Action Research Project Report on Study of different media for best recovery of *Pseudomonas aeruginosa* in Packaged Drinking water

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Introduction

Pseudomonas aeruginosa is a free-living bacterium, commonly found in soil and water. However, it occurs regularly on the surfaces of plants and occasionally on the surfaces of animals. *Pseudomonas aeruginosa* has become increasingly recognized as an emerging opportunistic pathogen of clinical relevance.

Pseudomonas aeruginosa is a Gram-negative rod measuring 0.5 to 0.8 µm by 1.5 to 3.0 µm. Almost all strains are motile by means of a single polar flagellum.

P. aeruginosa possesses the metabolic versatility and it can use more than seventy-five organic compounds for growth. Its optimum temperature for growth is 37 degrees, and it is able to grow at temperatures as high as 42 degrees.

P. aeruginosa strains produce two types of soluble pigments, the fluorescent pigment pyoverdin and the blue pigment pyocyanin.

It is tolerant to a wide variety of physical conditions, including temperature. It is resistant to high concentrations of salts and dyes, weak antiseptics, and many commonly used antibiotics.

Pseudomonas aeruginosa has a preference for growth in moist environments, which is probably a reflection of its natural existence in soil and water.

These natural properties of the bacterium undoubtedly contribute to its ecological success as an opportunistic pathogen. They also help explain the ubiquitous nature of the organism and its prominence as a nosocomial pathogen.

Pseudomonas in Drinking water

Pseudomonas bacteria can be found naturally in the ground and within drinking water sources such as aquifers. Conventional drinking water treatment systems can remove or inactivate these bacteria, but they may continue to multiply within finished drinking water attachments and can cause negative health effects in humans under certain conditions.

Elevated numbers of *Pseudomonas* may indicate the development of a bacterial layer on surfaces within a distribution system. Such surface areas may include home water treatment devices that utilize carbon filters or membranes. However, the presence of disinfectants such as chlorine or chloramines, when applicable, can control (but not prevent) such growth. Furthermore, *Pseudomonas* species' ability to slow their metabolism allows them to survive in bottled or distilled waters for months at low densities.

The presence of high numbers of P. aeruginosa in potable water, notably in packaged water, can be associated with complaints about taste, odour and turbidity.

Methods and media for detection of *Pseudomonas aeruginosa* in Drinking water

Membrane filtration technique is one of the most widely used method for detection of *Pseudomonas aeruginosa* in Drinking water. Main advantage of this technique is higher recovery from the sample as the entire sample is filtered through the membrane and detection of the organism present in lower numbers is also possible and results are more reliable compared to pour plate and spread plate technique due sample size limitation.

IS 13428 (AnnexD)recommends use of Aspargine Proline broth along with ethanol for detection of fluorescence and further confirmation on Skimmed Milk agar with cetrimide.

APHA recommends aspargine broth for presumptive test and acetamide broth/agar for confirmatory test(Method 9213 F) and M-PA agar for presumptive test and Milk agar for confirmation(Method 9213 E).

ISO 16266:2006 recommends Pseudomonas agar base/CN agar for presumptive presence and further confirmation on Kings B media,Acetamide broth and oxidase test.

Why alternative media ?

As per IS13428:2005 the method for detection of *Pseudomonas aeruginosa* relies upon the presumptive presence in Asparagine Proline broth with ethanol.

Presumptive presence is indicated by the development of fluorescence as observed under UV light(98 % of *Pseudomonas aeruginosa* produce pigment .In some cases only turbidity is observed.

Both cases (exhibiting fluorescent turbidity and turbidity without fluorescence) are to be confirmed further on Milk agar with cetrimide for development of pigment and casein hydrolysis.

There have been several instances where the development of fluorescence is quite weak and not detectable at all. It is only at the confirmatory stage that such cases are confirmed for the presence of *Pseudomonas aeruginosa.*Sometimes weaker strains do not exhibit characteristic fluorescence.

Therefore, there is a need for a more sensitive media for detection of the presumptive presence. It would be helpful for many BIS licensed water manufacturing units which operate on the basis of presumptive presence only for assessing the quality of Packaged Drinking Water or Packaged Natural Mineral Water as per IS 14543 and 13428 respectively.

Objective of the study

To do a comparative analysis between Asparagine Proline broth with ethanol and Pseudomonas agar base for detection of presumptive presence in Packaged drinking water.

Methodology

Spike and recovery studies with reference MTCC culture for *Pseudomonas aeruginosa* were carried out on two media (Asparagine Proline broth with ethanol and without ethanol and Pseudomonas agar base) for detection of presumptive presence. Further confirmation was also carried out on Skimmed Milk agar with cetrimide from both the media under study for detection of presumptive presence.

To study the sensitivity of two different media, samples of PDW received for testing over a period of time were spiked with reference culture which is known to produce characteristic growth in both the media. MTCC 2581 reference culture for *Pseudomonas aeruginosa* was inoculated on nutrient broth for 24 hours before spiking. The level of spike was used as 200µl per one litre of sample as per past experience. All positive culture related work was carried out in a Biosafety cabinet.

Only one litre sealed remnant were used for spiking. It was also ensured that such samples have been tested for the absence of *Pseudomonas aeruginosa* earlier. A total of 34 such samples were identified and used during the study. The use of samples for spiking with reference culture ensures that the sensitivity of the media is studied along with other background microflora depending on various chemical aspects of samples like pH, TDS, alkalinity etc. which differs from sample to sample and closely mimics the actual samples which can be observed during production of PDW or PNMW.

Membrane filtration technique was used and sample size was fixed as 250 ml as both IS 13428 and IS 14543 prescribe that *Pseudomonas aeruginosa* shall be absent in 250 ml.

The samples were passed through 0.45 micron membrane filter (nitrocellulose Pall make) and were placed on both the media(Asparagine Proline broth with ethanol and without ethanol and Pseudomonas agar base). Both negative and positive controls were kept during the study. Both media were incubated at 37±1 degree Celsius for 48 hours. After incubation subcultures were made on Skimmed Milk agar with cetrimide from both the media and were incubated at 42±0.5 degree Celsius for 24 hours.

Results and observation

Detailed results for all the samples under study are at annexure 1.Following observation are made as per available results:

Recovery on Pseudomonas agar (Presumptive presence)

- Recovery of *Pseudomonas aeruginosa* as presumptive presence on Pseudomonas agar is 67.6% .23 out of 34 samples showed characteristic growth.
- 19 samples exhibited Luxuriant growth greenish yellow and pigment production, Flourescence observed under UV light.
- 4 samples exhibited good characteristic growth with Isolated green coloured colonies, pigment production and fluorescence under UV light.
- One sample showed poor growth with a single isolated green coloured colony showing pigment production and fluorescence under UV light.
- 9 samples did not show growth at all.
- One sample did not exhibit characteristic growth, pigment production and fluorescence under UV light.

Recovery on Asparagine Proline broth with ethanol (Presumptive presence)

- Recovery of *Pseudomonas aeruginosa* as presumptive presence on Asparagine proline broth is 17.6%. Only 06 out of 34 samples showed weak characteristic growth.
- 23 samples exhibited No turbidity, No flourescence at all
- 5 samples exhibited turbidity in the media without any detectable fluorescence.

Recovery on Asparagine Proline broth without ethanol (Presumptive presence)

- Recovery of *Pseudomonas aeruginosa* as presumptive presence on Asparagine proline broth without ethanol is 52.9%. 18 out of 34 samples showed good characteristic growth with fluorescence under UV.
- One sample exhibited turbidity in the media without any detectable fluorescence.
- 15 samples exhibited No turbidity, No flourescence at all

Recovery on Skimmed Milk agar with cetrimide from Pseudomonas agar (confirmatory presence)

- Characterisitic growth on Pseudomonas agar (Luxuriant or isolated colonies with pigment production and fluorescence under UV ligtht) was further confirmed with pigment production and casein hydrolysis in all the 22 samples. Even single isolated colony exhibited good pigment production and casein hydrolysis
- Only two samples which showed good characteristic growth on Pseudomonas agar exhibited weak pigment production and casein hydrolysis.

Recovery on Skimmed Milk agar with cetrimide from Asparagine Proline broth with ethanol (confirmatory presence)

- Pigment production and casein hydrolysis was exhibited by all the six samples which exhibited weak characteristic growth.
- Pigment production and casein hydrolysis was also exhibited by three samples which exhibited only turbidity without any characteristic fluorescence in the presumptive presence.
- Pigment production and casein hydrolysis was exhibited by one sample which showed no turbidity and fluorescence in presumptive presence.

Recovery on Skimmed Milk agar with cetrimide from Asparagine Proline broth without ethanol (confirmatory presence)

- Out of eighteen samples which showed good turbidity and fluorescence at the presumptive stage, only three samples showed good pigment production and casein hydrolysis. Three samples showed weak pigment production and casein hydrolysis.12 samples which showed good turbidity and fluorescence at the presumptive stage did not show any pigment production or casein hydrolysis at all.
- Seven samples which showed no turbidity and fluorescence at presumptive stage did not show any pigment production or casein hydrolysis at all
- One sample which exhibited weak turbidity and no fluorescence also did not showed any pigment production and casein hydrolysis.

All controls both positive and negative were found satisfactory.None of the media under study were better than each other for those samples which showed no growth on Pseudomonas agar

Conclusions

Pseudomonas agar base/CN agar is a more sensitive media for detection of presumptive presence of *Pseudomaonas aeruginosa* in low densities.67.6% recovery rate was observed. Presumptive presence on pseudomonas agar is further supported by confirmatory characteristics on Skimmed Milk agar with cetrimide (91.3% samples showed characteristic growth and 8.6% samples showed weak characteristic on confirmatory medium.

Asparagine proline broth with ethanol is a less sensitive media for detection of *Pseudomaonas aeruginosa* in low densities. Only 17.6% samples showed weak characteristic growth.However,all samples with weak characteristic growth in presumptive stage exhibited pigment production and casein hydrolysis on Skimmed Milk agar with cetrimide. Even samples with turbidity only and without any turbidity and flourescnce on presumptive stage showed pigment production and casein hydrolysis on Skimmed Milk agar with cetrimide.Therefore, it is a less sensitive media for detecting presumptive presence.

In case of Asparagine proline broth without ethanol it was observed that 52.9% of the samples showed good turbidity and fluorescence at the presumptive stage. However, only 16.6% of such samples exhibited pigment production and casein hydrolysis on Skimmed Milk agar with cetrimide at confirmatory step.

Recommendations

Based on the results obtained during the study, Pseudomonas agar base/CN agar was observed as a more suitable media for detection of presumptive presence of *Pseudomonas aeruginosa* at lower densities as may be observed in case of Packaged Drinking Water or Packaged Natural Mineral water. Therefore, it can be recommended as an alternative media for detection of presumptive presence to the presently recommended Asparagine proline broth with ethanol in Annex D of IS 13428:2005. This would enable better understating of product quality for those PDW and PNMW manufacturing units which operate their licences based on the presumptive presence only.

Annexure1

Sl.No	**Sample ID**	**Growth on Pseudomonas agar**	**Growth on Asparagine proline broth with ethanol**	**Growth of Asparagine Proline Broth without ethanol**	**Further confirmation on Skimmed Milk Agar (From Asparagine proline broth with ethanol)**	**Further confirmation on Skimmed Milk Agar (From Pseudomonas agar)**	**Further confirmation on Skimmed Milk Agar (From Asparagine proline broth without ethanol)**
1	MB3254	Luxuriant growth greenish yellow and pigment production, Flourescence observed under UV light	No turbidity, No flourescence	Turbidity and flourescence	No pigment production and no casein hydrolysis	Pigment production and casein hydrolysis	No pigment production and no casein hydrolysis
2	MB3195	Luxuriant growth greenish yellow and pigment production, Flourescence observed under UV light	No turbidity, No flourescence	No turbidity, No flourescence	No pigment production and no casein hydrolysis	Pigment production and casein hydrolysis	No pigment production and no casein hydrolysis

3	MB3238	Luxuriant growth greenish yellow and pigment production, Flourescence observed under UV light	No turbidity, No flourescence	Turbidity and flourescence	Weak pigment production and weak casein hydrolysis	Pigment production and casein hydrolysis	No pigment production and no casein hydrolysis
4	MB3234	Luxuriant growth greenish yellow and pigment production, Flourescence observed under UV light	No turbidity, No flourescence	Turbidity and flourescence	Pigment production and casein hydrolysis	Pigment production and casein hydrolysis	Weak Pigment production and casein hydrolysis
5	MB3316	Luxuriant growth greenish yellow and pigment production, Flourescence observed under UV light	No turbidity, No flourescence	No turbidity, No flourescence	Weak pigment production and weak casein hydrolysis	Pigment production and casein hydrolysis	No pigment production and no casein hydrolysis
6	MB3282	Luxuriant growth greenish yellow and pigment production, Flourescence observed under	Turbidity,Weak flourencence	Turbidity and flourescence	Pigment production and casein hydrolysis	Pigment production and casein hydrolysis	No pigment production and no casein hydrolysis

		UV light					
7	MB3266	Luxuriant growth greenish yellow and pigment production, Flourescence observed under UV light	Turbidity,no flourencence	Turbidity and flourescence	Pigment production and casein hydrolysis	Weak Pigment production and casein hydrolysis	No pigment production and no casein hydrolysis
8	MB3311	No growth	No turbidity, No flourescence	No turbidity, No flourescence	No pigment production and no casein hydrolysis		No pigment production and no casein hydrolysis
9	MB3273	No growth	No turbidity, No flourescence	No turbidity, No flourescence	No pigment production and no casein hydrolysis		No pigment production and no casein hydrolysis
9	MB3279	No growth	No turbidity, No flourescence	No turbidity, No flourescence	No pigment production and no casein hydrolysis		No pigment production and no casein hydrolysis
11	MB3324	No growth	No turbidity, No flourescence	No turbidity, No flourescence	No pigment production and no casein hydrolysis		No pigment production and weak casein hydrolysis

12	MB3201	No growth	No turbidity, No flourescence	No turbidity, No flourescence	No pigment production and no casein hydrolysis		No pigment production and weak casein hydrolysis
13	MB3281	Isolated green coloured colonies, pigment production and fluorescence under UV light	No turbidity, No flourescence	No turbidity, No flourescence	No pigment production and no casein hydrolysis	Pigment production and casein hydrolysis	No pigment production and no casein hydrolysis
14	MB3272	Luxuriant growth greenish yellow and pigment production, Flourescence observed under UV light	Turbidity,no flourencence	Turbidity and flourescence	Pigment production and casein hydrolysis	Pigment production and casein hydrolysis	No pigment production and no casein hydrolysis
15	MB3213	Luxuriant growth greenish yellow and pigment production, Flourescence observed under UV light	Turbidity,Weak flourencence	Turbidity and flourescence	Pigment production and casein hydrolysis	Pigment production and casein hydrolysis	No pigment production and no casein hydrolysis
16	MB3255	Isolated green coloured single	No turbidity, No	No turbidity, No	No pigment production	Pigment production and	No pigment production and

		colony(poor recovery)	flourescence	flourescence	and no casein hydrolysis	casein hydrolysis	no casein hydrolysis
17	MB3278	Isolated green coloured colonies, pigment production and fluorescence under UV light	Turbidity,Weak flourencence	Turbidity and flourescence	Pigment production and casein hydrolysis	Pigment production and casein hydrolysis	No pigment production and no casein hydrolysis
18	MB3280	Isolated green coloured colonies, pigment production and fluorescence under UV light	Turbidity,no flourencence	Turbidity and flourescence	Pigment production and casein hydrolysis	Pigment production and casein hydrolysis	Pigment production and casein hydrolysis
19	MB3257	Luxuriant growth greenish yellow and pigment production, Flourescence observed under UV light	No turbidity, No flourescence	Turbidity and flourescence	Weak pigment production and weak casein hydrolysis	Pigment production and casein hydrolysis	No pigment production and no casein hydrolysis
20	MB3235	No characteristic growth	Turbidity,no flourencence	No turbidity, No flourescence	No pigment production and no casein hydrolysis		No pigment production and no casein hydrolysis

21	MB3231	Luxuriant growth greenish yellow and pigment production, Flourescence observed under UV light	No turbidity, No flourescence	Turbidity and flourescence	No pigment production and no casein hydrolysis	Pigment production and casein hydrolysis	No pigment production and no casein hydrolysis
22	MB3326	Luxuriant growth greenish yellow and pigment production, Flourescence observed under UV light	Turbidity,Weak flourencence	Turbidity and flourescence	Pigment production and casein hydrolysis	Pigment production and casein hydrolysis	Pigment production and casein hydrolysis
23	MB3232	Luxuriant growth greenish yellow and pigment production, Flourescence observed under UV light	No turbidity, No flourescence	Turbidity and flourescence	No pigment production and no casein hydrolysis	Pigment production and casein hydrolysis	No pigment production and no casein hydrolysis
24	MB3241	Luxuriant growth greenish yellow and pigment production, Flourescence observed under	Turbidity,Weak flourencence	Turbidity and flourescence	Pigment production and casein hydrolysis	Pigment production and casein hydrolysis	Weak pigment production and casein hydrolysis

		UV light					
25	MB3220	Luxuriant growth greenish yellow and pigment production, Flourescence observed under UV light	Turbidity,Weak flourencence	Turbidity and flourescence	Pigment production and casein hydrolysis	Pigment production and casein hydrolysis	Weak pigment production and casein hydrolysis
26	MB3258	Luxuriant growth greenish yellow and pigment production, Flourescence observed under UV light	No turbidity, No flourescence	Turbidity and flourescence	Weak pigment production and weak casein hydrolysis	Pigment production and casein hydrolysis	No pigment production and weak casein hydrolysis
27	MB3315	Luxuriant growth greenish yellow and pigment production, Flourescence observed under UV light	No turbidity, No flourescence	Turbidity and flourescence	Weak pigment production and weak casein hydrolysis	Pigment production and casein hydrolysis	Pigment production and casein hydrolysis

28	MB3325	Luxuriant growth greenish yellow and pigment production, Flourescence observed under UV light	No turbidity, No flourescence	Turbidity and flourescence	Weak pigment production and weak casein hydrolysis	Pigment production and casein hydrolysis	No pigment production and no casein hydrolysis
29	MB3199	Isolated green coloured colonies, pigment production and fluorescence under UV light	No turbidity, No flourescence	No turbidity, No flourescence	No pigment production and no casein hydrolysis	Weak Pigment production and casein hydrolysis	No pigment production and no casein hydrolysis
30	MB3228	Luxuriant growth greenish yellow and pigment production, Flourescence observed under UV light	No turbidity, No flourescence	weak turbidity and no fluorescence	No pigment production and no casein hydrolysis	Pigment production and casein hydrolysis	No pigment production and no casein hydrolysis
31	MB3327	No growth	No turbidity, No flourescence	No turbidity, No flourescence	No pigment production and no casein hydrolysis		No pigment production and no casein hydrolysis
32	MB3289	No growth	No turbidity, No	No turbidity, No	No pigment production		No pigment production and

			flourescence	flourescence	and no casein hydrolysis		no casein hydrolysis
33	MB3323	No growth	Turbidity,no flourencence	No turbidity, No flourescence	No pigment production and no casein hydrolysis		No pigment production and no casein hydrolysis
34	MB3250	No growth	No turbidity, No flourescence	No turbidity, No flourescence	No pigment production and no casein hydrolysis		No pigment production and no casein hydrolysis