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Designing of Culture Media for Increased Recovery of Total Heterotrophs From Water Samples

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Abstract

Bacteriological examination of water is one of the most important areas of investigation because upto 80% of all sickness and diseases in the world are water related. Heterotrophic bacteria are the least studied indicator for water quality determination. The present investigation encompasses two different approaches to estimate water quality.e.g a) to modify the conventional culture media for maximal recovery of heterotrophs and b) to assess the load of the total aerobic heterotrophs as an indicator from drinking and surface water. Six samples from different sources were examined with nine different media such as NA, MNA, PCA, PCA-6, SDA-8, R2A, SE, SOE, and SSE for comparative evaluation. Among them,SE,SOE and SSE are newly formulated media using soil extract, soybean extract and the combination of both of them respectively.SDA-8 was found to be superior in most cases in relation to its comparative efficiency.SSE medium was also found to be superior in case of two samples. The range of heterotrophic bacterial count on different media varied from 3.0 x104 cfu/100ml to 8.6x 105 cfu/100ml and 9.5x106 cfu/100ml to 7.5x107 cfu/100ml in case of drinking water and surface water respectively.

Key words: Heterotrophs, culture media, recovery, water sample

Introduction

The necessity of water in life can no way be denied. But the tiny and invisible microorganisms pose a serious threat to the safety of the world's drinking water including water for household, recreational and industrial use. Water, contaminated by microorganisms, particularly by the pathogenic ones, can become a growing peril with the potential to cause significant outbreaks of various types of infectious diseases[5].

The recognition that microbial contamination can be water borne has led to the development of methods for routine examination to ensure that water intended for human consumption is free from environmental pollution. These methods depend on the detection of organisms normally present in the faeces of humans and other warm-blooded animals as indicators of excremental pollution, as well as efficiency of water treatment and disinfections. The organisms most commonly used as primary bacterial indicators of fecal infection are the coliform group as a whole. Heterotrophic plate count (HPC) bacteria may sometimes be of value as a secondary indicator.

The heterotrophic group of bacteria encompass an extremely broad range of genera and these bacteria are defined by the medium utilized in their enumeration[3].Heterotrophic plate count is important because the large number of bacteria may suggest the presence of opportunistic pathogens of non faecal origin, potential interference with the detection of coliform bacteria and increased possibilities of taste, dour and corrosion problems in the distribution system. Several investigators have demonstrated the incidence and importance of heterotrophic bacteria in drinking water .Although the source of water

vary such as ground water, mineral water or tap water, certain genera of heterotrophs are frequently detected.Most of the heterotrophic bacteria in drinking and mineral water are autochthones flora such as **Acinetobacter spp, Aeromonas hydrophila ,Enterobacter spp, Hafnia spp and Pseudomonas sp**..Whether the autochthones flora has the potential for causing diseases is not clear. There is no doubt that some of the bacteria isolated from drinking water have the potential to cause diseases[2]**.**

Heterotrophic Plate count (HPC), previously known as Standard plate count has been employed to evaluate water quality and sanitation standard. This measure is an attempt to provide single values that express the number of aerobic and facultative anaerobic organisms in water sample[8].Regulatory agencies and environmental microbiologists have suggested that the heterotrophic bacterial count in finished drinking water should not exceed $5.0x\ 10^4$ cfu/100ml.

A high number of heterotrophic bacteria may interfere with the detection of Coliform bacteria. Total Coliform(TC) and HPC have been used for many years to assess the microbiological and sanitary quality of water. Although many investigators have examined the presence of either heterotrophs or TC isolated from drinking water treatment plants, few investigators have attempted to relate that.

Epidemiological studies of the relationship between HPC and TC have shown mixed results.In one of the first studies to examine the relationship between these two indicators ,Geldreich et al,1972 demonstrated that there appeared to be an increased occurrence of TC above an HPC count of 31/ml. Reilly and Kippin (1983) showed that HPC had no relationship with coliform numbers below 50 cfu/uml.When the counts exceeded 50 cfu/ml,TC number increased, although there were a few observations in this category.They concluded that low HPC count did not affect the frequency of total coliform recovery and that coliforms may become part of the endogenous flora of the distribution system and act like HPC,and that high or low HPC densities do not indicate either the presence or absence of coliforns.However,as the total bacterial population rises above 500 cfu/ml,it adversely affects detection of coliform drops to 5%.This supports a previous statement that standard plate count above 500cfu/ml adversely affects detection of coliform organisms.

Proper composition of growth medium enabling the recovery of appropriate or desired number of organisms is very difficult to formulate. The balancing of mass according to cell composition alone is only one aspect. Selecting the suitable form of natural salts and precursors are also important. Finally, assessment of the limiting components must be demonstrated. Organic carbon is utilized by heterotrophic bacteria for production of new cellular material and as an energy source. Most organic carbon in water supply is natural in origin and is derived from vegetation. These compounds may include humic and fulvic acid, polymeric carbohydrates, proteins and carboxylic acids.

Media presently in use have limitations to recover the maximum number of heterotrophs.Differences in bacterial populations in water supplies in different geographical area may result in a failure of a medium to produce higher plate counts than other media[8].Various studies on heterotrophs suggest that a low nutrient medium with easily utilizable substrate such as in semi defined medium is suitable for the recovery of heterotrophic organisms from potable water as an indicator of water quality and hygienic significance.

However, the recovery of microbes by the conventional microbiological culture system is limited and it mostly gives only 1-10% of the actual number of bacterial cells indigenously present in water owing to the property of viable but non culturable (VBNC) characteristics. Along with these new approaches, the conventional techniques should be carried out to develop a suitable medium for the recovery of the maximum number of heterotrophic organisms.

As compared to other indicators, there are few reports about the investigations on Total Heterotrophs (TH) as an indicator for drinking water quality. For the recovery of heterotrophic bacteria from potable water, various types of complex media

are presently used, which have limitations. Depending on media types, the total isolation/recovery rate of heterotrophs may greatly vary. Since the environmental bacteria are usually exposed to low concentration of nutrients, to avoid artifacts media having low nutrient concentration might recover more heterotrophs.Thus the present study investigates into following specific aims to design a suitable heterotrophic plate count(HPC) agar medium that allows the recovery of maximum number of heterotrophs as compared to the presently available recommended media and to determine the density of total heterotrophic bacteria present in drinking water supplied and in surface water.

Materials and Methods

Sampling Sites

Water samples were collected from the metropolitan city water distribution system. The sampling sites were Lalbag, Mirpur, University Residential area and Dhaka University campus. Water samples were also collected from Romna Lake, Shahidullah Hall pond and filtered water from Microbiology department.

Size of Sample

About 300 ml of water was collected for analysis from a single site.

Modification of conventional media

Many investigators have observed that conventional media contain excessive amount of organic nutrients. In piped water supply bacteria undergo nutrient starvation. So, on first isolation they are often sensitive to media with high nutrient concentration and therefore poorly recovered on standard laboratory media.

Plate Count Agar (PCA) and Modified Plate Count Agar (PCA-6)

Among the conventional media, plate count Agar (PCA) medium is widely used for the isolation of heterotrophic organisms from various sources of water. It appears that the concentration of tryptone (5.0g/l) and yeast extract (2.5 g/l) in PCA are higher which might impede the recovery process, since the organisms in water are never exposed to such high concentration of nutrients. So, attempts were taken to modify PCA particularly the concentration of tryptone and yeast extract 3.0g/l and 0/5 g/l respectively.

Nutrient Agar (NA) and Modified Nutrient Agar (MNA)

Nutrient agar (NA), a conventional medium used for isolation of heterotrophic organisms from water and other environmental sources also contains higher concentration of beef extract (3.0 g/l), peptone (5.0 g/l) and Sodium chloride (5.0 g/l). The exact composition of peptone and yeast-extract has not yet been fully known which may impose stress on the recovery of heterotrophs.Sodium Chloride also at high concentration may be toxic to some metabolically injured organisms. So attempts were taken to modify NA, particularly with the concentration of peptone, beef extract and Sodium chloride 1.5g/l, 1.0 g/l and 2.0 g/l respectively.

R2A

R2A is a low nutrient medium which has been developed for recovery of a higher number of heterotrophic bacteria.

Semi Defined Agar Medium-8(SDA-8)

SDA-8 is a semi-defined agar medium with low nutrient concentration. Depending on the minimal but adequate concentration, various attempts have been taken to develop media with organic and inorganic nutrients.A basal medium was designed with carbon and energy source, e.g mannitol and glucose which are utilized by various organisms in the environment. (**Table-1)**

Soil Extract Agar (SEA)

A medium was designed with carbon and energy source e.g mannitol and glucose which are easily utilized by various organisms in the environment. The concentration of inorganic nutrients was0.2(g/l) and 0.5(g/l) in case of NH_4Cl and K_2HPO_4 .protease peptone was added to enhance the growth of some fastidious organisms and opportunistic pathogens.

Though the exact composition of soil extract has not yet been known, it contains a lot of nutrients and growth factors for the recovery of heterotrophs. (**Table- 2)**

Soybean Extract (SOE) Agar Medium

Another medium was designed using Soybean extract in stead of soil extract. The concentration of other ingredients was as same as Soil Extract medium.

Soil and Soybean Extract (SSE) medium

Both soil extract (100ml/l) and soybean extract (100ml/l) were used to design a new medium for the maximum recovery. The concentration of other ingredients was the same as SE and SOE media.

Total Viable Bacterial Count

Selection of method

Drop plate method was employed for economy and accuracy**.**

Results

Heterotrophs recovery from various sources of water

Firstly, six media were selected for their relative efficiency to recover the maximum number of heterotrophs from various sources of water. The test media were PCA, PCA-6, NA, MNA, R2A and SDA-8, the details of which have been described earlier. Among them PCA and NA are conventional media used quite frequently. PCA-6 and MNA are the modified medium of PCA and NA. R2A is a low nutrient medium developed by Reasonar and Geldreich in 1985. SDA-8 is a semi defined medium newly formulated that yielded the maximum number of heterotrophs. Then another new semi defined media were designed using soil extract and soybean extract. These extracts contain a lot of nutrients for bacterial growth. The media formulated using soil extract was designated as SE media and the media formulated using soybean extract was designated as SOE media. Both soil extract and soybean extract were used in equal proportion to formulate a media, which was designated as SSE media.

Heterotrophs recovery from supplied drinking water

Water samples were collected from three areas of city water distribution system. These areas were Lalbag, Mirpur and Dhaka University residential area. Comparison in the mean numbers of colony forming unit recovered on the six medias revealed significant differences in the load of heterotrophic bacteria, on this medium highest count $8.6x10^5$ cfu/100 ml was recorded in Mirpur area and lowest count $2x10^5$ cfu/100 ml was found in Dhaka University residential area.(**Table-3)**

Heterotrophs recovery from surface water

Water samples were collected from ponds situated beside Shahidullah Hall and from Ramna Lake. The test media selected for pond water were PCA. PCA-6, NA, MNA, SDA-8 and R2A. But in case of water of Ramna Lake the newly formulated SE, SOE and SSE media were used with SDA-8, PCA-6 and R2A for the relative maximal recovery. (**Table-4,5)**

Heterotrophs recovery from filtered (by reverse osmosis) water

Filtered water was collected from the Microbiology Department of the Dhaka University. The test media for the recovery studies were PCA-6, SDA-8, R2A, SE, SOE and SSE. (**Table-5)**

Comparison among different media based on the recovery rate of HPC bacteria

In case of drinking water tested, SDA-8 medium recovered highest number of heterotrophs. The highest count on SDA-8 was 8.6 x 10^5 cfu/100 ml. R2A was also a good medium for the recovery of HPC bacteria. The highest count on R2A was 7.5 x 10^5 cfu/100 ml. Modified PCA-6 and MNA recovered a greater number of heterotrophs than conventional PCA and NA. Overall, the count was one log higher than the permissible load recommended by WHO which is 5.0 x 10^4cfu/100 ml.

In case of the pond water SSE recovered the highest number of heterotrophs. The highest count on SSE medium was 7.0 x 10^7 cfu/100 ml. SOE was better than SEA medium in relation to its efficiency. The count on SOE was 6.0 x 10^7 cfu/100 ml whereas on SEA the count was 5.0 x 10^7 cfu/100 ml.

So, it was evident from this investigation that SDA-8, a semi-synthetic medium with low but adequate nutrients and SSE with both soil extract and soybean extract were better than other media for the recovery of heterotrophic bacteria.

Discussion

In the present investigation, attempts were taken to modify the conventional culture media for an increased recovery of heterotrophs with minimal but adequate concentrations of nutrients. The media having potential to recover a higher number of heterotrophs were selected to evaluate their relative efficiency and to assess water quality for the incidence of total heterotrophs – a least studied indicator organism.

Several studies have shown not only different growth responses in the same dehydrated media from different manufactures, but also variations in growth responses between culture media lots from the same manufactures[5]. Peptone/tryptone is an important constituent in making complex media which exhibit differences in sodium, potassium, phosphate, trace metal (which may vary in the range of 100-1000 ppm), carbohydrate and vitamin contents due to biological variation in protein substrate and enzyme preparations. Heterotrophic organisms when grown on such complex media with high concentration of nutrients could pose stress on their recovery. Particularly injured cells will be specifically adversely affected, resulting in poor recovery. Excess nutrients may impose shock syndrome and result in a lower recovery.

Attempt was taken to modify the Plate Count Agar (PCA) and Nutrient agar (NA), which are most widely used to isolate heterotrophic organisms. Tryptone, peptone, yeast extract, beef extract and sodium chloride were used at low concentrations in modified media which resulted in improved recovery of heterotrophs.

Although complex media are used for isolation of micro-organisms from environmental samples, they do not provide consistent and reproducible result.This is mostly due to the unknown variables that are found in complex media components. Environmental conditions may have a profound effect upon the final results. Considering these, attempts have been taken to design a semi-defined medium for the maximal recovery of heterotrophic organisms. This medium contained minimal but adequate concentrations of added organic and inorganic nutrients.

To assess the recovery studies of heterotrophs, relative efficiency of the promising modified media were conducted with test media. Of these, the semi defined medium SDA-8 recovered the maximum number of viable heterotrophic bacteria. The growth of heterotrophs on conventional media was significantly lower than the modified SDA-8 with low nutrient concentrations. This is possible because a number of environmental bacteria are able to grow better in lower concentration of organic or inorganic nutrients[10]. Another low nutrient medium named R2A was also used and it gave satisfactory recovery.

Besides the six media tested, another three media such as SE, SOE and SSE were designed in this investigation using soil extract and soybean extract. These extracts contain a lot of water-soluble nutrients that support the maximum recovery of

heterotrophs. Among these three media SSE, which contain equal proportion of both soil and soybean extracts, recovered the maximum number of heterotrophs compared with SE and SOE media,

The incidence of total heterotrophs as an indicator of water pollution has varied significance and importance. Limitations of appropriate media, most often than not precludes the recovery and detection of target group of microbes. Hence, search is on to strike a suitable HPC medium that would give the maximal recovery with consistency. It is evident from this investigation that SDA-8, a semi synthetic media with low but adequate nutrients and SSE with both soil and soybean extracts have successfully been designed which consistently recovered significantly a higher number of total aerobic heterotrophs. This will certainly have an edge over the conventional media used for similar purposes.

It was also observed that most drinking water samples failed to meet internationally accepted limit set for total heterotrophs as an indicator. However, the water sample collected from the filter (reverse osmosis system) contained much lower HPC bacteria and was within the internationally accepted limit values.

Conclusion

Heterotrophic plate count (HPC), has been used as an indicator of water quality as the methods of isolation and identification of pathogenic bacteria are often complex, time consuming and expensive . This group of bacteria often includes opportunistic pathogens. So, efforts to reduce heterotrophs will reduce the potential danger to high-risk individuals in the population. The conventional HPC medium is fraught with discrepancies and needs attention for modifications.

In fact, media constraint appears to be one of the major impediments in relation to the maximal recovery of the heterotrophic bacteria. Media modifications with decreasing concentration of nutrients, compatible with the environmental concentrations should be attempted. Efforts should be taken to recover the stressed or injured organisms present in water which do not recover easily on conventional media and also to recover organisms which are “viable but non culturable” (VBNC).

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Table 1: Composition of Semi-defined Agar Medium-(SDA-8)

Ingredients	Amounts(g/l)
Mannitol	3.0
Glucose	2.0
Protease Peptone	0.5
NH_4Cl	0.5
K_2HPO_4	1.0
KH_2PO_4	0.75
$MgSO_4.7H_2O$	0.2
$CaCl_2.2H_2O$	0.02
$MnSO_4.H_2O$	0.01
$Fe(NH_4)_2(SO_4)_2$	0.005
Na-pyruvate	0.25
Yeast extract	0.5
Agar	20

Table 2: Composition of Soil Extract Agar (SEA) medium

Ingredients	Amounts(g/l)
Mannitol	5.0
Glucose	2.0
Protease Peptone	0.5
NH_4Cl	0.2
K_2HPO_4	0.5
Soil extract	200 ml
Agar	20

Table 3: Heterotrophic Plate count of drinking water

Sample site	Media(cfu/100ml)					
	PCA	PCA-6	NA	MNA	SDA-8	R2A
Lalbag	5.1×10^5	6.1×10^5	3.6×10^5	4.1×10^5	7.2×10^5	7.0×10^5
Mirpur	5.1×10^5	6.4×10^5	3.4×10^5	3.9×10^5	8.6×10^5	7.5×10^5
University	3.4×10^5	3.8×10^5	2.0×10^5	3.1×10^5	4.1×10^5	4.0×10^5

Table 4: Heterotrophic plate count of surface water:

Sample site	Media(cfu/100ml)					
	PCA	PCA-6	NA	MNA	SDA-8	R2A
Shahidullah hall Pond	9.5×10^6	1.0×10^7	4.5×10^6	6.5×10^6	1.4×10^7	1.2×10^7

Table 5: Heterotrophic plate count of both surface water and filtered water:

Sample site	Media(cfu/100ml)					
	SDA-8	PCA-6	R2A	SE	SOE	SSE
Ramna lake	4.5×10^7	5.0×10^7	7.5×10^6	5.0×10^7	6.0×10^7	7.0×10^7
Microbiology department	1.9×10^4	1.5×10^4	2.2×10^4	1.6×10^4	1.8×10^4	2.4×10^4