Anaerobes of Clinical Significance

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Edited by

Padmaja A. Shenoy, Aruna Poojary
and Om Prakash

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TABLE OF CONTENTS

PREFACE

The areas of anaerobic pathogenesis and gut microbiota are currently very interesting to clinicians and researchers. Common infection sites include deep wounds, brain abscesses, lung and abdominal infections, liver abscesses, periodontal pockets, traumatic and bite wounds, cellulitis, and empyema. Due to their survival ability without oxygen, facultative and obligate anaerobes are the primary causative agents of anaerobic infections. Cultivating, handling, and preserving obligate anaerobes present significant challenges, and further research on anaerobic pathogens of clinical significance is necessary.

In separate chapters, the book provides comprehensive information on obligate anaerobic pathogens isolated from various infection sites. Additionally, it covers the tools and techniques used in cultivating obligate anaerobes and focuses on safeguarding them for future reference, research, and training. The book covers various topics, including human gut microbiota, anaerobic probiotics, and fecal microbiota transplantation. It is aimed at bachelor's and master's students in medical fields and researchers in anaerobic microbiology. To ensure the book is engaging and accessible to students and academicians, we have designed each chapter to follow a consistent format and structure. We have invited contributions from experts with extensive experience with specific groups of anaerobes. Additionally, our editorial team includes expert microbiologists from clinical and research backgrounds, who will ensure the scientific accuracy of each contribution. This book will capture the interest of students and researchers in clinical research and will gain widespread popularity.

This book sets itself apart by providing readers with the most recent and up-to-date information on the topic. For instance, the human gut, being anaerobic, predominantly contains facultative and anaerobic microbes that play a crucial role in human health and disease. While many of these microbes are beneficial, some can act as human pathogens in immunocompromised individuals. Recognizing the significance of the gut microbiota in gut-health, the book includes a chapter on anaerobic probiotics, fecal microbiota transplantation, agents of anaerobic infections,

infection control practices in *Clostridioides difficile* infections, filling a gap in the existing literature on anaerobes.

The eukaryotic nature and presence of mitochondria initially led to the belief that fungi cannot grow anaerobically. However, recent findings have shown that several fungal pathogens can thrive in anaerobic environments, challenging this notion. As a result, we have included a dedicated chapter on anaerobic fungal pathogens in this book, a topic lacking in previous literature. Further research is needed to understand the role of obligate anaerobic Archaea, such as methanogens, in human health, given the challenges in cultivating and preserving them. In recent years, advancements in culture-independent OMICS have demonstrated their indirect involvement in various infections and health-related issues.

Given the growing interest among clinicians and biomedical researchers, we have included a comprehensive chapter on the clinical significance of Methanoarchaea as a model in this field. This book will also feature a dedicated chapter on collecting and handling anaerobic samples of clinical importance for cultivation and diagnosis. The chapters are organized from basic to advance to facilitate a thorough understanding of the concept and theme, with each chapter connecting to the next. They will maintain a consistent pattern, design, and length.

As we prepared and finalized the contents, our editorial team carefully selected chapter titles and themes to address the gaps in available books on this topic. We aimed to create a book that caters to the needs of medical students, medical microbiologists, clinicians, and researchers working with obligate anaerobic pathogenesis. Our book will stand out in terms of presentation, organization, and content, effectively meeting the needs of undergraduate, postgraduate, and PhD students in medical field and clinical research.

FOREWORD
DR. CHIRANJAY MUKHOPADHYAY

Discovery of butyric fermentation led Louis Pasteur understand the nature of a certain group of bacteria whom he later termed as anaerobes in 1863. Since then, it is understood that anaerobes are the most predominant normal human skin and mucous membrane flora and a frequent cause of endogenous life-threatening bacterial infections. Anaerobic clinical microbiology remains a challenge due to lack of awareness, specialist culture requirement and increasing antimicrobial resistance. The recent development due to metataxonomic and metagenomic sequencing has created interest in the potential role of the microbiota in a plethora of other aspects of human health, from obesity to mental health. At present days, the successful use of fecal microbiota transplants for the treatment of antibiotic associated diarrhea raises potential unchartered long-term consequences and possibilities.

While I have been requested to write the foreword note for the textbook on "Anaerobes of Clinical Significance", I accepted it as a privilege, since I have witnessed an upsurge in the incidence of anaerobic infections and antimicrobial resistance that has created an enormous challenge for the microbiologists to diagnose and for the clinicians to treat infections. Many laboratories do not perform anaerobic bacterial detection routinely and hence the awareness is less among the clinicians and microbiologists.

Department of Microbiology at Kasturba Medical College, Manipal is well equipped with state-of-the-art facilities to carry out anaerobic diagnostics. I know one of the editors of this book, Dr. Padmaja A Shenoy as my colleague for more than a decade. Her dedicated approach to the diagnosis of anaerobic infections has made her an expert in anaerobic bacteriology at the national level.

I recommend this book, not only for the students and the microbiologists, but also for the clinicians, since this book gives an excellent overview of anaerobic etiologies, diverse infections, and mainly the elaborative diagnostic workup for suspected anaerobic infections which may be very essential for regular practices. It also highlights the importance of antimicrobial susceptibility testing and antimicrobial resistance patterns among the virulent anaerobic pathogens.

I sincerely appreciate the efforts made by the editors Dr. Om Prakash, Dr. Aruna Poojary along with Dr. Padmaja A Shenoy and all the authors of this book for this commendable work. I would like to see this book as a routine textbook for the postgraduate students and researchers who working in the field of anaerobic bacteriology, for the microbiologists who want to build their career as a specialist anaerobic bacteriologist and for the clinicians who encounter similar challenging cases at the bedside.

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FOREWORD
DR. KAIOMARZ BALSARA

I am privileged to write a foreword on this informative book on "Anaerobes of Clinical Significance". Anaerobic organisms are in abundance within the human body. They are found on all mucocutaneous junctions, on the skin, the female genitalia and most importantly, in the gastrointestinal tract. They increase in numbers in the gut as one proceeds down the gut. The largest number is in the distal colon, where they outnumber aerobes in a ratio of 100000 to 1. These organisms thrive and replicate in environments without oxygen, though some can survive for several days in low-oxygen environments but cannot replicate.

Louis Pasteur was the first to grow anaerobic bacteria over a hundred years ago but their importance was only recognized in the middle of the nineteenth century. The reasons probably were that collection methods for culture needed to be standardized; they had fastidious growth requirements

that limited recovery, and separation from aerobic bacteria was difficult. By the 1960s, taxonomy had evolved along with methods of creating an anaerobic environment for bacterial growth, which led to a renewed interest in anaerobic bacterial infections. However, most clinicians suffer a lack of knowledge of anaerobic infections, and the use of one or two drugs as a panacea for all suspected anaerobic infections is standard practice.

This book edited by three authorities in microbiology is a welcome publication. The chapters are in good chronological order and enable the reader to understand the methods of collection, transportation and cultivation of material. Anaerobic fungal infections is a new subject and important in today's era of widespread use of chemotherapeutic and other immune-suppressive drugs. Gram-negative anaerobic bacilli which are most important in sepsis have been described in detail. Finally, a whole chapter on the human microbiome in health and disease. This has gained great importance in all disease states from dementia to gut malignancies. This will be a welcome read.

I congratulate the editors and the authors and encourage surgeons, physicians and medical students to use this text as a regular read and a referral book.

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FOREWORD
LT COL (DR) T VIJAYA SAGAR

Anaerobes constitute a substantial part of the human microbiome. According to current data, about 90% of human gut microbiota are anaerobes. The oral cavity, vaginal tract, deep wound and abscess are mainly anaerobic. It has been realized that anaerobic infections are the leading cause of morbidity and mortality across the globe. Still, due to the challenging nature of the cultivation and handling of anaerobes, they are neglected, and major attention is given to aerobes only. Considering the substantial role of anaerobic infections in human diseases, Scientists and clinicians are currently diverting their attention toward anaerobic pathogens, and anaerobic pathogenesis is an emerging area of research. I am glad to write about the content and Editors of the current book entitled "*Anaerobes of Clinical Significance*", which will be published by Cambridge Scholars Publishing, United Kingdom. The Editors of the book Dr Om Prakash (PhD-Microbiology), former scientist, NCMR-NCCS and deputy head and Associate Professor, Symbiosis Centre for Climate Change and Sustainability (SCCCS), Symbiosis International (Deemed University) Pune, India, Dr Aruna Poojary (MD. Microbiology), laboratory director, department of pathology and microbiology, Breach Candy Hospital Trust, Mumbai and Dr Padmaja A Shenoy (MD. Microbiology), Associate Professor in the department of microbiology at Kasturba Medical College, Manipal, has extended experience of anaerobic microbiology and they tried to fill the existing gap in contents published on this subject in other books available in the market. I have gone through

the book's content and found it extremely useful for students of medical discipline as well as researchers in the area of anaerobic infections, public health and antimicrobial resistance. I appreciate the editors' efforts and hard work in designing this book's contents and subject matter, which include from basic to advance in the field, including state-of-the-art methods of anaerobic cultivation, identification and preservation of anaerobic microorganisms. In addition, one complete chapter is dedicated to sampling, transportation and handling of the specimen, is an essential component of anaerobic cultivation and diagnosis. Furthermore, the book contains separate chapters on each anaerobic pathogen, which include detailed descriptions of organisms, methods of cultivation and handling which provide an in-depth understanding of microbiology and the clinical significance of strains. Although several books are available with information about anaerobic microbes, their contents are brief and need updating. Considering the recent development in the area of anaerobic microbiology related to anaerobic probiotics, human gut microbiome, and archaea, editors tried to include separate chapters on these untouched aspects. I found that this book contains separate chapters on fungal anaerobiosis and pathogenesis, the role of archaea in human-health and diseases, anaerobic probiotics and human-gut microbiota and the recent trend in fecal microbiota transplant to promote human health etc. These are the unique contents of this book and will definitely fill the existing gap, improve the knowledge and attract the audience's attention. Finally, I would like to appreciate the vision and hard work of the editors for the compilation of this book and wish them all the best for this and also for their future endeavors.

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Dr. Padmaja Ananth Shenoy

The head of the department, faculty, and technical staff are from the Department of Microbiology, Kasturba Medical College, Manipal, Manipal Academy of Higher Education, Manipal. Also, Manipal Academy of Higher Education, thank you for giving me the opportunity to carry out this project. Special thanks to family members for their invaluable support.

Dr. Om Prakash

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Dr. Aruna Poojary

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CHAPTER 1

ANAEROBIC WAY OF LIFE AND TOOLS USED IN CULTIVATION AND PRESERVATION OF OBLIGATE ANAEROBES

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Abstract

Anaerobes play crucial roles in various ecosystems and significantly contribute to human health and disease. Recently, the cases of anaerobic infections have been increasing in human and animal communities, and several anaerobes have been cultivated and characterized from various clinical specimens. Unlike aerobic microorganisms, anaerobes require special handling during cultivation, identification, and preservation due to their unique oxygen requirements. Knowledge of the anaerobic way of life, their metabolic pathways, and methods of cultivation, isolation, and identification is a prerequisite for adequately handling anaerobes. This chapter aims to provide state-of-the-art knowledge about the oxygen relationship of microorganisms, the technicalities of anaerobic media preparation, and how to cultivate, isolate, identify, and preserve them using available resources. We also discussed the tools used for cultivation and anaerobic media preparation and how to avoid excess exposure to oxygen for better viability and yield.

Background

The Earth's initial environment was completely anoxic, gradually transitioning from microoxic to its current oxygen-rich conditions. Anaerobes are believed to be the first form of life that originated on Earth. During evolution, aerobic microorganisms descended from anaerobes. Based on the oxygen relationship, microorganisms are classified into obligate aerobes, facultative aerobes/anaerobes, aerotolerant/ microaerophiles, and obligate anaerobes. Obligate aerobes only grow in the presence of oxygen, whereas obligate anaerobes cannot survive or grow in the presence of environmental oxygen. Facultative anaerobes can thrive in oxygen-rich and oxygen-depleted environments, while microaerophiles thrive in hypoxic or microoxic conditions. Similarly, aerotolerant microorganisms can tolerate environmental oxygen but do not grow when exposed.

Obligate anaerobes are categorized into two subcategories based on oxygen toxicity: strict obligate anaerobes and moderate obligate anaerobes. Strict obligate anaerobes are highly sensitive to oxygen exposure and cannot tolerate levels exceeding 0.5%, such as methanogenic archaea. In contrast, moderate obligate anaerobes can survive and tolerate oxygen levels ranging from 2-8% in the environment. Although the subdivision of obligate anaerobes may seem confusing, it is often classified within the microaerophiles category.

Anaerobes are present in all three domains of life, including Bacteria and Archaea (prokaryotes) and Fungi (eukaryotes). They inhabit diverse ecological niches devoid of oxygen, including deep seawater and sediments, hydrothermal vents, human and insect gut, and cattle rumen. Anaerobes exhibit a wide range of temperature preferences, including psychrophilic (0 to 20°C), mesophilic (20 to 40°C), thermophilic (40 to 70°C), and hyperthermophilic (70 to 110°C) groups, showcasing immense morphological, physiological, and genetic variations. (Holdeman and Moore 1977; Bintrim and Goodman 1997, 277–282; Boone and Whitman 1988, 212–219; Costa and Leigh 2014, 70–75; Jabolanski and Łukaszewicz 2015, 1360-1368). Anaerobes play significant roles in various ecosystems, contributing to processes such as wastewater treatment, pollutant degradation, solid-waste management, greenhouse gas emission and climate change mitigation, biofuel and green energy production, and the occurrence of anaerobic infections.

Ecological and Clinical Importance of Anaerobes

Anaerobes, encompassing bacteria, archaea, and eukarya (Fungi), constitute integral components of the normal microbiota in humans and animals, predominantly inhabiting sites like the oral cavity, human gut, rumen, and female genital tract, with occasional presence in other body regions. Anaerobic infections predominantly occur in oral, abdominal, and pelvic regions. However, the involvement of lungs, brain, and soft tissues also occurs, often arising from immunosuppressive conditions, trauma, surgery, or mucocutaneous breaches. Commonly studied anaerobic bacteria include *Prevotella*, *Veillonella Fusobacterium*, *Bacteroides*, *Peptococcus* and *Peptostreptococcus Actinomyces*, *Propionibacterium*, *Bifidobacterium*, *Lactobacillus* and *Clostridium*. This chapter focuses on cultivation and preservation techniques for obligate anaerobic microorganisms, with subsequent chapters detailing pathophysiology, specific handling requirements, media and incubation conditions, and virulence factors of different groups organized by site and organism.

Anaerobic Way of Life

Based on the evidence, anaerobic microorganisms utilize two main energy generation methods: fermentation and anaerobic respiration, each with distinct mechanisms for ATP formation. Fermentation primarily relies on substrate-level phosphorylation, while anaerobic respiration utilizes alternative electron acceptors such as nitrate, sulfate, and carbon dioxide (CO_2). In contrast to aerobic respiration, fermentation and anaerobic respiration yield less energy, resulting in slower growth and longer incubation times for visible colony formation or turbidity in broth cultures.

Inside cellular cytoplasm, oxygen metabolisms or oxidation-reduction processes generate Reactive Oxygen Species (ROS), including hydroxyl radicals (OH), hydrogen peroxide (H_2O_2), and superoxide anion radicals (O_2). ROS can damage cellular components such as DNA, proteins, and lipids. To counteract the detrimental effects of ROS, aerobes and aerotolerant organisms possess antioxidative defence systems comprising enzymes like superoxide dismutase (SOD), superoxide reductase (SOR), catalase, and peroxidase, as well as non-enzymatic antioxidants like ascorbic acid, glutathione, vitamin E, riboflavin, and cysteine.

Previously, obligate anaerobes were believed to lack enzymatic antioxidants like catalase, superoxide dismutase, and peroxidases to defend against ROS. However, recent studies have shown that anaerobes have developed

mechanisms to protect themselves from ROS, albeit to a lesser extent than aerobes. Anaerobic habitats like thermal vents, the human gut, and sludge often experience episodic oxygenation, exposing anaerobic populations to oxygen stress. Despite this, anaerobes can re-establish themselves due to their defence mechanisms.

Research indicates that the resilience of anaerobes to oxygen stress depends on the duration and intensity of oxygenation. The addition of antioxidants to transport and cultivation media has been shown to assist anaerobes in coping with oxygen stress and neutralizing the effects of ROS. This practice has resulted in better cultivating highly oxygen-sensitive anaerobes from the human gut, such as *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, and *Christensenella minuta*. Antioxidants prevent cellular damage by ROS, promote viability and culturability, and enhance longevity during long-term preservation, making their inclusion in anaerobic preservation protocols an emerging practice.

Consideration for Anaerobic Media Preparation

Anaerobes are common residents of soil, rivers, seawater sediment, sewage, anaerobic digesters, wetlands, insects, animal gut, rumen, rice, and paddies. Besides the above habitats, the human gut, oral cavity, deep anoxic wounds, and lung and brain abscesses are prime habitats of anaerobes. The oxygen sensitivity of these microbes is the foremost concern during sampling, transportation of samples, cultivation, isolation, purification, preservation, and handling of obligate anaerobes. Minimum oxygen exposure of the sample during these processes should be the best strategy. Hungate's technique is the best pre-reduced media preparation method for cultivating obligate anaerobes. The Hungate method employs boiling and bubbling the media to expel dissolved oxygen and make the media component anoxic. Boiling removes the dissolved oxygen and bubbling the medium with inert anoxic gases like nitrogen, CO_2, or a mixture of nitrogen and CO_2 (80:20 v/v) is the recommended practice. Adding reducing agents in media assists in removing the remaining dissolved O_2. Cysteine hydrochloride and sodium sulfide are the commonly used reducing agents in the culture medium. Avoid adding heat-labile media components like amino acids, vitamins, and antibiotics during boiling and autoclaving. Initially, it was believed that the absence of oxygen was not essential for certain groups of microorganisms like methanogens and obligate anaerobic fungi. However, they need specific redox potential to grow. In recent studies, this concept has changed, and it

is considered that the redox equivalent plays an essential role in anaerobic cultivation.

Redox indicator dye indicates the level of redox potential of the medium. Resazurin is the most common redox indicator dye used in anaerobic cultivation medium. Its colourless stage (redox potential around -150 to 300 mV) indicates the ideal stage for inoculation. In order to avoid toxicity, 1 mg/litre (1 ppm) of resazurin in the culture medium is considered ideal. The composition of the medium plays a vital role in cultivating microorganisms. Therefore, knowledge of microbial nutrition and metabolism is essential. Consideration should be given to the type of metabolism (anaerobic respiration or fermentation) organisms use for growth and colony formation. If they generate energy using anaerobic respiration, then the appropriate source of alternative electron acceptors should be used in the medium to promote its growth. In the fermentative type of metabolism, no alternative source of electron acceptors is required, and the organism generates its energy requirement (ATP) using substrate-level phosphorylation. Buffer is another critical component for cultivation and physiological characterization. Using inorganic buffers in culture medium is a common practice, but sometimes it exerts toxic effects on cells; therefore, it is always recommended to use biological buffers or Good's Buffers during cultivation and physiological studies.

Bicarbonate buffer is suitable for physiological studies (pH 7.2 to 7.4). During colony pickup and purification/isolation, the plate should be incubated for 24 hours inside an anaerobic chamber. Perform all the activity inside the chamber. If the chamber is unavailable, avoid extra exposure of the plates for not more than 15-20 minutes with environmental oxygen. Alternatively, transferring and opening a gas jar inside the anaerobic chamber is the best practice for isolating obligate anaerobes. Alternatively, the roll tube or roll bottle technique also helps to isolate anaerobes under suitable conditions. The isolated colonies can be transferred to liquid broth using an anaerobic chamber or a gassing manifold under a positive pressure of sterilized anoxic gas in the roll tube or bottle plate.

Challenges of Anaerobes Cultivation

Tools of Anaerobes Cultivation

Unlike aerobes, anaerobes need special care during cultivation and handling. The removal of dissolved oxygen from media components and water and precautions to prevent external environmental oxygen from entering the cultivation environment are essential. Several methods and tools have been invented to handle the anaerobes with minimum exposure to oxygen. Cultivation techniques for anaerobes vary in detail among laboratories as per the requirement. Traditional anaerobic techniques, introduced by Hungate and further refined by Bryant, Miller, and Wolin, involve the preparation of pre-reduced media to create anoxic conditions for cultivation (Hungate 1950, 1-49; Hungate 1969, 117-132; Bryant 1972, 1324–1328; Miller and Wolin 1974, 985-987).

Further, due to the development of procedures where anaerobes were cultured under a pressurized atmosphere, contamination or loss of reducing potential was eliminated (Balch and Wolfe 1976, 781–791; Holdeman and Moore 1977; Balch and Wolf 1979, 260–296). The detailed methodologies for anaerobic cultivation can be found in literature sources such as those by Sowers and Noll (Sowers and Noll 1995, 15–48). Essential tools for anaerobic cultivation include gas workstations or gassing manifolds for preparing pre-reduced media and anaerobic chambers, also known as anaerobic glove boxes, which have revolutionized anaerobic microbiology by simplifying the handling of obligate anaerobes (Fig.1).

GasPak or anaerobic gas jars (Fig.2), as well as anoxomat or anaerobic gas jar systems, facilitate sample transportation and plate incubation at different temperatures, enabling the cultivation of facultative anaerobes, aerotolerant organisms, microaerophiles, and capnophiles. Additionally, small tools such as crimpers, decappers, butyl stoppers, aluminium crimps, serum vials of various capacities, Hungate tubes, Balch tubes, hypodermic needles, and syringes are essential for cultivation, isolation, and transfer of anaerobic cultures (Fig.3).

For further in-depth discussions on tools and techniques in anaerobic microbiology laboratories, readers are referred to "Anaerobes and Anaerobic Processes" (CRC-Press), edited by Om Prakash and DR Ranade.

Fig. 1: Anaerobic Chamber / Anaerobic glove box used for cultivation and handling of anaerobes.

Fig. 2: Anaerobic gas jar system used to create an anaerobic and microaerophilic environment in Gas-Jar.

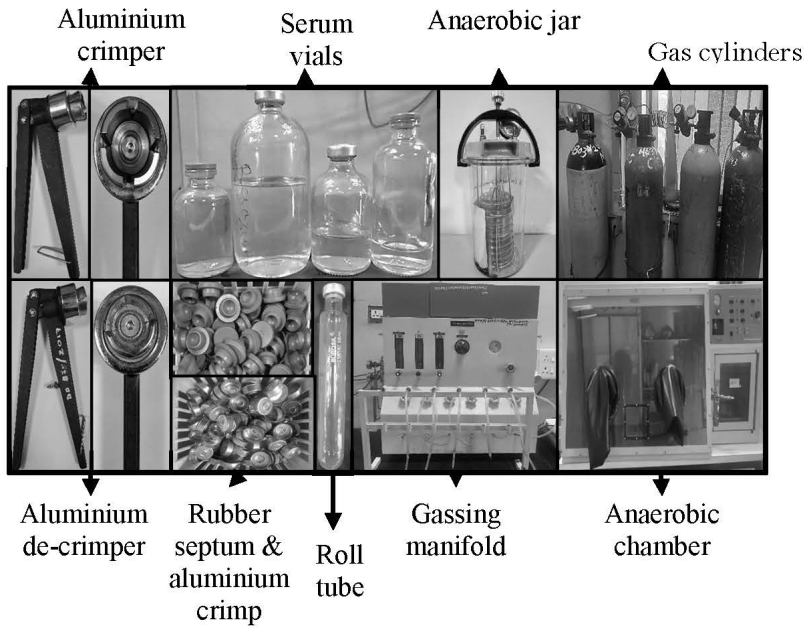

Fig. 3: Images of common tools used in anaerobic microbiology laboratories.

Usage of Oxygen Scrubbers and Reducing Agents

Certain anaerobes, like Methanogenic Archaea, are known to be very sensitive to oxygen and require a reduced environment for cultivation. Hence, scrubbing the traces of oxygen using a suitable scrubber from commercially available compressed gases is mandatory to cultivate such anaerobes. A gassing manifold or gas workstation used for pre-reduced media preparation can be equipped with a suitable oxygen scrubber to remove traces of oxygen. Generally, anoxic gases like Nitrogen (N_2) and CO_2, a mixture of 80% N_2 and 20% CO_2 (N_2: CO_2) or 80% H_2 and 20% CO_2 (H_2: CO_2), are used for flushing the medium and generating an oxygen-free environment. Before use, these gases pass through a gassing manifold, which acts as an inexpensive oxygen scrubber. It includes a heating glass column containing reduced-copper filings heated with electric heating tape connected to a variable transformer. Gas pressures (maximum 2 atm. or 2 kg/cm2) inside the column can be controlled using rotameters equipped with the gassing manifold. Therefore, constructing a sturdy gas-tight system with copper or stainless-steel tubing and connectors

is essential. The oxidized copper filings are reduced by slowly passing H_2 or H_2: CO_2 through the hot filings and recharging for media preparation. The oxygen-free gas is distributed through 5 to 6 outlet probes extending from the manifold using silicon tubing (one end of the tubing is connected to the manifold; another is connected to the gassing jet. The gassing jets are fabricated using a 3-cc glass Leur-Lock syringe filled with glass wool /non-absorbent cotton and are further connected with an 18-gauge (G) SS needle 12 cm long at the Leur-Lock end.

Precaution During Sampling and Cultivation

Selection of suitable samples, appropriate sampling, and transportation of samples are prerequisites for cultivating and isolating desired obligate anaerobes. It is recommended that the sample from oxygen-free environments like deep-aquatic-sediments, human faeces, faecal matter of animals, anaerobic digestor effluent, and clinical samples (Mehta and Lanjekar, Deshmukh., 2021, 100864; Lanjekar and Ranade 2015, 4749 – 4756; Ayesha and Sobia, 2022,145), should be collected aseptically in a sterile, nitrogen-flushed container or serum vial or sterile pack bags. The sample should be filled up to the brim of the container to minimize oxygen exposure. During transportation, to maintain the sample's temperature, it should be placed in ice packs until it is brought to the laboratory, and in the laboratory, it should be flushed with oxygen-free nitrogen to remove traces of oxygen and, if possible, immediately used for cultivation or enrichment. Commercially available buffers and oxygen-free transport media can also be utilized. Minimizing oxygen exposure from sampling to handling or serial dilution preparation is essential. Given the significance of sampling, transportation, and storage, a separate chapter dedicated to this theme is included in this book.

Preparation of Anoxic Medium

Anaerobes primarily utilize fermentation and anaerobic respiration for growth and reproduction. While most obligate anaerobes rely on fermentation for energy generation and do not require alternative electron acceptors, microorganisms such as denitrifiers, sulfate reducers, metal reducers, and methanogens need alternative electron acceptors to thrive in oxygen-free habitats. These fastidious anaerobes often require trace elements and vitamins to support metabolic pathways.

Several different kinds of media like Minimal media (Hungate, 1950,1-49; Miller and Wolin 1974, 985-987), Bi-Carbonate Yeast Extract Trypticase (BCYT (Touzeland Albagnac 1983, 241-245), or Mineral Salt (MS) (Hi et al., 1999), or Bi-carbonate Yeast Extract (BY) (Makkar and McSweeney, 2005) are commonly used media for cultivation of anaerobes. These are only a limited number of media, but researchers can select and expand the window based on the requirement; they can modify the media components to mimic the actual conditions. Also, if one wants to cultivate gut microbiota, one can use Peptone Yeast Extract Glucose (PYG) and Brain Heart Infusion (BHI) media (Holdeman and Moore 1977; Lanjekar 2014, 2250-2256). All the required heat-labile ingredients are initially dissolved in distilled water during media preparation. The medium is boiled and then cooled under the stream of oxygen-free nitrogen using a gassing manifold to ensure that the medium is oxygen-free. One millilitre of resazurin stock solution (1g/L) is added to the medium in one litre. Resazurin is one of the most sensitive redox indicators, indicating the medium's oxidation-reduction level. The medium with dissolved oxygen gives the blue colour to the indicator dye, and it gradually turns pink to colourless upon depletion of oxygen and complete reduction at neutral pH.

Another critical component of an anaerobic medium is the reducing agent. Only L-cysteine hydrochloride (1g/L) or a mixture of sodium sulfide (0.5g/L) and cysteine hydrochloride (0.5g/L) can be used to get the desired redox potential. A mixture of sodium sulfide and L-cysteine hydrochloride is generally preferred over cysteine hydrochloride for better results. Several other reducing agents are used in anaerobic medium, but it depends upon the nature of the microbes. The gas phase of the medium changes at this stage if it is different from nitrogen. The headspace gaseous phase is significant and plays a vital role in cultivation and enrichment. For example, gases such as specially calibrated mixtures of 80% N_2 and 20% CO_2 (N_2: CO_2) for acetoclastic and methylotrophic types of methanogens and 80% H_2 and 20% CO_2 (H_2: CO_2) for hydrogenotrophic variety of methanogens are used. The temperature of the medium is maintained using a serological water bath. If organisms use respiratory metabolism, the desired electron donor-acceptor combination can be added after cooling the medium from the prepared anoxic stocks. The pH of the medium should be adjusted using 1 N HCl/NaOH when the gas phase is only N2, and 1N HCl/sodium bicarbonate (10% w/v) should be used when only CO_2 or N_2: CO_2 or H_2: CO_2 is used as the gas phase. As mentioned, one should prefer biological buffers over inorganic buffers for better results. After preparation, the medium should be dispensed in the desired vessel, like serum vials, Hungate-tube, or Baltch-tube, using strict anoxic