

THE INTERNATIONAL JOURNAL OF SCIENCE & TECHNOLEDGE

Review On Anaerobic Media Cultivation

S. Sreeremya

Department of Biotechnology, Mercy College, Palakkad, Kerela, India

Abstract:

Anerobic method of cultivation of bacteria is an intense job. The anaerobic culturing can be carried out by using various techniques, along with various media compositions. Majorly chemical components such as Rezasurin, Thiohlycollate etc are prominent chemicals used for the growth of anaerobic organisms, majorly methanogenic bacterial species. Among the techniques implemented for the growth of anaerobic bacteria, the significant one is Hungate technique. The compositions of anaerobic media preparation and the various techniques involved are discussed in this paper.

Keywords: Anaerobic media

1. Introduction

Anaerobic method of cultivation of organisms can be carried out. Majorly if the isolates is obtained from the environmental sources such as from the pond or river water sources (D unbar. J et.al., 1997). The methods to cultivate the organisms include the use of membranes containing solidified agar (Joseph S.J et.al., 2003). Thus there is chance for isolating the novel anaerobic microorganisms. Majorly the media used for these kind of analysis is freshwater (FW) and a saltwater (SW) base media with or without added electron acceptors and or donors. Two different electron donors were used: H₂ or trimethylamine (TMA). BES was added to some media to inhibit methanogens. In the freshwater media, Na₂SO₄ was added to media. The samples like street marsh and pond water sample, and other environmental samples can be taken for analysis. One gram was diluted in a 10-fold dilution series in either FW or SW sterile broth without adding BES, Na₂SO₄ or TMA (Kaberlin .T. 2002). Two hundred microliters of each dilution was spread on each media. All plates were incubated at room temperature in an anaerobic intrachamber incubator with the chamber containing an atmosphere composed of 66% Gas A (80% N₂/20% CO₂/0.1% H₂S) and 33% Gas B (80% H₂/20% CO₂).

In the biofuel technology, biogas has a major role, among that the synthesis of methane from complex organic compounds involves the interaction of many microorganisms because the narrow substrate range of methanogenic bacteria restricts their metabolism to a few compounds such as H₂-CO₂ and acetate (Toerien.D.F.et.al., 1969). The fermentation of complex organic compounds occurs in various stages and the formation of hydrogen by nonmethanogenic bacteria may be coupled to methane production by H₂ transfer reaction (Wolin.M.J.1990) via the oxidation of H₂ by methanogenic bacteria. The interactions involving end-product formation and removal may be mutualistic, e.g., the metabolism and removal of metabolic end products of nonmethanogens by methanogens may improve the energy and growth yields of nonmethanogens and provide an energy source for the methanogens (Bryant M.P.et.al., 1967). Diffusion of nutrients from methanogenic to nonmethanogenic bacteria may also occur. So these transition from nonmethanogenic to methanogenic bacteria passes through different phases the end product methane is produced (Chung K.T. 1976).

Dichloromethane (DCM) is utilized by the strictly anaerobic, acetogenic mixed culture DM as a sole source of carbon and energy for growth (Stucki G et.al., 1988). Several aerobic bacteria capable of growth with dichloromethane (DCM) can act as a key source of carbon and energy

have been isolated over the past decade (Brunner W.et.al., 1980). Some of these organisms were identified as representatives of the genera *Methylobacterium*, *Methylophilus*, and *Hyphomicrobium* (Leisinger.T et.al., 1993) while others are unidentified facultatively methylotrophic bacteria. DCM dehalogenase, the key enzyme in aerobic DCM degradation, has been purified from some of these organisms (Berzanak.J.A 1982). Characterization of this enzyme, of its structural gene, and of the regulatory gene governing its expression (Brunner W.et.al., 1980) has led to a considerable understanding of aerobic DCM metabolism. The mixed culture which converts DCM to carbon dioxide, methane, and acetate (Stromeyer.S.A. et.al., 1991).

2. Methanogenic

Methanogens exist in the Archaea family and are characterized by their ability to produce methane under highly anoxic conditions (Guo et al. 2005). The rumen of the ruminants is considered as a bioreactor, this is due to the ample aerobic and anaerobic chambers present with in the intestine of ruminant. In rumen, methane emission accounts for the loss of 2–15% of ingested energy (Moss et al. 2000). Therefore, reduction of methane release could be a significant area for ensuring the sustainability of ruminant-based agriculture production. Methane is a normal product of rumen fermentation, representing a pathway for the disposal of metabolic hydrogen produced during microbial metabolism. In the major metabolic pathways like TCA cycle, EMP pathway, basically in the EMP pathway NAD^+ is reduced to NADH, which has to be reoxidised to NAD^+ to allow fermentation to continue. Under the anaerobic conditions prevailing in the rumen, where electron transfer to acceptors other than oxygen must regenerate NAD^+ , the major sink is the reduction of carbon dioxide to methane (although the sink include sulfate, nitrate and fumarate). Because methanogens present in the mixed microbial ecosystem use hydrogen, it does not accumulate in the rumen.

3. Mode of Action

The reducing agents thioglycollate and cystine ensure an anaerobiosis which makes-balance-even for fastidious anaerobes. The sulfhydryl groups of these substances also inactivate arsenic, mercury and other heavy metal compounds. The thioglycollate media are thus suitable for the examination of materials which contain heavy metals or heavy metal preservatives. The higher viscosity of the Fluid Thioglycollate Medium prevents rapid uptake of oxygen and helps maintain the stratification of organisms growing in different layers of the broth. Oxygen is driven out of the broth by autoclaving, but as the broths sit at room temperature, oxygen begins to diffuse back into the tube. Any increase in the oxygen content is indicated by the redox indicator sodium resazurin which changes its color to red. Obligate aerobes will only grow in this oxygen-rich top layer. Obligate anaerobes will only grow in the lower areas of the tube. Microaerophiles will grow in a thin layer below the richly-oxygenated layer. Facultative or aerotolerant anaerobes can grow throughout the medium but will primarily grow in the middle of the tube, between the oxygen-rich and oxygen-free zones.

3.1. Typical-Composition (g/liter)

Peptone from casein 15.0; yeast extract 5.0; D(+)-glucose 5.5; L-cystine 0.5; sodium chloride 2.5; sodium thioglycollate 0.5; sodium resazurin 0.001; agar-agar 0.75.

3.2. Preparation

Suspend 30g/liter, dispense into tubes, autoclave (15 min at 121°C), pH: 7.1 ± 0.2 at 25°C .

The prepared medium is clear and yellowish. The culture medium should always be freshly prepared. Fluid Thioglycollate Medium cannot be used if more than the upper third of the butt has turned pink due to the presence of oxygen and if this coloration does not disappear after boiling once.

3.3. Experimental Procedure and Evaluation

Inoculate the culture medium with the sample material taking care that the sample reaches the bottom of the tubes. In order to ensure anaerobiosis, the medium can then be overlaid with 1 cm of sterile liquid paraffin or agar solution. Incubation: several days at the optimal incubation temperature ($35\text{--}37^\circ\text{C}$). Anaerobes grow in the lower part of the culture.

4. Thioglycollate Medium with Resazurin

4.1. Intended Use

Thioglycollate Medium with Resazurin is used for sterility tests of biological products and for the culture of aerobic, anaerobic and microaerophilic bacteria. The formulation of the medium complies with the requirements of the European Pharmacopoeia, the United States Pharmacopoeia and the AOAC for the bacteriological analysis of antibiotics and the determination of the sporicidal effect of disinfectants. This medium is also used for the confirmation of *Clostridium perfringens* in food products.

4.2. History

Brewer demonstrated the value of this medium, containing a small quantity of agar and of a reducing substance, for the culture of anaerobic bacteria in the presence of sodium thioglycollate. Nungester, Hood and Warren then showed that sodium thioglycollate neutralized the inhibitory effect of mercuric compounds present in the samples analyzed. Malin and Flynn observed that in the presence of carbohydrates, thioglycollate was slightly inhibitory for several species.

4.3. Principles

- Thioglycollate Medium with Resazurin formulated with pancreatic digest of casein, yeast extract, cystine and glucose assures the growth of a large variety of aerobic and anaerobic bacteria.
- Sodium thioglycollate at the concentration of 0.05% decreases the redox potential without having a toxic effect. It also neutralizes the antibacterial power of mercuric derivatives used as preservatives in biological products.
- Agar favors the development of anaerobic bacteria by stabilizing the medium against convection currents so anaerobiosis is maintained in the lower part of the recipients.
- Resazurin, less toxic than methylene blue, is used as a redox indicator: it is colorless in a reducing medium and becomes pink in an oxidized medium.

4.4. Preparation

- Suspend 29.7 g of dehydrated medium (BK017) in 1 liter of distilled or deionized water.
- Slowly bring to boiling, stirring with constant agitation until complete dissolution.
- Dispense in tubes or flasks.
- Sterilize in an autoclave at 121°C for 15 minutes.
- 2/5

4.5. Instructions for Use

- Cool the medium to 25°C.
- Inoculate with the sample to analyze into tubes or vials prepared as above, or the ready-to-use media in tubes BM082.
- Incubate at least 2 weeks at the desired temperature for the sterility control or for 20 hours at 37°C when confirming *Clostridium perfringens* that can occur in food products.

4.6. Typical Composition

(can be adjusted to obtain optimal performance)

For 1 liter of medium:

- Tryptone.....15.00 g
- Yeast extract5.00 g
- Glucose.....5.50 g
- Sodium chloride2.50 g
- Sodium thioglycollate0.50 g
- L-cystine.....0.50 g
- Resazurin.....1.0 mg
- Bacteriological agar0.75 g

pH of the ready-to-use medium at 25°C : 7.1 ± 0.2.

4.7. Quality Control

- Dehydrated medium: cream-white powder, free-flowing and homogeneous.
- Prepared medium: semi-solid medium, slightly opalescent, light amber with a pink ring on the surface.
- Typical culture response after 3 jours of incubation at 30-35°C or 5 days of incubation at 20-25°C(*), according to the European Pharmacopeia ; typical culture response after 24 hours incubation at 37°C(1) :

4.8. Storage / Shelf Life

4.8.1. Dehydrated medium: 2-30°C.

- The expiration date is indicated on the label.

4.8.2. Prepared medium (benchmark value*) :

- Media in tubes or vials: 6 months at 2-8°C, shielded from light.

4.8.3. Ready-to-use media in tubes:

- Store between 2-8°C, shielded from light.
- The expiration date is indicated on the label.

4.9. Note

If after conservation, the media presents a rose coloration (a sign of oxidation) greater than 1/3 of the tube measuring from the surface downward, then restore anaerobiosis by heating at 100°C for 10minutes. Do not carry out this operation more than once (Brewer, J.H. 1940).- 5 kg drum BK017GC

Media used for: Sterility control for biological products, confirmation of *Clostridium*.

4.10. Thioglycollate Broth with Resazurin

Ref : BM08208

Incubation 24 hours at 37°C (anaerobic condition)

Characteristics : good growth with turbidity, under anaerobic conditions

Clostridium perfringens. Technique par comptage des colonies.

Pharmacopée Européenne 5.6. 01/2007:20613. 2.6.13. Contrôle microbiologique des produits non stériles : Recherche de microorganismes spécifiques. Solution et milieux de culture recommandés, 4679-4682.

United States Pharmacopeia 30. 2007. Microbiological Tests / Sterility Tests. Media, 98

*Benchmark value refers to the expected shelf life when prepared under standard laboratory conditions following manufacturer's instructions. It is provided as a

guide only and no warranty, implied or otherwise is associated with this information.

The information provided on the package take precedence over the formulations or instructions described in this document.

The information and specifications contained in this technical data sheet date from 2010-07-13.

They are susceptible to modification at any time, without warning.

Code document : BK017/A/2003-01 : 9.

4.11. Cultivation of Anaerobes

The DSMZ holds a large collection of prokaryotes that thrive only under anaerobic conditions

In the broadest sense obligate anaerobes can be defined as microorganisms which are unable to utilize molecular oxygen for growth. A further differentiation is possible based on their relationship to the presence of oxygen. Aerotolerant anaerobes are only slightly inhibited by significant levels of oxygen in the atmosphere. For instance *Clostridium intestinale* DSM 6191T can grow well on the surface of agar plates incubated in air at atmospheric pressure.

The other extreme is represented by strict anaerobes, which die, or immediately stop growing, upon exposure to low levels of oxygen. It is therefore important to retain anoxic conditions during all steps of handling of these microorganisms. Most strict anaerobes require not only the absence of oxygen to initiate growth, but also a redox potential below -300 mV, which can be only achieved by the supplementation of media with reducing agents (see section on *Reducing agents and resazurin*). Between both extremes all kinds of adaptation exist. The majority of anaerobic microorganisms is fastidious and requires complex media with many supplements.

In the DSMZ catalogue of strains (Internet: <http://www.dsmz.de/catalogues/catalogue-microorganisms.html>) each DSM strain is linked with a specific medium number. It is strongly recommended to use the respective media formulations given for each strain, because only those media were tested at the DSMZ for culturing and a transfer to alternative media may cause a delay or complete failure of growth. Before ordering an anaerobe from the DSMZ it is advisable to have a look on the recipe of the medium necessary for growing this strain and to read relevant publications dealing with its cultivation.

It only makes sense to purchase a strain of interest, if you are convinced to be able to handle it properly!

A large number of strict anaerobes are available from the DSMZ only as actively growing cultures. We recommend to use the Hungate technique to culture these strains. Some general remarks on this cultivation technique and required laboratory equipment follow below. Excellent descriptions of the Hungate technique can be found in the reviews of Hungate (1969) and Wolfe (1971), whereas the contribution of Breznak and Costilow (1994) contains more general information on anaerobiosis. However, please keep in mind, that even if described in detail, some steps of the handling of anaerobic cultures are frequently difficult to master without demonstration. For beginners in anaerobic microbiology it is therefore the best to visit a laboratory where anaerobic cultivation techniques are routinely in use. Anaerobic strains that are available from the DSMZ as lyophilized cultures are normally not sensitive to a short exposure to low oxygen concentrations (nonstringent anaerobes). For instance, a majority of the clostridia and sulfate reducers, but not all of them, belong to this group of strains. If you have received an ampoule from the DSMZ with a vacuum dried sample of a nonstringent anaerobe please read also the instructions given in the section: *Handling of vacuum dried anaerobic cultures*. Further special instructions on difficult to handle microorganisms, like methanogens or hyperthermophiles are available at the DSMZ web pages. Recommended vials for culturing strict anaerobes Suitable containers for pre-reduced media are an important prerequisite for the successful culturing of strict anaerobes. For this purpose special glassware has been developed which enables the easy use of completely gas-tight closures. Of crucial importance is the material of the rubber stoppers. Only stoppers or septa made of butyl rubber can efficiently prevent permeation of air into the vial. Nevertheless, a repeated puncturing of stoppers with injection needles could make them become permeable to oxygen. As a rule, the thicker the stopper the more often it is possible to reuse it without loss of impermeability. Two types of vials are commercially available for anaerobic culturing. The Hungate-type tubes are closed with a flange-type butyl rubber septum and a screw cap with 9 mm opening to allow puncturing of the septum with injection needles. Balch-type tubes are more stable than Hungate-type tubes and recommended if an overpressure of 2 to 3 bar can be expected during culturing. They are closed with a thick butyl rubber stopper which is held in place by sealing with an aluminum crimp. For sealing and removing of the aluminum crimp special devices (crimper/decapper) are necessary. Serum bottles which are available in various sizes can be used alternatively to Balch-type tubes. However, serum bottles are less stable than Balch tubes and should be handled with special care when strains are cultured that are expected to produce significant amounts of gas during incubation. Pre-reduced media can be stored in both types of vials at room temperature in the dark for several weeks without becoming oxidized.

Caution: Some microorganisms produce a considerable amount of gas during growth (e. g., Clostridia by fermentation). The formed gas can lead to a substantial overpressure during growth in closed vials. Strains which are known to accumulate gas during growth should be incubated in vessels that are filled only up to 25% with liquid medium. In addition, cultures of fast growing strains should be vented at least on a daily basis to avoid overpressure. Wear protective goggles during handling of glass vessels that might have overpressure!

4.12. Gassing of Media and Cultures with Oxygen-Free Gas

When vials of pre-reduced media or anaerobic cultures are opened a constant flow of oxygen-free gas over the surface of the medium is necessary to avoid exposure to oxygen. The used oxygen-free gas should have the same composition as that used for medium preparation. We recommend to use oxygen-free gasses of high purity (containing less than 5 ppm oxygen), that are delivered as compressed gas cylinders. Normally, oxygen-free gasses of high quality do not require an additional oxygen removal system (e. g., heated copper column) and can be used directly for culturing a broad spectrum of anaerobes.

The Hungate technique is based on the use of Gassing cannulas. Usually, several cannulas are connected by butyl rubber tubing to a manifold supplying oxygen-free gas with an overpressure that should be adjusted to approx. 0.5 bar. At least two cannulas are needed: one for the vessel to be inoculated or filled with medium and one for the vessel containing the inoculum or the medium to be dispensed. When an aseptic gassing of media or cultures is necessary a barrel of a glass syringe is packed with cotton and fitted between the gassing needle and the butyl rubber tubing. Assembly of cannulas used in the Hungate technique for aseptic gassing.

(A) Cannula used for aseptic gassing of opened vials with oxygen-free gas. (B) Overpressurizing of anaerobic cultures with sterile gas mixtures. After assembly, autoclave the cotton-filled glass syringe and needle, dry in a drying oven at 100 °C, allow to cool, and connect to the manifold. Prior to the first use flush the gassing cannula for approx. 15 min with oxygen-free gas to make it anoxic and then flame the needle to sterilize it. Caution: Make sure that needles sterilized by flaming are cooled down prior to using gas mixtures containing H₂. Hydrogen gas is highly combustible, and even only contact with hot surfaces may cause ignition. Wear protective goggles while overpressurizing vials. For the overpressurizing of cultures with H₂ or H₂/CO₂ gas mixtures use disposable, sterile injection needles (i. d. 0.4 mm or 27G) connected to cotton-filled glass syringes as described above. To keep the pressure within the glass syringe barrel at a constant level during overpressurizing it is necessary to avoid an imbalance between the inflowing gas stream and the outflow. This can be achieved by puncturing the rubber stopper of the cotton-filled syringe with a steel needle (approx. 20G) which is connected to the rubber tubing by an appropriate valve with Luer-Lock fittings. Adjust the gas pressure to the desired value (in most cases 0.5 to 2 bar overpressure). Turn the vial with the culture up side down and puncture the sterilized septum with the injection needle (Fig 2B). A sputtering of gas bubbles indicates the inflow of gas into the medium and can be observed as soon as the tip of the cannula enters the liquid. When the flow of bubbles slows down the pressure within the vial reaches equilibrium with the external pressure of the gas supply. Withdraw gassing needle immediately when the gas flow stops. Handling of vacuum-dried anaerobic cultures The DSMZ delivers lyophilized (freeze-dried) cultures of anaerobic strains exclusively in double-vial preparations, heat-sealed under vacuum. Double-vial preparations have the advantage that a contamination of the atmosphere by aerosols that can be produced by sudden release of the vacuum in single-vial preparations is efficiently prevented. In addition, the cell pellet is protected from contamination, because inflowing air filters through the sterile cotton plug of the inner vial. Before opening of the ampoule please identify the culture by the label on the inner vial which indicates the DSM strain number and date of preservation. Confirm that the ampoule is under vacuum by checking the color of the desiccant at the bottom of the outer vial.

Note: The DSMZ has changed the indicator stain of the desiccant. From January 2002 on the used desiccant is red, instead of blue. The indicator stain will change its color if the outer vial is damaged due to an increase of humidity inside the ampoule. The red color changes to orange and the blue to pink.

It is important to retain anoxic conditions during all steps after the opening of ampoules with freeze-dried anaerobes. This can be achieved in several ways depending on the used anaerobic technique and available equipment in the laboratory. For general information about vacuum dried cultures please visit the following site [Opening of ampoules and rehydration of dried cultures](#).

The freeze-dried pellet of most anaerobic strains available from the DSMZ is protected against short exposure to oxygen by amorphous ferrous sulfide (FeS) which confers a black color to the pellet. However, certain nonstringent or spore forming anaerobes are suspended prior to lyophilization in skim milk without addition of FeS, so that the ampoules display a white pellet.

If the Hungate technique is routinely used in the laboratory, open the ampoules as described in [Opening of ampoules and rehydration of dried cultures](#). After opening keep the inner vial under a flow of oxygen-free gas by inserting a gassing cannula. Add approx. 0.5 ml of the recommended anoxic medium to the vial and resuspend the cell pellet completely (in some cases this may take several minutes). Transfer the cell suspension either by using a 1 ml syringe with hypodermic needle (length at least 38 mm) or a sterile Pasteur pipette, which was made anoxic by flushing with oxygen-free gas. If a Pasteur pipette is used, the Hungate tube containing the appropriate cultivation medium (5 to 10 ml) has to be opened and gassed with a second cannula during transfer of the inoculum.

If an anaerobic gas chamber is available it is recommended to score the ampoule with a sharp file at the middle of its shoulder about one cm from the tip. Transfer the ampoule with the file mark in the anaerobic chamber and strike the ampoule with a file or large forceps to remove the tip. If necessary, wrap the ampoule in tissue paper and enlarge the open end by striking with a file or pencil, then remove the glass wool insulation and the inner vial. Gently raise the cotton plug and sterilize the upper part of the inner vial using an incandescent flaming device (alternatively wipe the upper part of the inner vial with tissue paper soaked in 70% ethanol). Add approx. 0.5 ml of anoxic medium to resuspend the cell pellet and transfer the suspension to a vial with the recommended cultivation medium (5 to 10 ml).

If possible the last few drops of the resuspended cell pellet should be transferred to an agar plate or slant of the recommended medium to obtain single colonies in order to check the purity of the strain. Anaerobic incubation conditions for agar plates can be

achieved by placing plates in an anaerobic chamber or an activated anaerobic Gas Pak jar or similar system (e. g., Anaerocult® bags available from Merck; <http://www.merck.de>).

We recommend to prepare also 1:10 and 1:100 dilutions of the inoculated medium, because some ingredients of the freeze-dried pellet may inhibit growth in the first tube. Inoculation of only one tube may prevent successful resuscitation of certain lyophilized strains (e.g., *Geobacter* spp.).

In most cases freeze dried cultures of anaerobic strains exhibit a prolonged lag period upon rehydration and should be given at least twice the normal incubation time before regarding them as non-viable. © Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH Handling of actively growing anaerobic cultures For the aseptical injection and removal of samples from anaerobic cultures it is recommended to use the Hungate technique which is essentially based on the use of disposable syringes and has the advantage that it allows the use of defined, oxygen-free atmospheres for culturing. The anoxic removal of a sample from an Hungate tube is demonstrated in. First, sterilize the butyl rubber septum by flaming it using a drop of ethanol which has been placed on the septum. If overpressure in the vial can be expected due to microbial growth (e.g., gas production by fermentation) remove excess gas by puncturing the septum with a sterile injection needle Then a sterile, disposable 1 ml syringe with a 25G to 23G hypodermic needle (i. d. 0.50 to 0.65 mm) is made anoxic by displacing the dead space with sterile oxygen-free gas or a reducing agent. Anoxic removal of a sample from a Hungate tube: Penetrate the septum and inject the same volume of oxygen-free gas into the vial as will be subsequently removed as sample from the culture. By doing this the development of an underpressure in the culture tube will be prevented. Then, turn the vial with the culture up side down and fill the syringe with the needed amount of liquid. Finally, withdraw needle and filled syringe carefully. Reducing agents and resazurin A redox sensitive dye is usually included in media used for culturing anaerobes to monitor the redox potential. The most commonly used redox indicator is resazurin, because it is generally non-toxic to bacteria and is effective at very low concentrations of 0.5 to 1 mg/l. This indicator dye is dark blue in its inactive form and first has to undergo an irreversible reduction step to resorufin, which is pink at pH values near neutrality (the color may change to blue under alkaline conditions). This first reduction step normally occurs when media containing an excess of organic nutrients are boiled for a few minutes or mineral media are heated under an oxygen-free atmosphere. In a second reversible reduction step hydroresorufin is formed which is colorless. The resorufin/hydroresorufin redox couple becomes totally colorless below a redox potential of about -110 mV and regains a pink color at a redox potential above -51

Please note, that some organisms require redox potentials lower than -110 mV and hence may not start to grow even if the medium is colorless. On the other hand, a pink color of the medium does not automatically imply that it became oxidized by oxygen (e.g., through an gas permeable rubber septum). For instance, certain nitrate reducers produce nitrite during growth which acts as potent oxidant and so may raise the redox potential above -51 mV. Reducing agents are added to most anaerobic media to depress and poise the redox potential at optimum levels. The most common reducing agents are sodium thioglycolate, cysteine x HCl, Na₂S x 9 H₂O, FeS (amorphous, hydrated), dithiothreitol and sodium dithionite. Sodium thioglycolate is often used in combination with ascorbate and mainly incorporated in some traditional media for culturing anaerobes (e.g., Postgate's media for sulfate reducers, DSMZ medium 63). Thioglycolate as reducing agent has the advantage that it is relatively stable at room temperature and can be therefore included in media prior to flushing with oxygen-free gas. It is only activated by heating above 100 °C and then efficiently removes oxygen. Hence, not so much care has to be taken in avoiding exposure to oxygen of the prepared medium prior to dispensing in anoxic vials. For this reason many commercially available media contain thioglycolate as reducing agent. However, the standard redox potential of thioglycolate alone (around -100 mV) is generally not low enough to allow initiation of growth of a majority of strict anaerobes which need highly reduced media. For maximum effectiveness of reducing agents other than thioglycolate, stock solutions under nitrogen gas should be prepared. While preparing stock solutions of reducing agents avoid insertion of the gassing cannula into the liquid, because this can have negative effects on the reducing capacity. Sodium dithionite, which reacts extremely fast with oxygen, has to be dissolved in oxygen-free water and sterilized by filtration. Freshly prepared stock solutions of dithionite can be stored only for up 2-3 weeks at room temperature in the dark. Add appropriate concentrations of reducing agents to the autoclaved medium just prior to use and allow the medium to sit until it becomes colorless (incubation at 37 °C may accelerate this process). If the medium stays pink despite addition of reducing agent exchange the septum of the vial under a flow of oxygen-free gas, because it might have become permeable to oxygen. Finally, add a small amount of dithionite for final adjustment of the redox potential to a value below -300 mV, if this does not help discard the medium tube.

5. Conclusion

The study on anaerobe and its cultivation is a very vivid process. For the experimenting on the anaerobes are also vast. So there are various techniques adopted by the researchers to culture the anaerobes. Different techniques and different media's are being used.

6. References

1. Brewer, J.H. 1940. Clear Liquid medium for the "aerobe" cultivation of anaerobes. JAMA, 115: 598. Journal Officiel du 25 octobre 1978. Essai de stérilité, 8233-8237.
2. Breznak, J. A. 1992. The genus *Sporomusa*, p. 2016-2021. In A. Balows, H. G. Truper, M. 363. In J. C. Murrell and D. P. Kelly (ed.), *Microbial growth on Cl compounds*. Intercept, Andover, United Kingdom.
3. Dworkin, and K.-H. Schleifer (ed.), *The prokaryotes*, 2nd ed., vol. II. Springer, New York.
4. Brunner, W., D. Staub, and T. Leisinger. 1980. Bacterial degradation of dichloromethane. *Appl. Environ. Microbiol.* 40:950-958.
5. Bryant, M. P., E. A. Wolin, M. J. Wolin, and R. S. 1967. *Methanobacillus omelianskii*, a symbiotic association of two species of bacteria. *Arch. Mikrobiol.* 59:20-31.
6. Chung, K. T. 1976. Inhibitory effects of H₂ on growth of *Clostridium cellobioparum*. *Appl. Environ. Microbiol.* 31:342-348.
7. cultivation of widespread and previously uncultured soil bacteria. *Appl. Environ. Microbiol.* 69:7210- 7215.
8. Dunbar, J., S. White, and L. Forney. 1997. Genetic diversity through the looking glass: effect of enrichment bias. *Appl. Environ. Microbiol.* 63: 1326-1331.
9. Ferry, J. G., and R. S. Wolfe. 1976. Anaerobic degradation of benzoate to methane by a microbial consortium. *Arch. Microbiol.* 107:33-40.
11. Guo YQ, Hu WL, Liu JX (2005) Methanogens and manipulation of methane production in the rumen. *Wei Sheng Wu Xue Bao* 45:145-148
12. Hobson, P. N., S. Bousfield, and R. Summers. 1974. Anaerobic digestion of organic matter. *CRC Crit. Rev. Environ. Control* 4:131-191.
13. Joseph, S. J., P. Hugenholtz, P. Sangwan, C. A. Osborne, and P. H. Janssen. 2003. *Laboratory*
14. Kaeberlein, T., K. Lewis, and S. S. Epstein. 2002. Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. *Science.* 296:1127-1129.
15. Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH **Literature** Hungate, R. E. 1969. A roll tube method for cultivation of strict anaerobes, pp. 117-132. In J. R. Norris and D. W. Ribbons (eds.),
16. Leisinger, T., S. La Roche, R. Bader, M. Schmid-Appert, S. Braus-Stromeier, and A. M. Cool 1993. Chlorinated methanes as carbon sources for aerobic and anaerobic bacteria, p. 351-
17. Moss AR, Jouany JP, Newbold J (2000) Methane production by ruminants, its contribution to global warming. *Ann Zootech* 49:231-253
18. Scholtz, R., L. P. Wackett, C. Egli, A. M. Cook, and T. Leisinger. 1988. Dichloromethane dehalogenase with improved catalytic activity isolated from a fast-growing dichloromethane utilizing bacterium. *J. Bacteriol.* 170:5698-5704.
19. Stromeier, S. A., W. Winkelbauer, H. Kohler, A. M. Cook, and T. Leisinger. 1991. Dichloromethane utilized by an anaerobic mixed culture: acetogenesis and methanogenesis. *Biodegradation* 2:129-137.

20. Stucki, G., R. Gilli, H. R. Ebersold, and T. Leisinger. 1981. Dehalogenation of dichloromethane by cell extracts of *Hyphomicrobium* DM2. *Arch. Microbiol.* 130:366-371.
21. Toerien, D. F., and W. H. J. Hattingh. 1969. Anaerobic digestion. I. The microbiology of anaerobic digestion. *Water Res.* 3:385-416.
22. Watanabe, K., M. Teramoto, H. Futamata, and S. Harayama. 1998. Molecular detection, isolation, and physical characterization of functionally dominant phenol-degrading bacteria in activated sludge. *Appl. Environ. Microbiol.* 64:4396-4402.
23. Wolfe, R. S. 1971. Microbial formation of methane. *Adv. Microb. Physiol.* 6:107-146.
24. Wolfe. 1967. *Methanobacillus omelianskii*, a symbiotic association of two species of bacteria. *Arch. Mikrobiol.* 59:20-31.
25. Wolin, M. J. 1900. Metabolic interactions among intestinal microorganisms. *Am. J. Clin. Nutr.* 27:1320-1328.