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A review on challenging experimental approach for methanogenic archaea cultivation.

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ABSTRACT

Methanogenic archaea is a large, well-diverse, and a poorly understood group of archaebacteria that are found in various anoxic environments such as anaerobic digesters, marshy areas, sewage, and black sea sediments. It has some of the unique features that separate it from bacteria and other organisms. Recently, it has received an increased attention due to its biological applications and use in effective biofuel production. Methanogens are poorly characterized with respect to isolation and metabolic, biochemical, and molecular properties. In our experience, the beginners often encounter difficulties in media preparation, handling, and culturing techniques. This review outlines the procedure for enumerating, isolating, culturing, and preserving methanogens from different anoxic environments. This review is also designed to encourage and help the study of methanogens and to increase the effectiveness in culturing methods in laboratories. The main objective of this review was to carry out a specialized research in the field of methanogens cultivation and biogas technology and to popularize it. We mainly focus on our laboratory studies conducted in the field of anaerobic cultivation and biogas production.

Keywords: Methanogens, Anoxic environment, Biofuel, Culturing methods

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INTRODUCTION

The methanogenic bacteria are a fastidious microorganism that has received attention of many researchers for its stimulating laboratory analysis and the possibility of its use in biofuel production. It is a diverse group and a unique candidate among prokaryotes because they produce valuable alternate biofuel-methane, a major product of anaerobic digestion. They are very slow-growing, strict anaerobes in the sense that they are unable to use molecular oxygen for their growth. They are nutritionally fastidious with redox potential below −300 mV. So it is important to retain anoxic conditions during all steps of methanogenic cultivation.

Cultivation of methanogens is a laborious and time-consuming process. For a long time, methanogens remained in the backwash of microbiology and biochemistry simply because they were very difficult to isolate and cultivate. Today, the available technologies for their cultivation are scarcely equal to the task it requires to cultivate because it needs highly specialized tools and glassware. These are some of the peculiarities of anaerobes, especially methanogens, that discouraged many scientists from studying them and only a few researchers had the dedication to attempt to study them.

At present many laboratories have started to work on methanogens and biogas production but even these studies are still in infancy with regard to the methanogens and its cultivation [1]. We have conducted a comprehensive study regarding easy cultivation of methanogens.

CULTIVATION OF METHANOGENIC ARCHAEA

In the last three decades, methanogenic archaea are the only special candidate in Archaeabacterial kingdom that has stimulated research because of their unique biochemical and genetic properties [2]. Generally, methanogens require significant time for growth due to their slow-growing character. The first methanogen was not obtained in pure culture until 1947. After 1947, many techniques have evolved including the basic Hungate techniques. But we are still facing some difficulties in methanogen cultivation because mostly knowledge about methanogenic cultivation is quite complex and limited. The following are needed for cultivation of methanogenic archaea.

Anaerobic Accessories

The cultivation procedures of Hungate (1950) were followed in the initial experiments for media preparation and cultivation [3]. Hungate technique had been a breakthrough and created a revolution about anaerobe cultivation. Further modifications were carried out in the later steps. In this regard, it is important to note that the following things are necessary for the cultivation and establishment of strict anaerobic methanogenic operations: anaerobiosis, oxygen-free gases, specialized equipment (gassing manifold), specialized glasswares, redox dyes, and reducing agents.

Anaerobiosis

Cultivation of obligate anaerobic methanogens has always been aimed at exclusion of oxygen. The most important distinctive feature of methanogenic archaea is that they are extremely sensitive toward oxygen. The methanogens are strict anaerobes; those are poisoned by the presence of oxygen at levels as low as 0.18 mg/L of soluble oxygen (as O_2) [4]. Methanogens grow at redox potentials below −300 mV [5]. Hungate (1967) intended that O_2 concentration of the methanogens at that potential is theoretically 10–56 mol per liter, so it can be safely assumed that oxygen is not present in well-reduced habitats [6]. Such exclusion of oxygen can be most effectively accomplished in the laboratory using a closed system from which oxygen has been removed and further preventing the re-entry of the oxygen at all stages of processing.

Oxygen-Free Gases

Normally air contains oxygen. Gas flushing is one of the best methods for removing trace amount of oxygen from culture medium. Generally, nitrogen, carbon dioxide, hydrogen, or their mixtures were used. Mostly nitrogen gas was used because it is a blanketing gas (nonflammable) and is mostly inert in nature. Carbon dioxide can also be used because it is heavier than air, is relatively cheap, is valuable in buffering

activity, and also meets some of the nutritional requirements in certain anaerobes. Commercially available anoxic cylinders are contaminated with trace amounts of oxygen. But we recommended using oxygen-free gases of high purity (less than 5 ppm oxygen).

Preparation of oxygen scrubbers for scrubbing traces of oxygen from commercially available compressed gases such as hydrogen, carbon dioxide, and nitrogen can be done through a heated cylinder. This oxygen present in gases can be removed by passing the gas through packed copper turnings or through reduced copper filings, an inexpensive oxygen scrubber. The copper filings are initially reduced by passing hydrogen or H_2:CO_2 slowly through the hot filings. This reduction releases significant amounts of heat and should be performed carefully so as not to cause the copper filings to clump. After reduction, cool the filings by passing nitrogen through the cylinder. Very small amount of oxygen present in the cylinder gases react with the copper to yield copper oxide, turning the copper black as it is oxidized. Turn on the transformer and gradually increase the setting until the column temperature approaches 340 °C. At higher temperatures, significant reduction of CO_2 to CO may occur. In active laboratories, the transformer remains turned on continuously. After continued use, oxidized copper is regenerated by passing H_2:CO_2 through the column for 5 min [7]. When hydrogen is used in the mixture, there is no need for regeneration. Otherwise, removal of oxygen from these cylinders is carried out by passing the gas through a chromous acid solution or cold catalyst (e.g. DeOxo).

Gassing Manifold: Description of the System

Gassing manifold is one of the specialized culturing methods developed for growing the strict anaerobic methanogens (Fig 1 shows description of gassing manifold) . Nitrogen, hydrogen, and carbon dioxide cylinders were separately connected to a rotameter (used to measure flow rate of gases). The rotameter is connected to a mixing chamber and a copper column which is connected to a pressure/vacuum gauge. These are connected to separate valves that are connected to a gassing jet. Normally, 0.5–2.0 bar pressure is used. The gassing jet is directly connected to the probe (a sterilized glass syringe with needle packed with cotton and is fitted within the gassing jet) (Figure 2 Shows micrographs of gassing jet in a gassing manifold)

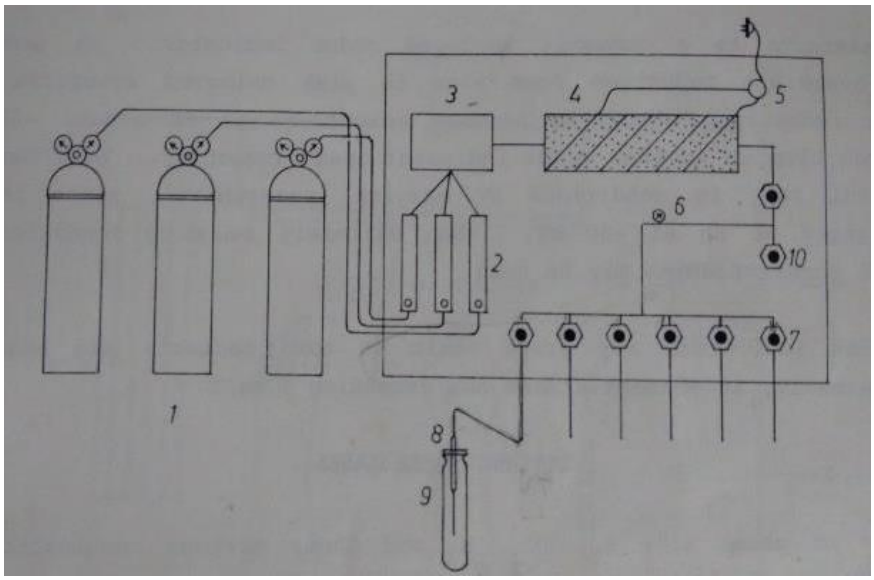

Figure 1: Description of gassing manifold from Balch *et al.* (1979)

1. Gas cylinders 2. Rotameter 3. Mixing chamber
4. Glass copper column 5. Temperature controller 6. Pressure/ vaccum Gauge
7. Valves 8. Gassing jet 9. Roll tube 10. Vaccum valve

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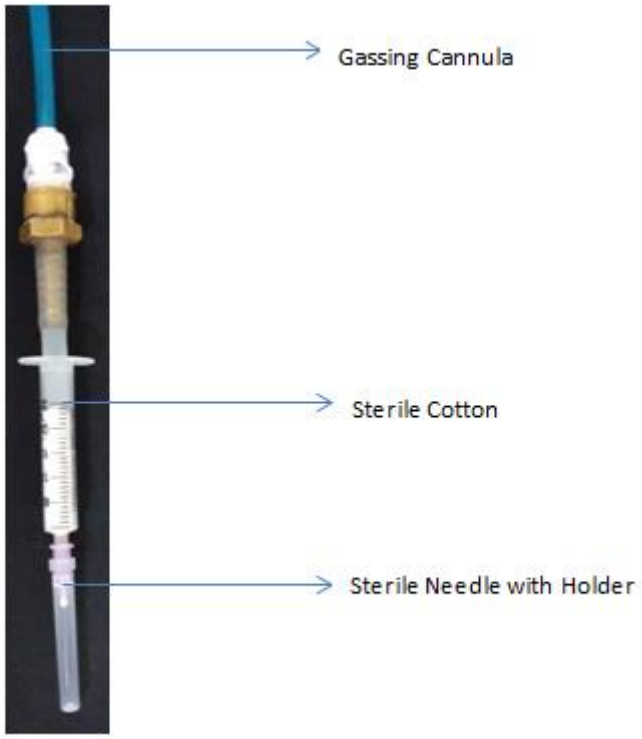

Figure 2: Micrographs of Gassing jet in a gassing manifold

Specialized Glasswares

Specialized glasswares have been developed for easy cultivation of methanogens. These enable the easy use of gas-tight closures (cultivation of anaerobes: DSMZ) to protect media from atmospheric oxygen. These are designed for maintaining anaerobic conditions required for the growth of anaerobic methanogens (Figure 3: shows specialized vessels used for culturing methanogens).

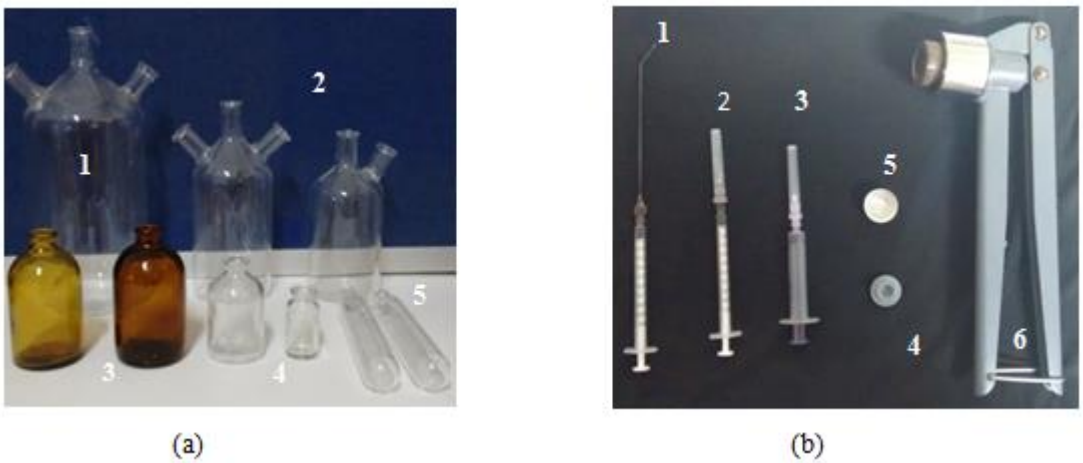

Figure 3: Specialized vessels used for culturing methanogens

3(a) Glassware used for methanogens cultivation (1.three armed, 2.double necked 3. Serum bottles (amber and brown) 4. Serum bottles (colourless) 5. Roll tube)
3(b) Specialized equipment used for methanogens cultivation **(**1. LP needle 2. Tuberculin syringe 3. 2ml syringe 4. Butyl rubber stopper 5. Aluminum crimps 6. Capper and Decapper)

Vials

Generally three types of vials- serum bottles, Hungate-type tubes, and Balch-type tubes—are used for methanogen cultivation. In our laboratory Balch-type roll tubes (25 × 150 mm), two-necked bottle, and three-necked bottle are used, which are more stable than Hungate-type tubes. These are used for easy enumeration and purification. They also bear excess pressure of 2–3 bar. Serum bottles, which are made up of borosilicate glass, are mostly used for liquid media preparation.

Butyl Rubber Septum, Aluminum Crimp, Glass Syringes and Needles, and Crimper and Decapper

Butyl rubber stoppers are mostly used for autosealing activity. They provide gas-tight closure for methanogenic cultivation by efficiently preventing the permeation of air (oxygen) into the bottles. The maintenance of anaerobic conditions necessitates the use of butyl rubber stoppers rather than stoppers made of other types of rubber. Generally, repeated puncturing of the septa with injecting needles could make them become permeable to oxygen. But butyl rubber stopper, because it is thicker than other stoppers, allows multiple punctures and can be reused without loss of impermeability [8]. Aluminum caps are crimped metal seal. They are also called center/hole/center tear-off caps and do not allow any air trap in the vials. So they are necessary for methanogen cultivation.

We used Luer lock syringes with hypodermic needles and needle holders because the entire work had to be done with minimum air exposure (oxygen). These needles are used to avoid air transfer during transferring of cultures. Generally, 1 ml tuberculin disposable syringes, Luer lock 2 ml disposable syringes are used. A hand crimper was used for crimping aluminum caps on vials and a decapper was used for removing aluminum caps from the vials [9].

Reducing Agents

Anaerobic archaea are extremely sensitive to the oxygen. Although oxygen is removed as much as possible, still some will be present because it adheres to glassware and other materials and is dissolved in rubber and plastic tubing and stoppers. Even if all oxygen is removed from a culture medium, addition of a reducing agent is necessary to remove traces of oxygen and to obtain low redox potential needed for growth of most obligate anaerobes [10, 11]. Low oxidation reduction (OR) potential is necessary for the growth of methanogenic archaea. Methanogens grow at redox potentials below −300 mV [5], which can only be achieved by supplementation of media with reducing agents. Reducing agents are added to the anoxic media to depress and poise the redox potential at optimum levels. A variety of reducing agents have been used, including thioglycolate, cystine, sodium sulfide, hydrogen sulfide, dithionite, and hydrogen, with a platinum catalyst [11]. The most common reducing agents used for methanogens are mixtures of cysteine hydrochloride and sodium sulfide. Reducing agents are toxic and normally used in concentration below 500 mg/L [11]. Prepare a 0.2 M solution of cysteine HCl by adding the powder to a volume of hot, boiled distilled water that is about one-third the volume of the container vial. Flush out the atmosphere above the liquid along with nitrogen from a gassing probe. Likewise, prepare 0.2 M solution of $Na_2S.9H_2O$ (sulfite on the surface of a crystal of sulfide is toxic to methanogens; so briefly rinse the surface of the crystal, held in tweezers, under flowing water and dry the crystals with a paper towel before weighing). Unfortunately, the strong reducing agent, sodium dithionite, is toxic to methanogens and cannot be used [7].

Oxidation Reduction and Redox Indicator

Redox dyes are widely used as indicators of an anaerobiosis environment. These dyes form color in oxidized state and are colorless in reduced condition. A commonly used redox indicator for methanogen cultivation is resazurin. It has an EO′ of −51 mV and it undergoes an irreversible reduction from blue to pink resorufin. On further reduction, resorufin becomes colorless at Eh approximately −110 mV. It is pinkish when oxidized and colorless when reduced at −330 mV[7, 12]. It is included in the media at 1 mg/L. Other redox indicators include ethylene blue; phenosafranine is also used for anaerobe cultivation (Figure 4 shows redox indicator and reducing agents used for methanogenic bacteria cultivation).

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Reduction		Reduction	
Resazurin ----------	Resorufin	----------	Dihydroresorufin
(purplish blue)	(pink)		(colorless)

4(a)

4(b)

Figure 4: Redox Indicator and Reducing agents used for methanogenic bacteria cultivation

4(a)Redox indicator (pink- oxidized state; colourless- reduced state) used for methanogenic bacteria cultivation

4(b) Reducing agent (cysteine hydrochloride plus sodium sulphide) used for methanogenic bacteria cultivation

Isolation of Methanogenic Archaea

Source Sites

Samples were collected from different ecological niches (water, soil sediments) and living samples like insects for isolation of methanogens. The samples were collected aseptically in sterile, pre- gassed (or filled with nitrogen to displace the existing air), sealed containers or bottles. These samples were immediately transferred and sealed to a container so that the exposure of oxygen contamination can be minimized. After sample collection, the samples were immediately processed with nitrogen gas, otherwise they were stored in an ice box.

Preparation of Pre-reduced (Anoxic) Culture Medium: PRAS [3, 11, 13]

Preparation of pre-reduced liquid media for anaerobic methanogenic cultivation is one of the very effective and simple methods. It achieves reduced condition when sterilized and kept reduced up to the time they are inoculated and incubated. The basic procedures of Hungate (1950) were followed in this experiment. The following basic steps were involved in media preparation [3].

The water is taken and it must be boiled to drive off dissolved oxygen in it. After that, the boiled water is continuously flushed with the help of anoxic gas (oxygen-free nitrogen gas) by cooling and replacement and removal of oxygen contaminants. One milliliter of resazurin stock solution (1 g/L) is added to the medium. After addition of resazurin, the medium will be appearing purple due to oxidized state. It indicates the presence of oxygen. Nitrogen gas is continuously bubbled when the temperature is reduced. During this time, the ingredients of the medium are added one by one for thorough mixing and pH is adjusted and then the medium is ready to be dispensed. Sealing the bottles is done by a butyl rubber stopper and an aluminum crimp by using a sealer and a desealer, and by autoclaving. Autoclaving works in two ways—by sterilization and by purging oxygen. After sterilization (121°C, 103 KPa for 15 min), reducing agents, vitamins, trace elements, and type of substrates are added. Generally, in our laboratory, sodium bicarbonate and Mah's medium are used. Use of nature's buffer of carbon dioxide–bicarbonate–carbonate is recommended to maintain a pH near neutrality [7].

In methanogenic cultivation, some of the heat labile compounds (sodium sulfide, vitamins) are sterilized using membrane filters and other stocks such as precipitate-causing material (sodium, bicarbonate, calcium chloride) and redox indicator, and reducing agents are separately autoclaved and added to the medium.

Liquid Media

The serum bottles were pre-gassed (for replacement of the present air) with continuous nitrogen gassing. Do these at an aggressive nitrogen flow rate for 30–60 s. Methanogens produce a considerable amount of methane during growth metabolism, which can lead to a substantial overpressure during growth in closed vials. In that bottle, medium (approximately 20 ml) is filled (by either pipetting or peristalic pumping method), and it contains more head space for methane gas production. To avoid post-transfer oxygen contamination, we bubble the gas for few more minutes, maintaining anaerobic conditions as described earlier.

Solid Media: Roll Tube and Slope

Generally solid medium is prepared for purification operations. We prepared two types of solid medium in our laboratory- roll tube and solid slope- to add agar in serum bottles and double-necked bottles. Approximately 0.2 g agar was added to 10 ml medium in the roll tube. Maintaining anaerobic conditions as described earlier, we prepared slopes after completion of autoclaving.

Types of Substrates Chosen based on the Methanogens

On the basis of the type substrate to be chosen, methanogens can be separated into three main nutritional categories.

Carbon dioxide-type substrates: Carbon dioxide (with electrons derived from hydrogen, certain alcohols, or pyruvate), formate, and carbon monoxide.

Methyl substrates: Methanol, methylated amines (methylamine, dimethylamine, trimethylamine, tetramethylamine), and methylated sulfides (methylmercaptan, dimethylsulfide, methanethiol). *Acetotrophic substrates:* Acetate.

- *Hydrogenotrophic methanogens* (non-aceticlastic/CO_2-reducing/H_2-oxidizing/H_2-consuming methanogens): A mixture of 80% hydrogen and 20% carbon dioxide was used as substrate. These hydrogenotrophs (38 species) oxidize H_2 and reduce CO_2 to form methane, and among those some are able to oxidize formate for methane formation. So we use hydrogen or formate as a substrate for hydrogenotrophic methanogens.
- *Aceticlastic methanogens* (acetotrophic/acetate-splitting or acetate-fermenting methanogens). The last category, aceticlastic (or acetotrophic) methanogens (nine species), uses the methyl group of acetate to produce CH_4; only two species are obligate acetotrophs. Some species share nutritional characteristics and cannot be classified in a single group [14]. So these use acetate as a substrate.
- *Methylotrophic methanogens* (disproportionation of methanol and methylamines): The second nutritional group includes methylotrophs (20 species), which use methyl compounds such as methanol, methylamines, or dimethyl sulfides to produce methane. H_2 is also used here as an external electron donor. Thirteen species are obligate methylotrophs.

Gas Phase

On the basis of the substrate, the gas base has to be chosen. Hydrogenotrophs use hydrogen/carbon dioxide (80:20) as a gas phase. For other groups, either nitrogen alone or nitrogen/carbon dioxide (80:20) is used as a gas phase.

Sample Enrichment

After collection of samples, the serum bottles were quickly decapped with continuous nitrogen gas flushing with the help of the gassing jet. One milliliter of the sample was transferred to the serum bottles containing 20-ml liquid medium. Then, the bottles were sealed and incubated at desired temperature. Generally, 7–20 days are needed for satisfactory growth of the methanogens. We confirmed the growth of methanogens by observing turbidity, use of substrates, and formation of methane gas. Generally, many transfers (approximately 5–10 transfers) are needed for better enrichment. Before transferring the serum

bottles, first sterilize the butyl rubber stopper septum by flaming it using a drop of ethanol placed on the septum. After flaming, remove excess methane by puncturing the septum with a sterile injection needle before the isolation is done. Gas-flushing for enriched samples is done on a daily basis for displacing the dead space or removing excess methane gas, and also for substrate utilization with sterile oxygen-free nitrogen gas (Figure 5 shows photographs of the different enrichment cultures of the methanogenic archarea by using different substrates (acetate/formate/H_2+CO_2).

Figure 5: Photographs of the different enrichment cultures of the methanogenic archarea by using different substrates (acetate/ formate/ H_2+ CO_2)

Purification of Methanogenic Archaea: Roll Tube

A simple and effective technique for cultivation of methanogens was originally developed by Hungate (1967) [6]. Roll tube technique is one of the scientific foundations for cultivation of methanogens. The enriched samples are serially diluted up to 10^{-9} with the help of sterile syringes and continuous flushing of nitrogen gas. For that, we have to take 0.5 ml from 10^{-5} to 10^{-9} diluted samples into melted roll tube at 50 °C. The tubes were thoroughly mixed and rolled on ice spanch for equal distribution. Roll tubes are incubated in inverted position mainly for the easy removal of condensed water. This may be to avoid smearing or merging of the colonies.

Figure 6: Micrographs showing purification of serially diluted (10-^{5}) methanogens by using roll tube techique

To aseptically remove a stopper from a culture tube in which a methanogen had generated a negative pressure, the operator should have exceptional skill to prevent contamination or the entrance of oxygen into the tube [15, 16]. After proper incubation, the condensed water is present in the neck of the roll tube, which is removed using sterile syringes before sterilizing the roll tube with flame. Well-developed colonies appear on the surface of the roll tube. We mark it and deseal it with continuous flushing of nitrogen gas. The colonies are picked by bent LP needle and are transferred into a fresh medium. After proper incubation, we check the purity of the culture. Organisms were subcultured every 2 weeks on sodium bicarbonate or Mah's medium. The dead space of the culture was displaced by regularly flushing with oxygen-free nitrogen gas on a daily basis(Figure 6 shows micrographs showing purification of serially diluted (10^{-5}) methanogens by using roll tube techique).

Common tools for identification of methanogens

Some of the common tentative and confirmatory identification tools are indigenously introduced, which will help easy identification of methanogenic archaea.

Aerobic media test

In the anaerobic test first we inoculate our anaerobic culture into aerobic broth. Whether our culture is pure anaerobic is not growing in aerobic media. But an Anaerobic test is one of the tentative identification method.

Gas production

Generally in methanogenic metabolism, the obligatory production of unique gaseous hydrocarbon methane is an end product from a relatively narrow range of substrates for their energy metabolism [17]. After 15 days of incubation, the total methane gas production is checked. Following methods are used for checking gas production for tentative and confirmatory identification.

Moisture Syringe Method

Moisture syringe method is one of the tentative identification tools. After proper incubation period, the total amount of gas was analyzed using this method. In this method, a 5-ml syringe, which is moistened with a drop of sterile water with the plunger inserted to its full, is injected in the bottle. The excess gas in the culture bottle is allowed to flow in the syringe and the amount of methane gas is analyzed and measured.

Flame Test

Flame test is a tentative confirmative test for methane gas production. In this method, methane gas is sucked through a sterile 2-ml syringe with needle. In the culture bottle, the head space contains the methane gas, which is plungered and injected on flame. The culture containing pure methanogens, in its metabolism pure gaseous methane gas was produced and flaming with bluish color.

Water Displacement Method

Biogas (methane) production was also observed through a water displacement unit that is connected to the culture vials. Methane gas was produced during methanogenic process, which was observed through the bubble formation in the water displacement unit.

Gas Chromatography

Gas chromatography technique is one of the confirmatory tools for identification of methanogens. The methane gas in the head space of the culture is analyzed and estimated through gas chromatography. In this method, we use Porapak-Q column and thermal conductivity detector as detector.

Microscopic Method

Generally microscopic methods are used for tentative identification of methanogenic archaea. These are only primary screening methods. Such identification methods should always be corroborated with other confirmatory methods.

Phase-Contrast Microscopy

Some methanogens are tentatively identified by phase-contrast microscopy due to its distinct morphologies from natural samples. Most notable examples are *Methanosarcina*, *Methanothrix*, and *Methanospirillum.*

Epifluorescent Microscopy

Epifluorescent microscopy is one of the best techniques that have facilitated qualitative identification and also used for studying methanogenic ecology. It has remained a simple and limited microbiological laboratory tool for a long time. F_{420} is one of the electron carriers that are mainly found in methanogens. The presence of these unique biochemical markers, co-factor F_{420}, which is found more concentration in methanogens rather than other eubacteria (in eubacteria not in high concentration for autofluorescence). It is of the best useful tools for the detection of methanogens from samples [18]. The presence of F_{420} will fluoresce when observe under epifluorescent microscopy. The co-factor F_{420} is a low potenticofactor. It is responsible for a bluish green or greenish yellow appearance of cells. However, F_{420} levels vary widely in methanogens, and growth conditions may affect those levels [19]. Normally, fluorescence of young cultures was stronger than that of stationary cultures. Sometimes the absence of fluorescence in some cells may be due to aging. These old cells only show a weak greenish yellow fluorescence. Fluorescence at 420 nm excitation was greenish yellow and fluorescence at 350 nm excitation was bluish white. Normally, the fluorescence due to excitation at 420 nm was stronger than that at 350 nm. The fluorescence faded on illumination at both wavelengths (420 and 350 nm) but more rapidly at excitation at 350 nm, and fading of the fluorescence of F_{420} may result from photoreduction, which is also observed for other deazaflavins [20]. It is one of the viable tools. It gives a tentative viable count such as morphology of predominant methanogenic species. It is used for the identification of aggregated forms by association of surfaces or with protozoa.

Use of Antibiotics

Methanogens are cell wall-less bacteria. They do not contain murein, the peptidoglycan of bacteria, which is composed of muraic acid. Instead, they contain pseudomurein or protein subunits depending on the pseudomurein or protein subunits. So, methanogens are resistant to antibiotics, such as penicillin G, D-cycloserine, cephalosporin C, vancomycin, and mixtures of these antibiotics, which inhibit the synthesis of cell walls in bacteria. Also, the differences in protein synthesis between archaea and bacteria also make them insensitive to antibiotics such as kanamycin, which interfere with bacterial protein synthesis [17]. In some cases, rifampicin is also added, which influences the level of RNA polymerase and affects some methanogens but inhibits all the tested eubacteria [21]. This particular feature has been used to facilitate the isolation of pure strains of methanogens by elimination of contaminating non-methanogens [14]

MCR/COM (2-mercaptoethanesulfonic acid/2-sulfanylethanesulfonate, HS-CoM)

The presence of the methyl-coenzyme M reductase (MCR) is one of the important features of methanogenic archaea. Nickel-containing MCR acts as a methane activator, which is methylated to produce methane gas. Coenzyme M is the central part to the final step of methanogenesis pathway. It is either produced by methanogens (*Methanobacterium*) or is required from an external source (*Methanobrevibacter ruminantium*). Coenzyme M is used to reduce CO_2 to methane. The MCR can be specifically inhibited by the coenzyme M analogue, bromoethanesulfonic acid or chloroform [22].

Methods to Identify Isolated Methanogens

Morphological and Physiological Traits: Generally, methanogens are poorly characterized ones. However, some of the special morphological and physiological approaches are essential for their characterization.

Wet Mound Preparation: In this morphological trait, we have to identify the cell shape, size, and motility using a well-growing liquid culture under the microscope.

Gram Character: In this morphological trait, we have to check gram-positive, gram-negative, gram variable, or gram-nonreactive characters of methanogens. Most of the methanogenic strains are gram negative because those are cell wall-less bacteria.

Physiological traits are also used for identification of methanogens, which is based on their temperature, pH, and type of substrates used. Enrichment of methanogens with different defined substrates has been supportive of identifying the types of methanogens [23] (Figure 7 shows pictorial overview for cultivation of methanogenic archaea)

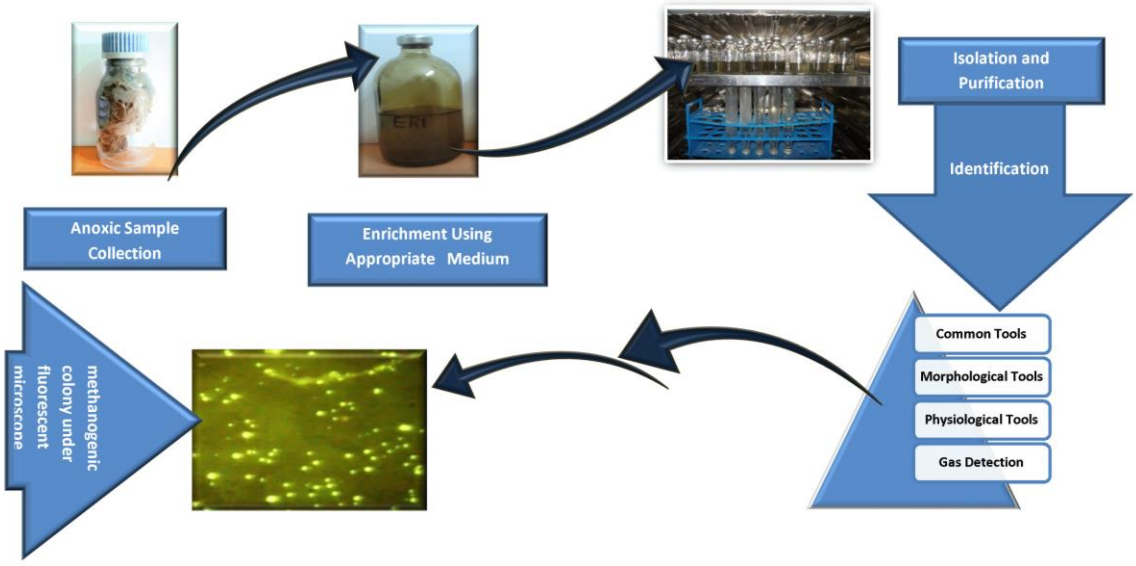

Figure 7: Pictorial overview for cultivation of methanogenic archaea

Current issue and prospective about methanogens cultivation

Presently, new culturing methods are emerging for the cultivation of methanogens. However, it is still challenging and time consuming. In the meantime, the speed analysis achieved through molecular approaches without having to grow them under difficult environmental conditions. These molecular studies have been speeded diverse. Various methanogenic species have been identified and their molecular characterization has been accomplished.

Molecular methods for detection of methanogens include microbiological methods like most probable number (MPN) and molecular methods such as quantitative real-time PCR (Qpcr), microarray, denaturing gradient gel electrophoresis, terminal restriction fragment length polymorphism, and fluorescence in situ hybridization [1].

CONCLUSION

The cultivation of methanogenic archaea is one of the challenging experimental techniques. Many methods are available for the cultivation of anaerobes. Among these, roll tube technique is one of the

standard, easy cultivatable techniques. All the anaerobe cultivation techniques are modified based on the principles of the Hungate technique.

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