International Journal of Technical Research and Applications e-ISSN: 2320-8163,
www.ijtra.com Volume 4, Issue 4 (July-Aug, 2016), PP. 86-93

Alternative Growth Media for Qualitative Analysis of General Aerobic Bacteria Counts at Specific PH Range

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Abstract— **An alternative Growth Media for Qualitative analysis of general aerobic bacteria counts (GAB) was determined and counterchecked with the ready-made media from HACH company Paddle testers are double-sided slides to proof the ability to use the Total Coliform media for two day at 35°C for qualitative determination of the GAB colonies in PH range 5.5-7.5 because both are Gram negative bacteria, several trials done using positive source of GAB and Coliform with blanks and all the results were accepted. However, as described by Kip and van Veen, microbial enhancement/inhibition of corrosion of metals take place through complex reactions and the mechanism of these processes is not well understood. Some microbes, particularly bacteria, colonize the metal surface forming thick non-corroding biofilms made of secreted Extracellular Polymeric Substances (EPS).**

Index terms- **Aerobic Bacteria, Coliform,Corrosion, Produced Water, Wash water.**

I. INTRODUCTION

Physical and Environmental Requirements for Microbial Growth

The procaryotes exist in nature under an enormous range of physical conditions such as O2 concentration, Hydrogen ion concentration (pH) and temperature. The exclusion limits of life on the planet, with regard to environmental parameters, are always set by some microorganism, most often a procaryote, and frequently an Archaeon. Applied to all microorganisms is a vocabulary of terms used to describe their growth (ability to grow) within a range of physical conditions. A thermophile grows at high temperatures, an acidiphile grows at low pH, an osmophile grows at high solute concentration, and so on. This nomenclature will be employed in this section to describe the response of the procaryotes to a variety of physical conditions. [1]

The Effect of Oxygen; Oxygen is a universal component of cells and is always provided in large amounts by H2O. However, procaryotes display a wide range of responses to molecular oxygen O2 (Table 1).[2]

Obligate aerobes require O2 for growth; they use O2 as a final electron acceptor in aerobic respiration.

Obligate anaerobes (occasionally called aerophobes) do not need or use O2 as a nutrient. In fact, O2 is a toxic substance, which either kills or inhibits their growth. Obligate anaerobic procaryotes may live by fermentation, anaerobic respiration, bacterial photosynthesis, or the novel process of methanogenesis.[3]

Facultative anaerobes (or facultative aerobes) are organisms that can switch between aerobic and anaerobic types of metabolism. Under anaerobic conditions (no O2) they grow by fermentation or anaerobic respiration, but in the presence of O2 they switch to aerobic respiration.[4]

Aerotolerant anaerobes are bacteria with an exclusively anaerobic (fermentative) type of metabolism but they are insensitive to the presence of O2. They live by fermentation alone whether or not O2 is present in their environment.

Table 1 Terms used to describe O2 Relations of Microorganisms

Group	Aerobic	Anaerobic	O_2 Effect
Obligate Aerobe	Growth	No growth	Required (utilized for aerobic respiration)
Microaerophile	Growth if level not too high	No growth	Required but at levels below 0.2 atm
Obligate Anaerobe	No growth	Growth Toxic	
Facultative Anaerobe (Facultative Aerobe)	Growth	Growth	Not required for growth but utilized when available
Aerotolerant Anaerobe	Growth	Growth	Not required and not utilized

The response of an organism to O2 in its environment depends upon the occurrence and distribution of various enzymes which react with O2 and various oxygen radicals that are invariably generated by cells in the presence of O2. All cells contain enzymes capable of reacting with O2.[5] For example, oxidations of flavoproteins by O2 invariably result in the

formation of H2O2 (peroxide) as one major product and small quantities of an even more toxic free radical, superoxide or O2.-. Also, chlorophyll and other pigments in cells can react with O2 in the presence of light and generate singlet oxygen, another radical form of oxygen which is a potent oxidizing agent in biological systems.[6]

All photosynthetic (and some non-photosynthetic) organisms are protected from lethal oxidations of singlet oxygen by their possession of carotenoid pigments which physically react with the singlet oxygen radical and lower it to its nontoxic "ground" (triplet) state. Carotenoids are said to "quench" singlet oxygen radicals.

The Effect of pH on Growth

The pH, or hydrogen ion concentration, [H+], of natural environments varies from about 0.5 in the most acidic soils to about 10.5 in the most alkaline lakes. Appreciating that pH is measured on a logarithmic scale, the [H+] of natural environments varies over a billion-fold and some microorganisms are living at the extremes, as well as every point between the extremes! Most free-living procaryotes can grow over a range of 3 pH units, about a thousand fold change in [H+]. The range of pH over which an organism grows is defined by three cardinal points: the minimum pH, below which the organism cannot grow, the maximum pH, above which the organism cannot grow, and the optimum pH, at which the organism grows best.[6] For most bacteria there is an orderly increase in growth rate between the minimum and the optimum and a corresponding orderly decrease in growth rate between the optimum and the maximum pH, reflecting the general effect of changing [H+] on the rates of enzymatic reaction (Figure 4).[7]

Microorganisms which grow at an optimum pH well below neutrality (7.0) are called acidophiles. Those which grow best at neutral pH are called neutrophiles and those that grow best under alkaline conditions are called alkaliphiles. Obligate acidophiles, such as some Thiobacillus species, actually require a low pH for growth since their membranes dissolve and the cells lyse at neutrality. Several genera of Archaea, including Sulfolobus and Thermoplasma, are obligate acidophiles. Among eukaryotes, many fungi are acidophiles, but the champion of growth at low pH is the eucaryotic alga Cyanidium which can grow at a pH of 0. In the construction and use of culture media, one must always consider the optimum pH for growth of a desired organism and incorporate buffers in order to maintain the pH of the medium in the changing milieu of bacterial waste products that accumulate during growth. Many pathogenic bacteria exhibit a relatively narrow range of pH over which they will grow. Most diagnostic media for the growth and identification of human pathogens have a pH near 7.[8]

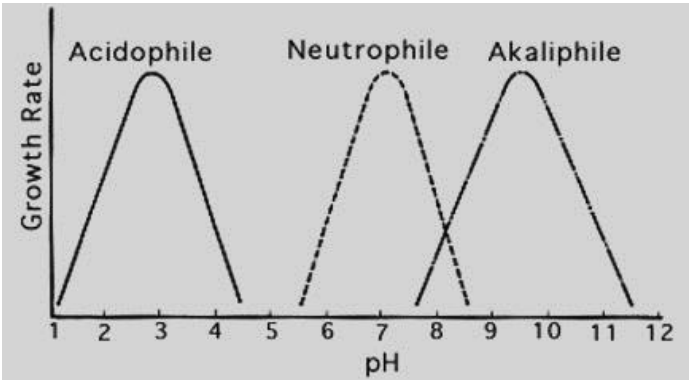

Figure 1 Growth rate vs pH for three environmental classes of procaryotes. Most free-living bacteria grow over a pH range of about three units. Note the symmetry of the curves below and above the optimum pH for growth.

Table 2 Minimum, maximum and optimum pH for growth of certain procaryotes.

Organism	Minimum pH	Optimum pH	Maximum pH
Thiobacillus thiooxidans	0.5	2.0-2.8	4.0-6.0
Sulfolobus acidocaldarius	1.0	2.0-3.0	5.0
Bacillus acidocaldarius	2.0	4.0	6.0
Zymomonas lindneri	3.5	5.5-6.0	7.5
Lactobacillus acidophilus	4.0-4.6	5.8-6.6	6.8
Staphylococcus aureus	4.2	7.0-7.5	9.3
Escherichia coli	4.4	6.0-7.0	9.0
Clostridium sporogenes	5.0-5.8	6.0-7.6	8.5-9.0
Erwinia caratovora	5.6	7.1	9.3
Pseudomonas aeruginosa	5.6	6.6-7.0	8.0
Thiobacillus novellus	5.7	7.0	9.0
Streptococcus pneumoniae	6.5	7.8	8.3
Nitrobacter sp	6.6	7.6-8.6	10.0

Gram stain and bacterial morphology:

Of all the different classification systems, the Gram stain has withstood the test of time. Discovered by H.C. Gram in 1884 it remains an important and useful technique to this day. It allows a large proportion of clinically important bacteria to be classified as either Gram positive or negative based on their morphology and differential staining properties. Slides are sequentially stained with crystal violet, iodine, then destained with alcohol and counter-stained with safranin.[9] Gram

positive bacteria stain blue-purple and Gram negative bacteria stain red. The difference between the two groups is believed to be due to a much larger peptidoglycan (cell wall) in Gram positives. As a result the iodine and crystal violet precipitate in the thickened cell wall and are not eluted by alcohol in contrast with the Gram negatives

where the crystal violet is readily eluted from the bacteria. As a result bacteria can be distinguished based on their morphology and staining properties.[10]

Some bacteria such as mycobacteria (the cause of tuberculosis) are not reliably stained due to the large lipid content of the peptidoglycan. Alternative staining techniques (Kinyoun or acid fast stain) are therefore used that take advantage of the resistance to destaining after lengthier initial staining.

Growth Requirements:

Microorganisms can be grouped on the basis of their need for oxygen to grow. Facultatively anaerobic bacteria can grow in high oxygen or low oxygen content and are among the more versatile bacteria. In contrast, strictly anaerobic bacteria grow only in conditions where there is minimal or no oxygen present in the environment. Bacteria such as bacteroides found in the large bowel are examples of anaerobes. Strict aerobes only grow in the presence of significant quantities of oxygen.[11]

Pseudomonas aeruginosa, an opportunistic pathogen, is an example of a strict aerobe.

Microaerophilic bacteria grow under conditions of reduced oxygen and sometimes also require increased levels of carbon dioxide. Neisseria species(e.g.,the cause of gonorrhea) are examples of micraerophilic bacteria.

Biochemical reactions:

Clinical microbiology laboratories typically will identify a pathogen in a clinical sample, purify the microorganism by plating a single colony of the microorganism on a separate plate, and then perform a series of biochemical studies that will identify the bacterial species.

Serologic systems:

Selected antisera can be used to classify different bacterial species. This may be based on either carbohydrate or protein antigens from the bacterial cell wall or the capsular polysaccharide.

(Group A streptococcal M proteins or O and H polysaccharide antigens of salmonella).

Environmental Reservoirs:

When considering likely pathogens it is also important to know which of the different species are found in different locations. Environmental reservoirs are generally divided into those that are endogenous (i.e., on or within the human body) and exogenous (somewhere in the environment). When considering the likely cause of an infection the likely source of the infection is important in your differential diagnosis. For example, an anaerobic organism resident in the large bowel is the likely cause of an abdominal abscess that develops after large bowel surgery. A skin rash developing in a hiker with a history of multiple tick bites is more likely to be borrelia, the agent of Lyme disease. An outbreak of food poisoning traced to imported unpasteurized cheese might be due to listeria.[12]

Endogenous reservoirs account for a large proportion of human infections. Many parts of the body have their own normal flora. S. epidermidis is found on the skin. Viridans streptococci are a part of the normal oropharyngeal flora and S. aureus is a commensal of the anterior nares.

II. MOLECULAR MECHANISM OF MICROBE-METAL INTERACTION

Bacteria metabolize the substrates via diverse mechanisms, depending up on the sources of energy, carbon and reducing equivalents available in their habitat. These substrates then undergo degradationinside the cell through a series of well-coordinated metabolic pathways. Usually, the final metabolic pathway inside the cell is Electron Transport Chain, wherein, the electrons are transmittedthrough a series of proteinaceous redox complexes situated along the cell membrane for thesynthesis of ATP. These electrons are then finally transferred to a terminal electron acceptor outside the cell, preferably oxygen (aerobic respiration), due to its high reduction potential. Bacteria transfer electrons to other electron acceptors with lower reduction potentials in oxygen deficient habitats (anaerobic respiration), and the respiration process becomes slower. In oxic-anoxic belts, they exist as facultative anaerobes that can alternate between aerobic and anaerobic respiration depending upon the availability of oxygen. Majority of bacteria inhabiting such environments belong to the group of chemolithotrophs that use inorganic sources for energy, such as Nitrifying bacteria, S-oxidizing bacteria, S-reducing bacteria and Fe-oxidising bacteria. In these bacteria, evident terminal electron acceptors are metal salts.[13]

These interactions result in changes in surface topography, chemical environment and electrochemical characteristics of the metals as an apparent outcome of microbial metabolic activities.

In addition, bacteria release a number of metabolites including organic and inorganic acids, alcohols, aldehydes and nitrates on to the metal surfaces. These metabolites may either be detrimental or beneficial for metals. [14]

III. BIOCHEMISTRY OF MICROBIALLY INDUCED CORROSION (MIC)

MIC is a complex process that happens when bacteria gain access to the metal surface and is governed by electrostatic, van der Waal's and Lewis acid-base interactions. In aerobic environment, oxygen acts as the terminal electron acceptor at the cathodic sites and form hydroxides. [15] This hydroxide layer protects the metal from further corrosion. Corrosive bacteria gain access to the metal surface either by damaging this protective layer or by forming a corrosive layer that prevents the formation of hydroxide coating. Bacteria corrode the metal surfaces by altering its chemical and electronic environment, and this process is dependent on the properties of metal surface and bacterial communities that are involved4. Different genera of bacteria exhibit different modes of corrosive action [16].

IV. MATERIALS AND METHODOLOGY:

I.Total Coliforms by Membrane Filtration Media Preparation and Testing (Scheme)

A. Apparatus and Materials

1. Incubator (35 OC)
2. Autoclave
3. Dry heat oven
4. Refrigerator
5. Water bath (44.5 OC)
6. pH meter
7. Balance
8. Binocular microscope (10x to 20x magnification)
9. Colony counter
10. Tally counter
11. Graduated cylinders, different sizes
12. Erlenmeyer flasks, different sizes
13. Petri dishes 50 x 10 mm tight-lid dishes
14. Thermometers, different ranges
15. Refilling dispense syringe, 5mL and 10mL sizes
16. Disposable pipettes, sterile and graduated
17. Flame Source
18. Sample bottles, autoclavable
19. Dilution bottles
20. Heating plate with magnetic stirrer
21. Millipore membrane filters, 47 mm diameter, 0.45 µ pore size
22. Ultraviolet UV sterilizers
23. Vacuum source
24. Glass filter holder
25. Filtering flask, 2 liter
26. 3-place manifold, stainless steel
27. Forceps, flat-tipped

B. Chemicals and Reagents

1. M-Endo agar
2. Phosphate buffer, potassium dihydrogen phosphate (KH2PO4)
3. Magnesium chloride (MgCl2)
4. Ethanol
5. Reagent water (filtered de-ionized water)
6. Sodium thiosulfate (Na2S2O3)
7. Sodium hydroxide (NaOH)

C. Culture Media and Reagents Preparation

1.m-Endo agar:
-Suspend 51 grams of the powder in 1-liter reagent water containing 20 mL ethanol and heat to boiling to dissolve completely. Cool to 45-50 OC. dispense 4 mL amounts into the lower halves of the petri dishes and allow to solidify.
2.Phosphate Rinse Buffer:
-Stock Solution I:
Dissolve 34.0 grams of potassium di-hydrogen phosphate (KH2PO4) in 500 mL of reagent water. Adjust the pH to 7.2 with 1N NaOH. Dilute to 1000 mL with reagent grade water to produce 1 liter of stock solution. Refrigerate stock buffer. Discard if it becomes turbid.
-Stock Solution II:
Dissolve 38 grams of magnesium chloride (MgCl2) in 1 liter of reagent grade water
-Working Solution:
Add together 1.25 mL of Stock solution I and 5.0 mL of Stock solution II and dilute to 1 liter with reagent grade water.
-Dispense the buffer into 100 mL glass or autoclavable plastic bottles. Loosely cap or cover and autoclave at 121 OC, 15 psi for 15 minutes (100 mL or less) or 30 minutes (500 mL to 1 liter)

D. Procedure and Analysis

The size of the sample to be filtered depends on the expected bacterial density. Use sample volumes that will yield about 50 coliform colonies and not more than 200 colonies of all types on 47mm membrane surface. For drinking water samples, 100 to 1000 mL volume can be filtered depending on the turbidity and the non-coliform background bacteria. Other waters are analyzed by filtering three different volumes (diluted or undiluted) depending on the expected bacteria density.
1.Prepare the filtration apparatus by connecting the filtration manifold to the vacuum source and filtrate disposal container. Sterilized the filter holder, support bases and funnels for 3 – 4 minutes in the UV sterilizer.
2.Load a sterile membrane filter, grid side up, evenly centered onto the filter holder support base, and then clamp the funnel into position. Handle the membrane with smooth-tipped forceps that have been dipped into alcohol, then quickly flamed to sterilize the tips.
3.Mix the water sample by vigorously shaking for several seconds. For samples larger than 20 mL, pour the sample directly into funnel. For samples less than 20 mL, first pour 20-30mL of sterile buffer into the funnel then pipette the sample into the buffer.
4.Apply vacuum to filter the sample. Rinse the funnel walls with at least 30 mL of sterile buffer and draw rinse buffer through the filter.
5.Turn off the vacuum and lift off the filter holder funnel. Remove the membrane using flame-sterilized forceps and transfer it immediately to the previously prepared m-Endo agar dish, with a rolling motion to avoid entrapment of air under the filter as this will prevent the nutrient media from reaching all of the membrane surfaces.
6.Incubate the inverted dishes at 35 OC for 24 hours.

E. Reading the Results

To view the dishes, use the binocular wide-field microscope with a cool white fluorescent light source directed from above with rays as nearly perpendicular as possible to the plane of the filter. Count the typical coliform colonies which have pink to dark red color with metallic surface sheen. The sheen area may vary in size from a small pinhead to complete coverage of the colony surface. A typical coliform colony can be dark red or nucleated without sheen. Colonies that lack sheen may be pink, red white, or colorless and considered to be non-coliform.
II.General Aerobic Bacteria Media Preparation and Testing (Scheme)

Apparatus and Materials
1.Autoclave
2.pH meter

3. Rubber stopper
4. Aluminum seal
5. Crimping tool
6. Sterile 3 mL disposable syringes
7. Serum vials, 10 mL

Chemicals and Reagents

1. Source water
2. Bacto-peptone
3. Yeast Extract

Procedure and Analysis

1. Filter the source water through a 0.45 µ membrane filter.
2.Dissolve 0.5 gram Bacto-peptone and 0.5 gram yeast extract in a liter of field water.
3.After mixing, check the pH and adjust to 7.0 – 7.4, if necessary, using diluted sodium hydroxide or hydrochloric acid.
4.Using an autoclave dispenser, dispense exactly 9 mL of medium into each serum vial.
5.Cap and seal the vials with rubber stoppers and aluminum seals. Crimp each seal with a crimping tool.
6.Sterilize in the autoclave for 15 minutes at 121 OC (15 psi).
7.After autoclaving, check the pH of the medium using 2 randomly selected vials. The final pH should
be 7.0 – 7.4.
8.Check the vials to see if the medium remains clear after autoclaving. Depending on the source of the water, some GAB media may become slightly turbid when autoclaved. Consequently, results may be difficult to read since a positive result (growth) is scored based on the development of turbidity in the medium.
9.If the medium becomes sufficiently turbid after autoclaving, so that the reading of results becomes difficult, the medium preparation should be repeated using field water diluted with distilled water by 50 % or 75 % (1:1 to 1:3 V/V) until the medium becomes clear after autoclaving. Alternatively, a blank medium vial (sterile, no growth) should be incubated along with the inoculated vials to use as a reference for comparison.

V. RESULTS & DISCUSSION:

As both General Aerobic bacteria and Coliforms derived from Gram Negative bacteria so they might have some similar properties and we can utilize one media to determine the other one in case of Qualitative and urgent analysis if the required chemicals for media preparation are missing.

the main factor is PH as per our process stream in the utility and the GOSEP (Gas Oil Separation Plant) units the PH range in the produced water and Wash water is 5-8. So we can focus on the Aerobic bacteria species that can growth in the optimum PH in the same range as per Table (4)

we can differentiate between the presence of the Coliforms colonies because they will be Shiny colonies while the Aerobic bacteria not and the Aerobic bacteria start to growth and multiply in the second day after the incubation while the coliforms can multiply within 24 hours only.

Several trials done on different water sources (produced water, wash water and oily water) in Safaniya and Tanajib areas to see the availability of using the Coliform and HPC media for GAB qualitative analysis.

Water sources determined based on the sample points history of presence or absence of the general aerobic bacteria from the quality and the corrosion department.

Same source of water analysed for blank using three different media (GAB, Coliform and HPC) and the results shows Nil.

Positive aerobic bacteria results sample point was selected to do the experiment based on the PH range (5.5-7.5) and the results shows the growth of the GAB in the three medias with 2% different in the final results which is neglect able due to the colonies formation.

Special bacteria that mentioned in Table 4 was injected separately in Coliform media in the.

Organism	**Minimum pH**	**Optimum pH**	**Maximum pH**
Zymomonas lindneri	3.5	5.5-6.0	7.5
Escherichia coli	4.4	6.0-7.0	9.0
Erwinia caratovora	5.6	7.1	9.3
Pseudomonas aeruginosa	5.6	6.6-7.0	8.0
Thiobacillus novellus	5.7	7.0	9.0
Nitrobacter sp	6.6	7.6-8.6	10.0

Figure 2 GAB Counts in Coliform Media after 48 Hours

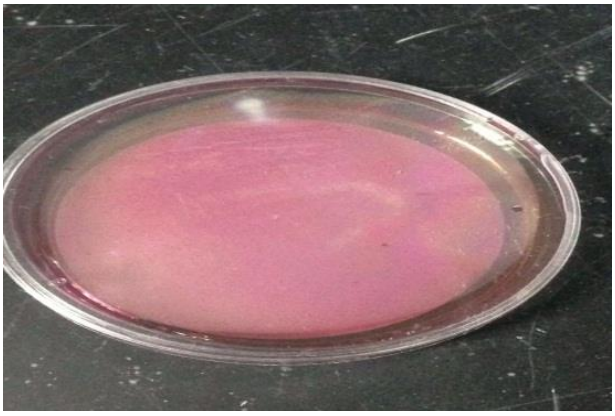

Figure 1 GAB Counts in Coliform Media after 48 Hours

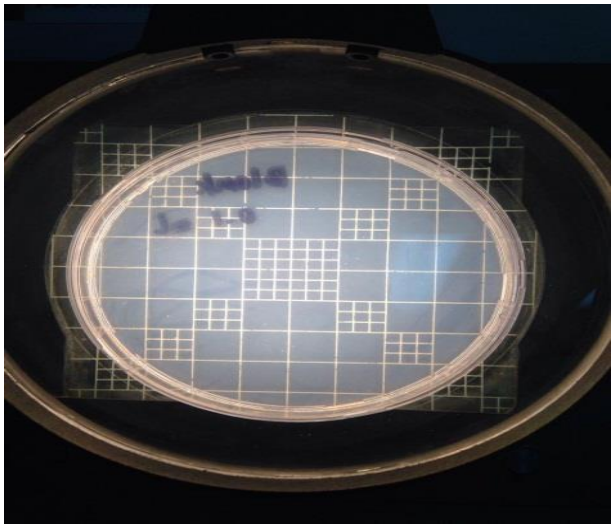

Figure 4 Blank Sample in Heterotrophic plat count media

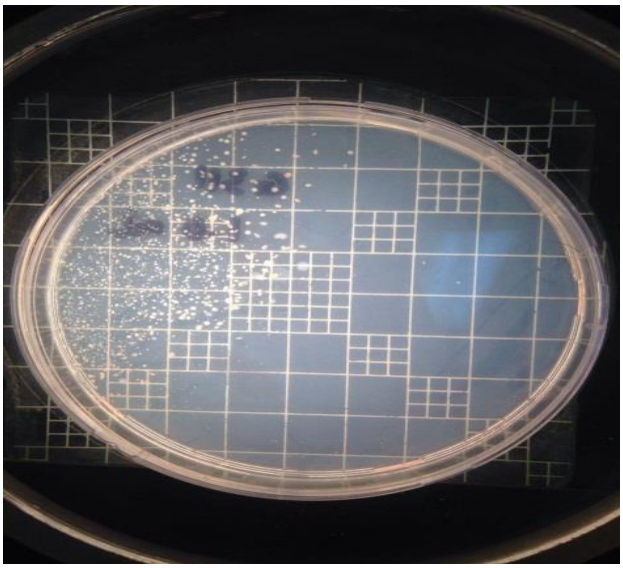

Figure 5 GAB Colonies in Hetero Trophic Plat counts media after 48 Hours

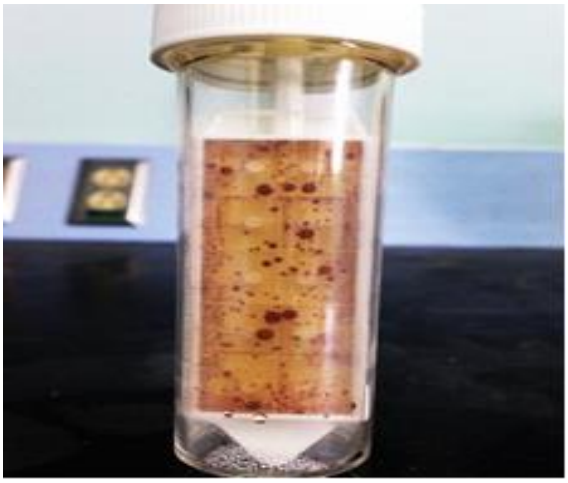

Figure 6 Blank Sample for GAB in GAB Rapid Kit after 48 hours

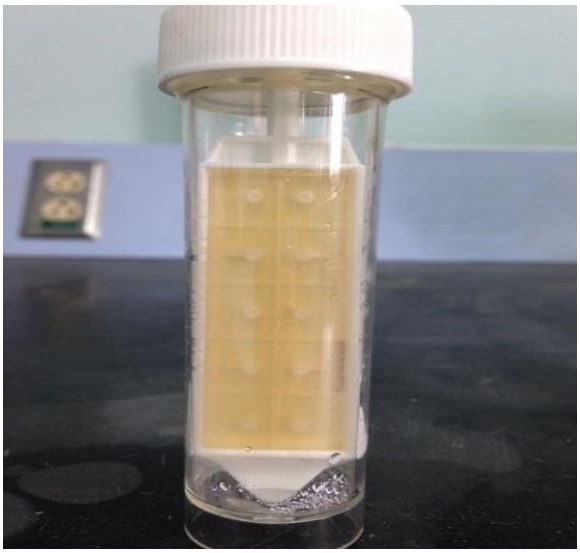

Figure 7 GAB Counts in Coliform Media after 48 Hours

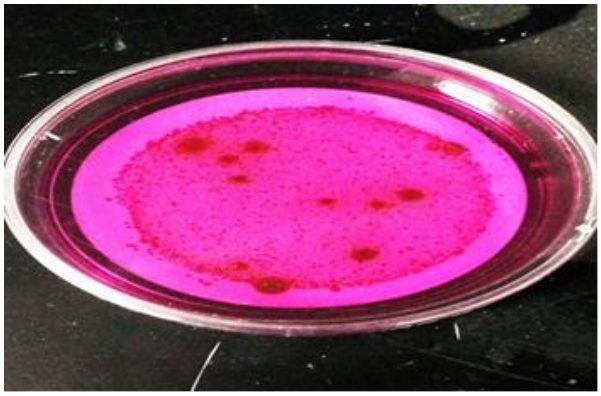

Figure 8 GAB & Coliform Counts in Coliform Media after 48 Hours

Table 3 Clearing Zone Inhibition of Gram Negative Bacteria with Coliform and HPC media

compounds	Clearing Zone inhibition (m.m)	
	Coliform Media	HPC Media
Zymomonas lindneri	-	-
Escherichia coli	-	-
Erwinia caratovora	-	0.1
Pseudomonas aeruginosa	-	-
Thiobacillus novellus	-	0.5
Nitrobacter sp	-	-

By seeing the above pictures and the final results of the colonies count we can utilize one of the available media to determine the GAB presence qualitatively because if we u

For first picture in (figure 1) it shows dark pink colonies aggregates after 48 hours which indicating the presence of Gram negative bacteria (General Aerobic bacteria) which are not coliforms colonies because Coliform colonies will be shiny grains.

The second picture in (figure 2) shows the absence of any bacteria aggregate by using the wash water source which doesn't have GAB colonies which already have history of absence of bacteria due to the good chemical injection and monitoring.

From (figure 3) showing the blank wash water which used with HPC media and shows no growth of the bacteria.

(Figure 4) showing the growth of the gram negative bacteria which indicate the GAB colonies.

(Figures 5 & 6) showing the test colonies growth of the general aerobic bacteria using the ready-made media for GAB testing and the blank for the same wash water sample.

We selected one sample point which having both GAB and coliforms to check if it possible to differentiate between them or some interference will occur.

After the first 24 hours Shiny Colonies formed with small amount of other dark pink colonies as per the picture in (figure 7) while after another 24 hours the huge number of the dark pink colonies (GAB) formed.

To proof the availability to use the Coliform media to determine the GAB colonies we requester the Microbiology Laboratory in Faculty of Science in Al-Azhar University, Cairo.

They have injected each kind of bacteria separately in both media that prepared in our Lab for Coliform and HPC counts.

The results shows that there is growth of the bacteria in the media after 48 hours at 35 °C and there is no any inhibition towards the bacteria except little inhibition for Erwinia caratovora & Thiobacillus novellus in HPC media.

VI. CONCLUSIONS

We can use either Heterotrophic plate count and Coliform medias for General aerobic bacteria Counts qualitatively in case of urgent requests or if we don't have one or more chemicals that required for media preparation, but the water source should be in PH range of 5.5-7.5 to determine the especial type of GAB such as Zymomonas lindneri, Escherichia coli, Erwinia caratovora, Pseudomonas aeruginosa , Thiobacillus novellus & Nitrobacter sp.

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