IJBPAS, November, 2025, 14(11): 5976-5988

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

DETECTION AND CHARACTERIZATION OF COLIFORM BACTERIA IN WATER SAMPLES: COMPARATIVE ANALYSIS USING MPN AND SPC METHODS

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Received 15th July 2024; Revised 20th Sept. 2024; Accepted 1st Nov. 2024; Available online 1st Nov. 2025

https://doi.org/10.31032/IJBPAS/2025/14.11.9565

ABSTRACT

The study focuses on detecting coliform bacteria in water samples from different sources using methods like the Most Probable Number (MPN) and standard plate count (SPC). These methods are essential for assessing water quality and identifying potential fecal contamination, which is critical for public health.

Keywords: Coliforms, Most Probable Number, standard plate count, enzymes

1. INTRODUCTION:

Coliform bacteria are a group of gram-negative, rod-shaped microorganisms characterized by their ability to grow in the presence of bile salts or other surfactants that inhibit growth of other bacteria. They can ferment lactose at temperatures between 35°C and 37°C, producing acid, gas, and aldehyde within 24 to 48 hours. Coliforms are oxidase-negative, non-spore-forming, and exhibit β-galactosidase activity, which is a key biochemical trait used in their identification **[1]**.

Coliform bacteria encompass three main groups: total coliforms, fecal coliforms, and

Escherichia coli. Total coliforms include a variety of genera such as *Enterobacter, Klebsiella, and Citrobacter,* which are not necessarily of fecal origin but can grow in similar conditions. Fecal coliforms, a subset of total coliforms, are more specifically associated with fecal contamination and are typically found in the intestines of warm-blooded animals. *Escherichia coli (*E. coli) is a well-known species within the fecal coliform group and serves as a specific indicator of fecal pollution, as its presence strongly suggests contamination by fecal matter and the possible presence of enteric pathogens **[2]**.

Enzymes, as biological catalysts, accelerate chemical reactions within living organisms and are indispensable for a multitude of metabolic processes and industrial applications. These protein molecules are produced by microorganisms, plants, and animals, and they exhibit a high degree of specificity for their substrates **[3]**.

In the textile industry, enzymes like cellulases and pectinases are employed for fabric finishing, where they improve softness and reduce pilling. In the detergent industry, proteases, lipases, and amylases are added to formulations to break down protein, fat, and starch stains, thereby enhancing cleaning efficiency **[4]**. Enzymes and organic acids play a crucial role in numerous industrial, medical, and environmental applications. The strategic use of these bioproducts continues to expand, highlighting their growing importance in modern science and industry **[5]**.

Table 1: polysaccharide degrading enzymes details

Enzymes	Microorganism	Function	References
Amylase	***Bacillus subtilis***	**Breaks down starch into simpler sugars**	**[6]**
Protease	***Bacillus licheniformis***	**Breaks down proteins into peptides and amino acids**	**[7]**
Lipase	***Pseudomonas aeruginosa***	**Breaks down fats into fatty acids and glycerol**	**[8]**
Cellulase	***Clostridium thermocellum***	**Breaks down cellulose into glucose**	**[9]**

In this research contributes valuable insights into microbial ecology, contamination assessment in water sources, and the biotechnological potential of bacterial isolates. Further genetic and molecular studies are recommended to elucidate species identities and explore their specific roles in environmental and industrial settings. Based on the detailed analysis of bacterial isolates from various environmental samples (drinking water, tap water, and soil), this research paper investigates microbial

diversity and functional capabilities, focusing on their ability to degrade different polysaccharides. The study employed rigorous methods including standard plate counts, presumptive tests, confirmatory tests, and biochemical analyses to characterize and identify bacterial isolates **[10]**.

2. MATERIAL AND METHODS:

2.1. Composition of media and reagents:

The various media and chemicals used in the present research work were of analytical grade and high purity from Hi-media laboratories in India. Such as MLBB, EMB agar, Nutrient agar, Safranin, starch agar, cellulose agar, lipase agar, Crystal violet, and Gram's iodine. The composition of various media used in given in here **[11] [12] [13]**.

2.2. Sample Collection:

Bacterial isolates were collected from a soil sample contaminated with agricultural crop wastes located in **Table 2**. To prepare the sample, 1 gram of soil was homogenized in 9 milliliters of physiological saline. For water samples, collections were made from both drinking water and tap water using sterile bottles. These samples were then transported to the laboratory for further processing. In the laboratory, each 1 milliliter of water sample was homogenized in 9 milliliters of distilled water.

2.3. Standard plate count (spc) or total viable count (TVC):

Prepare 10^{-1},10^{-2} and 10^{-3} to 10^{-6}dilutions of the water sample (if necessary). From each of the dilution transfer a fixed amount into sterile melted nutrient agar tube, mix it well and pour immediately in sterile petri dishes. Label plates clearly indicating the dilution and the volume plated respectively. Incubate all the plates at 37ºC for 24 hours. Count the total number of colonies that has developed on each of the plate. If necessary, use colony counter to help counting of colonies. Calculate final number of organisms present in the water sample as follows **[14]**.

$$\text{CFUs/ml} = \frac{\text{Average number of colonies}}{\text{dilution} * \text{volume plated}}$$

2.4. Presumptive test: Shake the water sample vigorously to ensure uniform distribution of organisms. With sterile graduated pipettes inoculate the water sample as follows, 5 MLBB double strength tubes with 10ml water, 1 MLBB single strength tube with 1.0ml water, 1 MLBB single strength tube with 0.1ml water. One tube of MLBB single strength is not inoculated and hence, serves as control. Incubate all the tubes at 37ºC for 24 hours. Examine tubes for the presence of acid and gas after 24 hours. If no gas formed,

reincubate all the tubes for another 24 hours (total 48 hours). Record the presence or absence of acid and gas at each examination and interpreted.

2.5. Confirmed test: Streak a loopful of suspension from a positive presumptive tube (which shows the highest amount of gas production), so as to get well isolated colonies. Incubate the plate at 37ºC for 24 hours. Record results and interpreted.

2.6. Completed test: Select and mark a well isolated typical/atypical colony on Eosin methylene blue (EMB) or Endo's agar plate. With the help of nichrome wire loop, pick up half of the previously marked typical/atypical colony and transfer it to Brilliant green lactose bile broth (BGLB) or nutrient lactose broth tube. From the remaining half of the same colony streak over the surface of the nutrient agar slant. Incubate slant and broth at 37ºC for 24 hours. Check lactose broth for presence of acid and gas. Prepare Gram's stain of the growth from the surface of agar slant and observe the slide. Look for the presence of gram-negative non-spore forming short rods. Record results and interpreted.

2.7. On various polysaccharides:

To assess the ability of bacterial isolates to degrade various polysaccharides, specific media were prepared using pectin, starch, cellulose as substrates. Each substrate was incorporated into a suitable agar medium to create distinct testing plates. Pour the sterilized medium into sterile Petri dishes and allow it to solidify. Inoculate the surface of the solidified medium with the bacterial isolates using a sterile loop. Incubate the plates at 37°C for 48 hours. Flood the plates with iodine solution. Observe for clear zones around bacterial colonies, indicating various substrate degradation **[15]**.

2.8. Identification of microorganisms:

Identifying microorganisms is crucial across various fields such as clinical diagnostics, environmental microbiology, food safety, and biotechnology. Accurate microbial identification aids in understanding microbial diversity, tracking disease outbreaks, ensuring food safety, and developing biotechnological applications **[16]**. Here's a detailed overview of the importance of microbial identification and the methods used to achieve it.

Colony Morphology: Analyzes the appearance of microbial colonies on agar plates, such as shape, color, texture, and edge characteristics. These features can provide

initial clues about the microorganism's identity.

Gram's Staining: Prepare a heat-fixed smear of the culture. Cover the smear with crystal violet stain for 2 minutes, rinse with water, and add Gram's iodine for 1 minute. Decolorize with alcohol until the violet color stops leaching, rinse with water, counterstain with safranin for 1 minute, rinse, and air dry.

Biochemical Tests: Biochemical tests detect specific metabolic and enzymatic activities of microorganisms, providing a biochemical profile that helps identify the organism.

Molecular Methods: Molecular methods provide highly accurate identification by analyzing the genetic material of microorganisms. 16S rRNA Gene Sequencing: The 16S ribosomal RNA gene is highly conserved among bacteria but contains hypervariable regions that provide species-specific sequences. Sequencing this gene allows for precise identification. Widely used in phylogenetic studies, microbial taxonomy, and identifying unknown bacterial isolates.

3. RESULT AND DISCUSSION:

3.1. Sampling sites:

These samples from distinct environments, including drinking water, tap water, and soil, were chosen to provide a comprehensive view of bacterial diversity and their polysaccharide-degrading capabilities. Sterile collection and preparation protocols were strictly followed to ensure sample integrity and reliability for subsequent analyses in this research study.

Table 2: Samples collection for research work

Sample name	Sample area	Location	
		Latitude	Longitude
Drinking water	Mehsana, Gujarat	23.529	72.457
Tap water	Mehsana, Gujarat	23.529	72.457
Castor soil	Chanasma, Gujarat	23.718	72.109

3.2. Standard plates count: The standard plate count method was utilized to determine the bacterial load in the collected samples. The results are showing the colony-forming units (CFU) per 100 mL for drinking water, tap water, and castor soil samples. The drinking water sample from Mehsana exhibited a bacterial load of 5160 CFU per 100 mL. According to standard water quality guidelines, coliform bacteria in drinking water should typically be absent or present in very low numbers, often less than 1 CFU per 100 mL, to ensure safety for human consumption. The tap water sample, also from Mehsana, showed a slightly lower bacterial count of 4291 CFU per 100 mL compared to the drinking water sample. This reduction might result from additional treatment processes

such as chlorination or filtration. Coliform counts in agricultural soils can vary widely, often ranging from hundreds to millions of CFU per 100 mL, depending on soil type, moisture, and contamination levels. The observed count reflects the expected high microbial activity in soil environments.

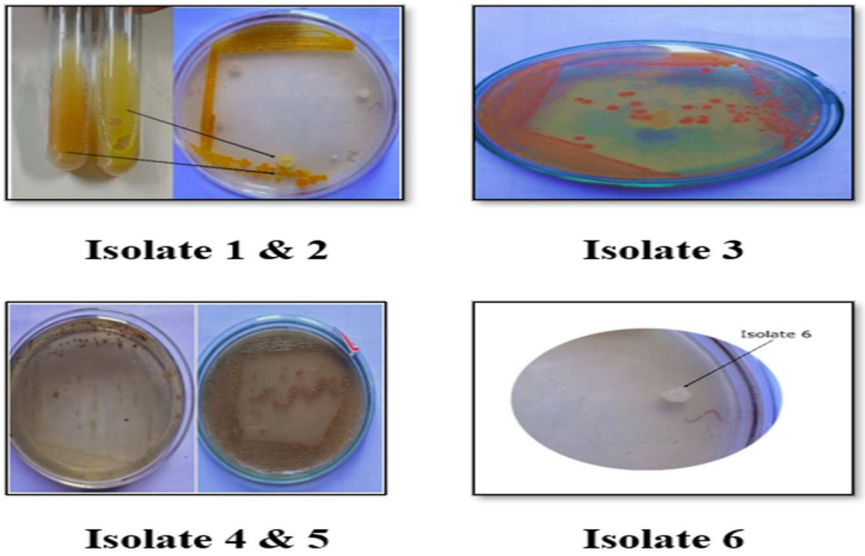


Figure 1: bacterial isolates from various samples

In comparison, a study conducted by Chitlapilly Dass *et al*. (2018) reported similar issues with high CFU counts in drinking water, emphasizing the need for stringent monitoring and purification systems. This is consistent with findings by Pandey *et al*. (2016), who observed high microbial loads in agricultural soils due to the presence of organic matter and nutrients. Some other similar studies mentioned by Patel *et al*. (2024) **[17]**.

3.3. Detection of coliform: Presumptive test in the coliform counts in both drinking and tap water samples from Mehsana significantly exceed safe drinking water standards, indicating a need for improved water treatment and contamination control measures. The drinking water sample exhibited a total coliform count of 5.5 CFU per 100 mL. This value exceeds the acceptable limit for potable water, which typically should have no detectable coliform bacteria per 100 mL. The presence of coliforms indicates potential contamination and poses a health risk if the water is consumed without proper treatment. The counts from different dilutions (2 coliforms from 0.1 mL and 1 coliform from 1 mL) suggest variability in contamination levels across the sample. This reinforces the

need for effective filtration and disinfection processes at the source to ensure water safety. Similarly, the tap water sample also presented a coliform count of 5.5 CFU per 100 mL. This result is concerning as tap water, intended for regular use, should be free from coliform bacteria to be considered safe. The coliform counts (1 from 0.1 mL and 2 from 1 mL) highlight inconsistent contamination, possibly due to issues in the water distribution system or treatment inefficiencies. Ensuring the integrity of the water supply infrastructure and enhancing treatment protocols are critical to addressing these contamination issues. Similar findings were reported by Paul *et al*. (2019), who highlighted the risks associated with coliform contamination in drinking water supplies and the importance of effective treatment processes.

3.3.1. Confirmed Test: To confirm the presence of coliform bacteria in the samples, a series of confirmatory tests were performed on selected isolates that showed positive results in the preliminary tests. The confirmatory tests involved using selective media and biochemical assays to verify the presence of coliforms. Positive samples from the preliminary tests were streaked onto Eosin Methylene Blue (EMB) agar plates. EMB agar selectively inhibits gram-positive bacteria and differentiates between lactose fermenters (coliforms) and non-fermenters. Coliforms typically produce dark colonies with a metallic sheen. After incubation, the plates were examined for the presence of typical coliform colonies (dark with a metallic sheen).

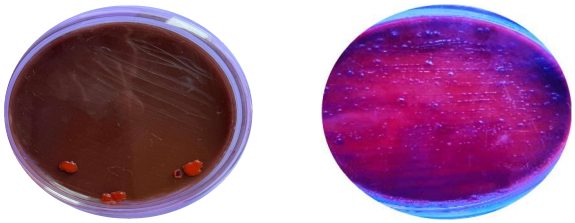
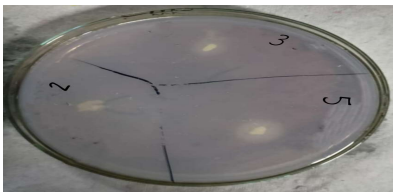


Figure 2: (1) is negative isolate, (3) is isolates give the positive results on EMB agar plates

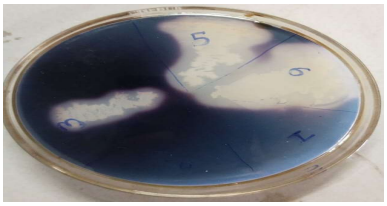
3.4. Completed test: In the identification section, it was noted that only isolate-3 tested positive for coliform detection, which was isolated from tap water. The biochemical tests that gave positive results for isolate-3 included the citrate utilization test and the methyl red test. Gram staining indicated that isolate-3 was composed of gram-negative rods.

3.5. Various substates: The provided images depict bacterial cultures grown on different substrates to evaluate their ability to degrade various polysaccharides. This image (A) shows bacterial cultures grown on a

substrate likely used to test pectin degradation. The plate is divided into several sections (isolates 1, 2, 3, 5). Visible colonies indicate bacterial growth in response to the substrate. The clear zones around some colonies suggest pectinase activity, where the bacteria have broken down pectin, creating a clear zone **[18]**. In image (B): This plate appears to be testing for cellulose degradation, possibly using Lugol's iodine dye to stain the cellulose. Sections (marked 1, 5, 6) show growth with significant clearing zones, indicative of cellulase activity. The presence of dark or discolored regions around the colonies suggests that the bacteria can hydrolyze cellulose, resulting in a distinct color change. In image (C): This image likely depicts a starch degradation test, possibly using iodine to indicate the presence of starch. Sections (marked 2, 5, 6) show bacterial growth with clear zones around colonies, indicating amylase activity. The clearing suggests that the bacteria have hydrolyzed the starch in the medium, leading to a reduction in the iodine-starch complex and subsequent clearing. In image (D): This plate might be used for testing lipase activity on a lipid-rich medium. The plate is labeled and shows bacterial growth with clear zones around some colonies. These clearing zones indicate lipase activity, where the bacteria have broken down the lipids in the medium, resulting in transparent areas around the colonies.

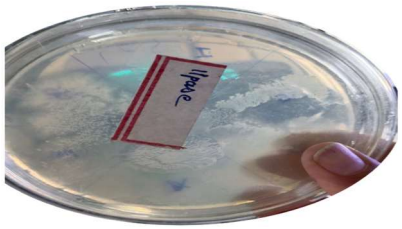


(A) Plate indicating zone of hydrolysis by isolate 3 & 5 on pectin Agar.



(B) Plate indicating zone of hydrolysis by isolate 3,5 & 6 on cellulose agar plate.

(C) Plate indicating zone of hydrolysis by isolate 5 & 6 on starch agar plate.



(D) Plate indicating zone of hydrolysis by isolate 2,3 & 5 on lipase agar plate.

Figure 3: clear zone of hydrolysis around colonies on various substates

The images illustrate bacterial growth on different substrates, demonstrating their enzymatic capabilities in degrading pectin, cellulose, starch. The clear zones around colonies in various sections indicate the presence and activity of specific enzymes, such as pectinase, cellulase, amylase confirming the ability of the bacterial isolates to break down these polysaccharides. **Identification:** The provided table summarizes the morphological and physiological characteristics of six bacterial isolates (1 to 6) cultured on various substrates. These characteristics include colony size, shape, margin, elevation, surface texture, odor, opacity, and pigmentation. Understanding these traits is essential for identifying and differentiating bacterial species, as well as inferring potential functional capabilities and ecological roles. Size: Most isolates (2 to 6) form large colonies, indicating rapid growth and possibly high metabolic activity. Isolate 1, with a medium size, may have slower growth or different nutrient requirements. Smooth surfaces are often found in non-motile or less aggressive strains, while rough and contoured surfaces might be associated with more resilient or stress-tolerant bacteria.

Table 3: Morphological characteristics of isolates

Characteristics	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6
Size	Medium	Large	Large	Large	Large	Large
Shape	Round	Irregular	Round	Round	Irregular	Irregular
Margin	Entire	Undulate	Entire	Undulate	Lacerate	Undulate
Elevation	Convex	Raised	Flat	Flat	Flat	Raised
Surface	Smooth	Contoured	Rough	Smooth	Smooth	Rough
Odor	Fecal	No	No	No	No	No
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Pigmentation	Violet	Red	Pink	Yellow	Golden yellow	White

All isolates are opaque, suggesting dense cellular structures and possibly high biomass production. Pigmentation: The isolates show diverse pigmentation: violet (isolate1), red (isolate 2), pink (isolate 3), yellow (isolate 4), golden yellow (isolate 5), and white (isolate 6). Pigmentation can be linked to specific metabolic processes, protection against environmental stresses (e.g., UV radiation), or pathogenicity. For instance, red and violet pigments might be associated with specific bacterial genera known for their distinctive coloration. Further biochemical and genetic analyses are necessary to confirm the identities and functional properties of these isolates**.**

Gram staining: The Gram staining results from the selected isolates reveal critical insights into the morphological and structural characteristics of the bacteria. Isolates 1, 2, 4,

5, and 6 are Gram-positive, indicating they have a thick peptidoglycan layer in their cell walls which retains the crystal violet stain. Isolate 3 is Gram-negative, characterized by a thinner peptidoglycan layer and an outer membrane, which does not retain the crystal violet stain and instead takes up the counterstain (safranin or fuchsine), appearing pink or red under the microscope. Coliforms, as indicators of fecal contamination, can signal the presence of other pathogenic microorganisms.

Table 4: Result of gram staining of selected isolates

Isolates	Gram's reaction	Arrangements	Shape	Size
1	Positive	Single, Pair	Bacillus	Small
2	Positive	Single, Pair	Bacillus	Big
3	Negative	Single, Pair	Rod	Small
4	Positive	Single, Pair	Bacillus	Big
5	Positive	Single, Pair	Bacillus	Small
6	Positive	Single, Pair	Bacillus	Big

3.5.1. Biochemical test:

Based on the results of the biochemical tests for the six isolates, we can analyze and compare their metabolic capabilities and identify potential similarities and differences among them. Here's a detailed discussion of each test and the overall interpretation.

Table 5: Results of biochemical test

Biochemical Tests	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6
Indole	+	-	-	-	-	-
Citrate utilization test	-	+	+	-	+	-
Methyl red test	+	-	+	-	+	+
Voges Proskauer test	-	+	-	-	+	+
Catalase tests	+	+	+	+	+	+
Nitrate reduction test	+	+	-	+	+	+
Urease test	-	+	-	+	+	-
Gelatin hydrolysis test	-	+	-	-	+	+

Isolate 1 is distinct with positive indole and methyl red tests but negative for citrate utilization, Voges-Proskauer, urease, and gelatin hydrolysis tests. Isolate 2 shows positive results for citrate utilization, Voges-Proskauer, urease, and gelatin hydrolysis tests but negative for indole and methyl red tests. These biochemical test results suggest that each isolate has unique metabolic characteristics, allowing for differentiation among them. These differences can be used for microbial by molecular identification and classification in further studies.

4. CONCLUSION:

The study highlighted the presence of coliform bacteria in water samples from Mehsana, emphasizing the need for enhanced water treatment and monitoring

protocols to ensure public health safety. The methods used, including SPC, MPN, and various biochemical tests, provided comprehensive data on microbial contamination and their potential applications.

Acknowledgments: The research was non-funded. The authors are thankful to the Mehsana Urban Institute of Sciences, Faculty of Science, Ganpat University, Gujarat for providing a laboratory facility.

Funding: The authors disclosed that the research is non-funded.

Conflict of interest: The authors disclosed no potential conflicts of interest, financial or otherwise.

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