen characteristic. Coenzyme B and Coenzyme M are oxidised to the corresponding heterodisulphide (Grabarse *et al.* 2001). The heterodisulphide is an important intermediate of the energy metabolism in methanogens since it is substrate of an energy conservation reaction catalysed by Heterodisulphide reductase/hydrogenase (Hdr) system. In this reaction heterodisulphide is reduced to Coenzyme M and Coenzyme B (Hedderich *et al.* 1994). The reaction steps along with the free-energy equivalents and the various catalytic factors involved in the process are shown in the **Fig. 2**.

Methyl-Coenzyme M Reductase

Methyl-Coenzyme M Reductase (MCR) is an enzyme that occurs in Archaea and catalyses the formation of methane by combining the hydrogen donor coenzyme B and the methyl donor coenzyme M. It has two active sites, each occupied by the nickel-containing F_{430} cofactor (Thauer 1998). The conversion is presented as CH_3-S-CoM + HS-CoB → CH_4 + CoB-S-S-CoM.

All known methanogens express the enzyme Methyl-Coenzyme M Reductase (MCR), which catalyses the terminal step in biogenic methane production (Reeve *et al*. 1997; Thauer 1998; Ferry 1999). The presence of MCR is considered a diagnostic indicator of methanogenesis (Ferry 1992; Reeve *et al*. 1997; Thauer 1998; Lueders *et al*. 2001; Luton *et al*. 2002; Yoshioka *et al*. 2010; Narihiro and Sekiguchi 2011). The genomes of all methanogenic archaea encode at least one copy of the *mcrA* operon (Reeve *et al*.

1997; Thauer 1998). Composed of two alpha (*mcrA*), beta (*mcrB*) and gamma (*mcrG*) subunits, the *mcrA* holoenzyme catalyses heterodisulphide formation between coenzyme M and coenzyme B from methyl-coenzyme M and coenzyme B and the subsequent release of methane (Ellermann *et al*. 1998). Recently when two strains were subjected to structural comparison of MCR, a thioglycine, a C2-methyl alanine, a C5-methyl arginine, an *N*-methyl histidine, and an *S*-methyl cysteine were found in the α-chain (Kaster *et al.* 2011). Functional constraints on its catalytic activity have resulted in a high degree of MCR amino acid sequence conservation, even between phylogenetically distant methanogenic lineages (Reeve *et al*. 1997; Luton *et al*. 2002). This conserved primary structure is used to develop degenerate PCR primers for recovering naturally occurring *mcrA* fragments from a variety of environments (Lueders *et al*. 2001; Luton *et al*. 2002).

BIOENERGETICS

Methanogens, energetically the simplest form of life, have survived since a very long time (Ueno *et al.* 2006). They have continued to exist and participate in several geochemical cycles, such as sulphur cycle, nitrogen cycle, methanogenesis and so on, over time (Canfield *et al.* 2006; Lane 2010). In whole of the pathway discussed above under biochemistry of methanogenesis, two of the reactions are coupled to the formation of chemical gradients that drive ATP synthesis, the membrane-bound N^5-methyltetrahydromethanopterin coenzyme M methyltransferase in the CO_2 reduction and acetate fermentation, and reduction of CoM-S-S-CoB. Methyltransferase is an integral membrane-bound complex that generates a sodium ion gradient across the membrane during methyl transfer. The complex contains factor III of which the Co^+ atom functions as a super-reduced nucleophile accepting the methyl group from CH_3-H_4MPT producing CH_3-Co^{3+} in the first of the two partial reactions catalysed by the enzyme. The second partial reaction involves transfer of the methyl group from CH_3-Co^+ to CoM, producing CH_3-S-CoM and regenerating the activated Co^+ form of the corrinoid. It is proposed that sodium ion translocation is accomplished by a permease associated with MtrA and that the energy for translocation is derived from a conformational change in MtrA during the methylation-demethylation cycle of Co^+/CH_3-Co^{3+} (Harms and Thauer 1996).

The second energy-generating step is the demethylation of methyl-coenzyme M and reduction of the heterodisulphide CoM-S-S-CoB catalysed by methyl-coenzyme M and heterodisulphide reductases. In cell extracts, the methyl-coenzyme M reductase is generally inactive and experiments suggest that activation occurs by reduction of the protein-bound coenzyme F_{430} to the Ni(I) state (Ferry 2002). The electron donor for activation of methyl-coenzyme M reductase is ferredoxin. A membrane-bound electron transport chain delivers electrons to the heterodisulphide, generating a proton gradient that drives ATP synthesis. The relative positions of CoM, CoB and F_{430} in the crystal structure of the methyl-CoM reductase is consistent with a nucleophilic attack of Ni(I) on CH_3-S-CoM and formation of a [$_{F430}$]Ni(III)-CH_3. In the next step Ni(III) oxidises HSCoM, producing CS-CoM thiyl radical and [F_{430}]Ni(II)-CH_3. Finally, protonolysis releases CH_4 and the thiyl radical is coupled to 2 S-CoB to form CoB-S-S-CoM with the excess electron transferred to Ni(II) forming Ni(I) (Ermler *et al*. 1997; Thauer *et al*. 2010).

CONCLUSION

Use of fossil fuel as an energy source is integral part of our daily life, but this is an unsustainable resource owing to their finite reserves and negative environmental effects. Recycling and minimising waste are two main and major objectives of waste management strategies globally. Biogasification seems to have a solution to both of these ever-growing global menaces. In a full-scale system, several environmental conditions will be varying constantly owing to the complexity and variability of organic wastes. It is therefore important to predict environmental conditions have the largest impact. Further, the molecular approach can help in identifying the ecology-, abundance- and/or activity-wise relevant microbes. These microbes can then be the subject of detailed studies or a target of directed cultivation.

Majority of prokaryotes living in natural environments are rather inconspicuous. Several molecular techniques are developed in order to overcome the lack of information about the bacterial function by cultivation-independent methods. Despite the progress made in linking the identification of distinct microbes with their functions *in situ*, it may still be necessary to isolate or enrich novel bacteria to reveal their metabolic potential under various environmental conditions. The results of molecular ecology research has established that experimental strategies based on the combination of molecular techniques with traditional cultivation-dependent methods have great potential in revealing some of the hidden complexity of natural microbial ecosystems. The opportunities for the discovery of new organisms and the development of resources based on microbial diversity are greater than ever before. Molecular sequences have finally given the microbiologists a way to define microbial phylogeny. The sequences are the bases of tools that will allow microbiologists to explore the distribution and function of environmental microbes.

Metagenomics, a new lens to screen the methanogens, has revolutionised the understanding of the entire living world. In Metagenomics, the power of genomic analysis is applied to entire communities of microbes, bypassing the need to isolate and culture individual microbial species. This new approach will bring to light the many abilities of the methanogens. A combined approach of high throughput metagenomics and massive environmental data monitoring is necessary to find correlations between the environment and community (Knights *et al*. 2010). In addition, ecological principles can aid in selecting for superior communities that, for example, are rich in parallel metabolic pathways (Hashsham *et al.* 2000), have high evenness (Wittebolle *et al.* 2009), and are either resistant, resilient, or redundant (Allison and Martiny 2008) to sustain a stable bioprocess.

Methanogens has been studied since long but still a lot await discovery. A lab-scale feasible technology needs scaling-up to commercial level with proper dissemination programmes for the rural and urban society. Beside molecular and biochemical aspects, there are other many means that help to understand and enhance biogas production, e.g., physical, physiochemical, nature of substrate and many more. Based on this knowledge, an engineer makes decisions on the designing, inoculation, and operation of the full-scale system to obtain the sufficient kinetic rates and yields for process viability. Breakthroughs like better processing technique for methane to be used as source of energy is also envisaged.

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