

# Chromatic Toxicity Bioassay of Water through Bacterial Ferricyanide Reduction

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## Research

**Keywords:** Bacterial ferricyanide reduction, Immobilization, Microbial Biosensor, Chromatic Assay, Heavy Metal Water Contamination

**Posted Date:** February 5th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-162816/v1>

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# Abstract

Water quality assessment demands a precise anatomization of specimens that comply with acclaimed water purity standards. Today, the growing number of toxicants and their synergistic consequences make it necessary to develop general toxicity assays able to examine and determine water pollution.

Contemporary general toxicity methods hinder specimen analysis due to their prolonged operation protocols. Also, the equipment involved is very expensive that not everyone can afford it. In an effort to resolve these drawbacks, a quick and cost effective toxicity bioassay based on chromatic changes related to bacterial ferricyanide reduction is introduced here. *E.coli* cells (Model Bacteria) were stably confined on four supports: Cellulose-based Paper Discs, Silica 60, Polystyrene, and Acrylic Beads, which remained useful for a long period at -20°C. Copper was used as a model toxic agent to perform Bioassay Assessment. Chromatic changes related to bacterial ferricyanide reduction were determined by visual inspection. Cellulose Paper Discs, Polystyrene, and Acrylic beads showed good results and better viability, while Silica 60 proved itself as a weak support and resulted in poor viability.

## Introduction

Waterborne and related diseases not only deteriorate the environment and cause pollution, but they are also the reason of increasing rates in morbidity and mortality. Though efforts are continuously being made to preserve water safety, waterborne outbreaks are still reported

worldwide. Diseases related to water cause more than 3.4 million deaths each year (Berman, 2009).

Water is a "Universal Solvent," as it is able to dissolve almost everything (Anne Marie Helmenstine, 2019). This makes water essential for the proper functioning of the Earth's ecosystem. All water-related problems are a major public health concern all around the world. The foremost source of water pollution are the industries. Industries use freshwater to carry the wastes from different sources into rivers, lakes, and oceans. Water pollution has a negative impact on all living creatures and consequently the food chain as well. It can negatively affect the daily use of water from drinking to bathing. To get the proper solution to all these water-related problems, the treatment of water is necessary. Therefore, for the proper analysis and control of water quality, the detection of the pollutants and toxic contaminants is a major step. To detect any type of contaminant, a wide variety of tools, as well as advanced analytical methods, are required. Specialized, appropriate, specific, and powerful diagnostic tools have been developed to detect various contaminants in water (Ramírez-Castillo, 2015), as well as chemical analysis techniques are also of great importance in determining specific substances, but most of these tools and the techniques are limited in analysing a complex sample that contains contaminants in a huge variety. Also, they are time-consuming, require lengthy protocols, and rely on very expensive instrumentation.

In recent years, the progress of biosensor has unlocked an extraordinary perspective to the onsite, easy, and money-saving monitoring of water quality (Chaubey A, 2002). In order to analyse contaminants of water, the innovative idea of biosensors is appropriate and convenient. By electrochemical means, an

immense proportion of enzymatic biosensors are operated. Towards the target analyte, high sensitivity is the benefit of Enzymatic Biosensor (Eggins, 2013). Nevertheless, due to enzyme deactivation, it also faces drawbacks like time consumption, expensive protocols of enzyme purification and immobilization; as well as less life span and deprived stability (Jaffrezic-Renault, 2011).

As compared to Enzymatic Biosensor, Microbial Biosensor is more beneficial as it has more simplicity associated with biocatalyst production, predominantly when huge quantities are needed. To a large variety of analytes, microbial biosensors are more flexible and tactful (Jaffrezic-Renault, 2011). Mainly Microbial Biosensors have been explored as water quality monitoring devices, and presently few prototypes used as water toxicity sensors are also commercialized. Microbes that remain alive under high alkaline, acidic, high temperature, and saline conditions give ways to great perspectives on water monitoring for industrial process waste monitoring (D'souza, 2001).

*E.coli* has the honour of being the most extensively studied microbial organism due to its various important roles in fields related to biotechnology, industrial sciences, medicine, and biological sciences; and in recent years, it has gained quite a positive reputation in development of Microbial Biosensors. Being a model organism for laboratories, almost every aspect of *E.coli*, from its genome to its protein-making capability, has been exploited (Lee, 2009). In bacterial metabolism, oxygen acts as the hydrogen acceptor in the TCA cycle under the aerobic condition for growth and respiration (Hadjipetrou, 1966). However, under anaerobic conditions, an artificial hydrogen acceptor can be utilized by the bacterial metabolism as well. This artificial hydrogen acceptor can be a nitrate, sulphate, benzoquinone, or ferricyanide. We used ferricyanide as a hydrogen acceptor because after accepting the hydrogen, it gets reduced into ferrocyanide, and this whole reaction is accompanied by a change in colour (Pujol-Vila, 2015). Bacteria are termed immortal because they do not die a natural death, but they only die because of abiotic factors like temperature, pH, and insufficient nutrient supply (Bickerstaff, 1997).

Several assays indirectly assess the activity of microbial cells by quantitatively analysing changes to the bacterial metabolic system (Buffi, 2011), specifically to the electron transport chain. The electron transport chain is selected because it maintains the basic integrity of the cell. There are various aspects of analysing the microbial metabolism, and it can be analysed by measuring inhibition of respiration, by qualitatively assessing the accumulation of ferrocyanide in ferricyanide mediated bioassays or by quantifying bioluminescence. The incubation of bacteria that can utilize ferricyanide as an alternate terminal electron acceptor is referred to as ferricyanide-mediated respirometry assay (FM-RES assay) (Catterall, 2010). Our experiment revolves around the fact that in the presence of a heavy metal such as copper, lead, mercury, chromium, cadmium, etc., this metabolic conversion of ferricyanide into ferrocyanide altogether stops. The bacteria are unable to reduce ferricyanide; thus, no ferrocyanide is produced. We are using this principle to detect the presence of heavy metals in water.

## Materials And Methods

### 3.1. Chemicals and Samples

Luria-Bertani Broth, MacConkey Agar, Potassium Ferricyanide, Copper Sulphate, Glucose, Potassium Dihydrogen Phosphate, Dipotassium Hydrogen Phosphate, Deionized Water, Silica 60 (high purity grade), 9mm Cellulose Paper Discs (0.7mm thickness), Acrylic Beads, and Polystyrene. All chemicals were of high systematic grade, and all solutions were prepared with distilled water under strictly sterile conditions. NaCl was used to rehydrate disc before streaking it on agar. Water samples were prepared with Copper Sulphate in 50mL Falcon tube at a ratio of 1:2 and 1:4. Concentrated CuSO<sub>4</sub> was diluted with water in the ratio 1:4 (one part of concentrate is mixed with four parts of water) and 1:2 (one part of concentrate is mixed with two parts of water).

## **3.2. Preparation of *E.Coli* Culture and Bacterial Paper Discs (BPDs)**

*E.coli* was grown aerobically in Luria-Bertani (LB) broth for 18 hours at 37°C. The grown culture was centrifuged at 10100 x g for 15 minutes and then re-suspended in 0.1M phosphate buffer containing 2% glucose to a  $2.0 \times 10^{10} \pm 0.5 \times 10^{10}$  cell mL<sup>-1</sup> bacterial concentration. By the adsorption method of immobilization, *E.coli* was entrapped in 9mm Cellulose Paper Discs (PDs) (0.7mm thickness), which were used as the supporting material. For *E.coli* cell entrapment on PDs, we inoculated one side of the disc with a bacterial suspension of volume 60 µl and dried it at room temperature (25 °C) for 2 hours in Laminar Flow Hood (F. Pujol-Vila, 2016). After being completely dried, PDs were stored at -20°C until further required.

## **3.3. Bacterial Viability Checking**

For evaluating bacterial viability in the immobilized paper discs, the immobilized paper discs were rehydrated by putting them in 0.9% (w/v) NaCl then shaken with vortex for 5 minutes to re-suspend the immobilized bacteria. Bacterial viability was determined by plating the suspension on MacConkey agar and then they were incubated for 24 hours. Similar protocol was followed for evaluating bacterial viability in *E.coli* immobilized Silica 60, Polystyrene, and Acrylic Beads.

## **3.4. Immobilization of *E.coli* on Silica 60**

For the *E.coli* entrapment, 20ml of *E.coli* culture (containing 1M phosphate buffer (PB) and 2% glucose) and 10ml of 50mM phosphate buffer (immobilization buffer) with pH 7.0 was added to 1g of silica 60. Silica 60 is the matrix for immobilization.

The immobilization was carried out in a 100 mL glass vessel placed on a magnetic stirrer at 200 rpm, for an hour, at 25°C. Then it was dried at room temperature (25 °C) for 24 hours in a Laminar Flow Hood and stored at -20°C until further required (Sugahara, 2014).

## **3.5. Immobilization of *E.coli* on Polystyrene**

For the immobilization of *E.coli* cells, Polystyrene was used as third support material, and we made small fragments of it prior to immobilization. Polystyrene was hydrated with *E.coli* culture (containing 1M

phosphate buffer (PB) and 2% glucose). Then it was dried at room temperature (25 °C), for 24 hours in a Laminar Flow Hood and stored at -20°C until further required (Hackel, 1975).

### **3.6. Immobilization of *E.coli* on Acrylic Beads**

For the entrapment of *E.coli* cells in Acrylic Beads, we placed the beads in an aqueous buffer containing *E.coli* cells culture (containing 1M phosphate buffer (PB) and 2% glucose). The immobilization was carried out in a 50mL Falcon tube, by placing it in a Laminar Flow Hood for 12–72 hours, at room temperature. After being completely dried, Acrylic Beads were stored at -20°C until further required (Boller, 2002).

## **Results & Discussion**

### **4.1. Analytical performance of *E.coli* immobilized cellulose paper discs in visual inspection of colour change**

The paper-based chromatic bioassay was specified in terms of sample volume, sample concentration, range, and limit of detection. In terms of sample volume and concentration, 10µl of potassium ferricyanide solution and 20µl of 1:4 diluted CuSO<sub>4</sub> solution (as test solution) when inoculated with the bacterial immobilized cellulose Paper Discs (PDs), produced a change in colour in the reaction mixture after 24 hours, which showed conversion of ferricyanide into ferrocyanide.

### **4.2. Analytical performance of *E.coli* immobilized silica 60 in visual inspection of colour change**

Amorphous *E.coli* immobilized powder of Silica 60 was inoculated in 20µl of 1:4 diluted CuSO<sub>4</sub> and 10µl potassium ferricyanide solution. After 24 hours, only a minor change in colour was observed in the reaction mixture. This was inferred to the low immobilization capacity of bacterial cells in silica 60.

### **4.3. Analytical performance of *E.coli* immobilized acrylic beads in visual inspection of colour change**

The *E.coli* immobilized acrylic beads were inoculated in 20µl of 1:4 diluted CuSO<sub>4</sub> and 10µl of potassium ferricyanide solution. After 24 hours, the reaction mixture showed a change in

colour.

We then checked for a change in colour with a reaction mixture that contained 20µl of 1:2 diluted CuSO<sub>4</sub> solution instead of 1:4 diluted CuSO<sub>4</sub> solution, and after 24 hours, the reaction mixture produced no change in colour. This was inferred to the increased concentration of copper in the reaction mixture which hindered the conversion of ferricyanide into ferrocyanide.

### **4.4. Analytical performance of *E.coli* immobilized polystyrene in visual inspection of colour change**

The *E.coli* immobilized polystyrene fragments were inoculated in 20µl of 1:4 diluted CuSO<sub>4</sub> and 10µl of potassium ferricyanide solution. After 24 hours, the reaction mixture showed an obvious change in colour.

We then checked for a change in colour with a reaction mixture that contained 20µl of 1:2 diluted CuSO<sub>4</sub> solution instead of 1:4 diluted CuSO<sub>4</sub> solution, and after 24 hours, the reaction mixture produced no change in colour. This was also inferred to the increased concentration of copper in the reaction mixture that hindered the conversion of ferricyanide into ferrocyanide.

## **4.5 Bacterial cell entrapment and adsorption efficiency and stability on cellulose paper discs, silica 60, acrylic beads, and polystyrene**

The entrapment capacity of bacterial cells on the chosen solid hydrophobic matrices was seen to be different. We checked it by putting each *E.coli* immobilized solid matrix through a growth test in Nutrient Broth.

For cellulose paper discs, we checked the entrapment capacity through inoculation in 10ml nutrient broth containing test tubes and later on incubating them at 37°C. We then checked for bacterial growth in the broth after 24 hours. The presence of *E.coli* pellicle in the broth confirmed the successful immobilization of *E.coli* on cellulose paper discs.

Entrapment capacity for silica 60 was also checked in the same way as for cellulose paper discs. After 24 hours of incubation of silica 60 in nutrient broth containing test tubes, we observed slight turbidity in the growth media, which confirmed that *E.coli* immobilization had occurred but at a much lower scale in contrast to the cellulose paper discs.

Likewise, *E.coli* immobilized acrylic beads and polystyrene inoculation in nutrient broth and their incubation at 37°C for 24 hours resulted in significant turbidity of the growth media, confirming successful entrapment of *E.coli* cells on the matrices.

The highest entrapment capacity was observed with cellulose paper discs as compared to the other three matrices as it produced a characteristic *E.coli* growth pellicle in nutrient broth.

## **4.6. Validation of results using two different concentrations of CuSO<sub>4</sub> solution as model toxic compound**

In order to validate our result, we used two different concentrations of CuSO<sub>4</sub>: 1:4 and 1:2. In the reaction mixture that contained 1:4 CuSO<sub>4</sub>, the conversion of ferricyanide into ferrocyanide occurred with a visible change in colour. Reaction mixtures containing 1:2 CuSO<sub>4</sub> did not produce any change in colour, which

meant that the conversion of ferricyanide into ferrocyanide did not take place. This can be validated by using different concentration ranges of other heavy metal compounds apart from copper.

## Conclusion

In this research, *E.coli* cells (used as model bacteria) were confined on cellulose paper discs, Polystyrene, Acrylic beads, and Silica 60; and chromatic changes corresponding to bacterial ferricyanide reduction reaction was used for toxicity diagnosis. *E.coli* cells were stably confined on Cellulose paper discs and showed good viability, whereas Polystyrene and Acrylic beads also showed good viability in 1:4 Copper Sulphate solution and much better viability in 1:2 Copper Sulphate solution. Silica 60 immobilization capacity was less than other supports, so it showed poor viability in a 1:4 copper sulphate solution. After validation of results with different concentrations of CuSO<sub>4</sub> solution and checking entrapment capacity of the supports in nutrient broth, we conclude that cellulose paper discs showed the best detection results compared to acrylic beads, polystyrene and silica 60. Silica 60's entrapment capacity was the least among other supports, that is why it showed poor heavy metal detection results hence the reason for being considered inferior as a Bacterial cell support matrix.

## Abbreviations

1. PDs: Paper Discs.

## Declarations

## Ethics Approval and Consent to Participate

Not Applicable.

## Consent for Publication

Not Applicable.

## Availability of Data and Materials

Not applicable. Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

## Competing Interests

The authors declare that they have no competing interests.

# Funding

Funding for this study was provided by Shaheed Zulfikar Ali Bhutto Institute of Science and Technology (SZABIST).

# Author's Contributions

PS, ZK and MS prepared all the chemical solutions needed in this study and carried out the immobilization processes together. MS analysed and interpreted the results regarding the chromatic assay. All authors contributed equally in writing the manuscript; and have read and approved the final manuscript.

# Acknowledgments

The authors of this study would like to acknowledge and express their sincere gratitude towards their supervisor Dr. Noman Syed for providing his esteemed guidance and assistance at every stage and in validating the results. Without his constant support and supervision, this research study would not have taken place.

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## Figures

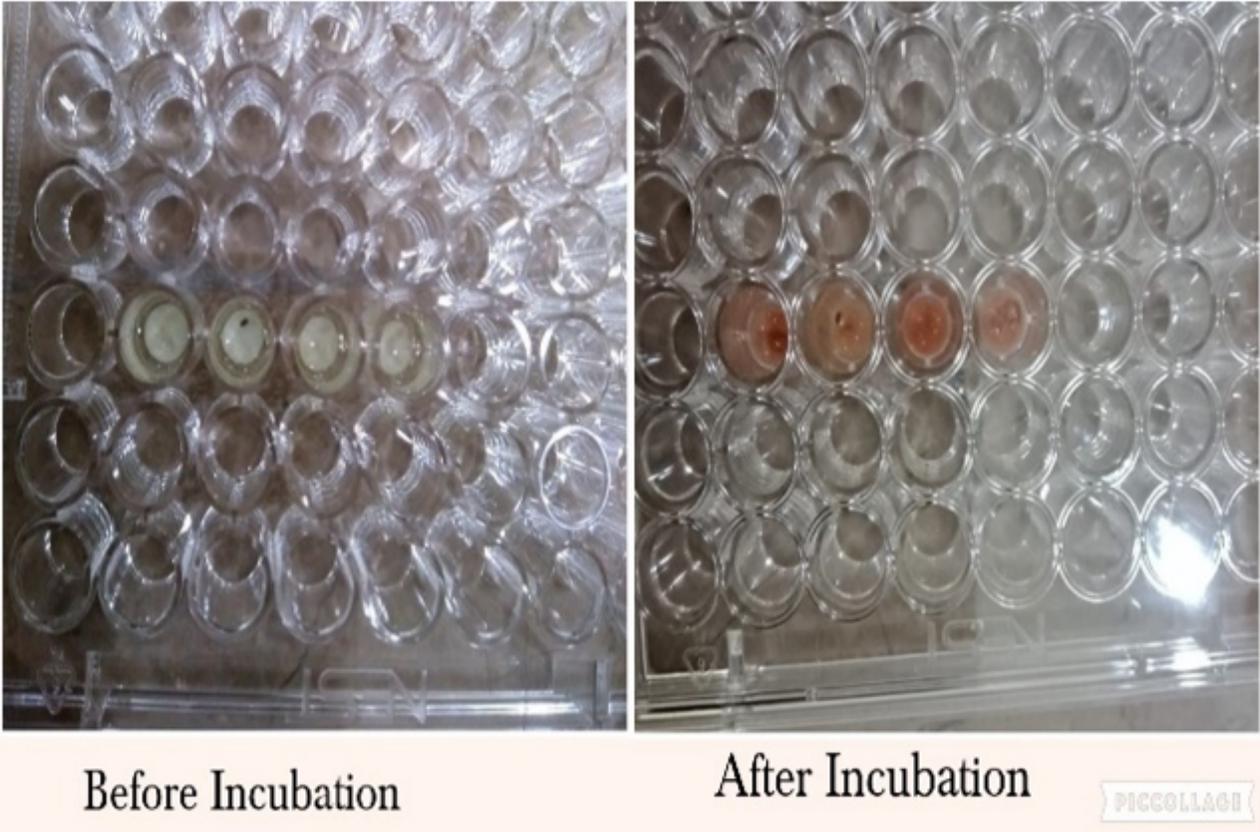


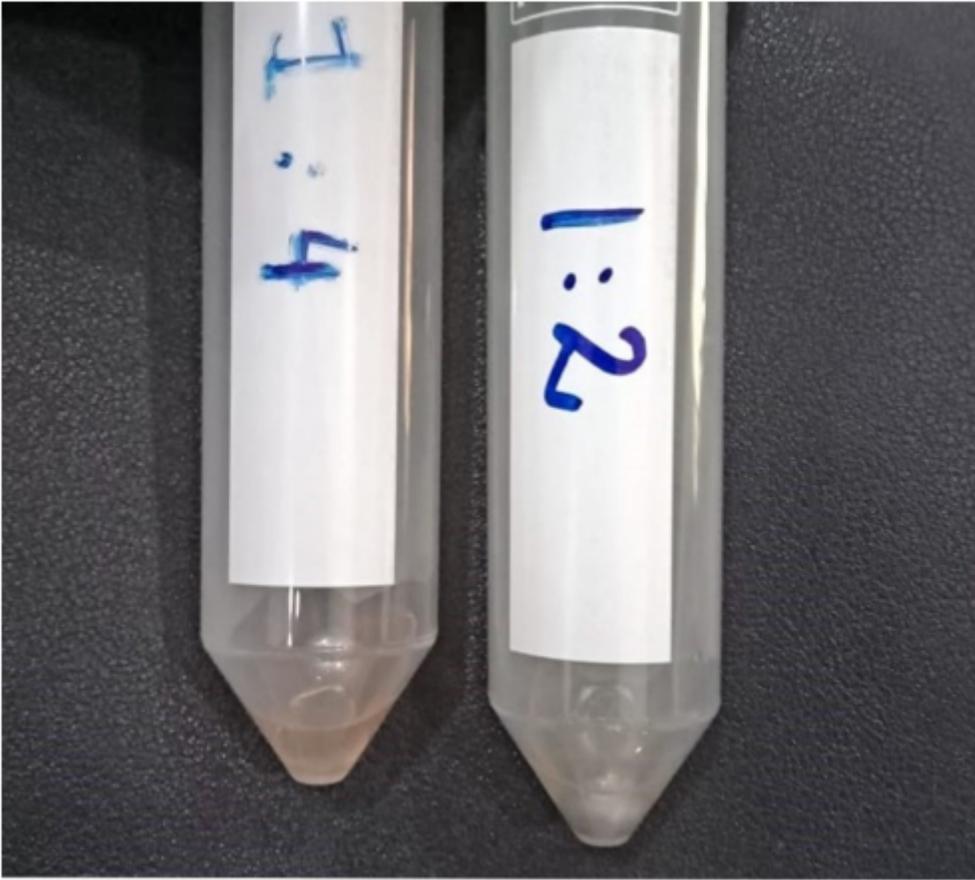
Figure 1

Visual detection of results with immobilized cellulose paper discs.



**Figure 2**

Visual detection of results with immobilized silica 60



**Figure 3**

Visual detection of results with immobilized acrylic beads.



**Figure 4**

Visual detection of results with immobilized polystyrene



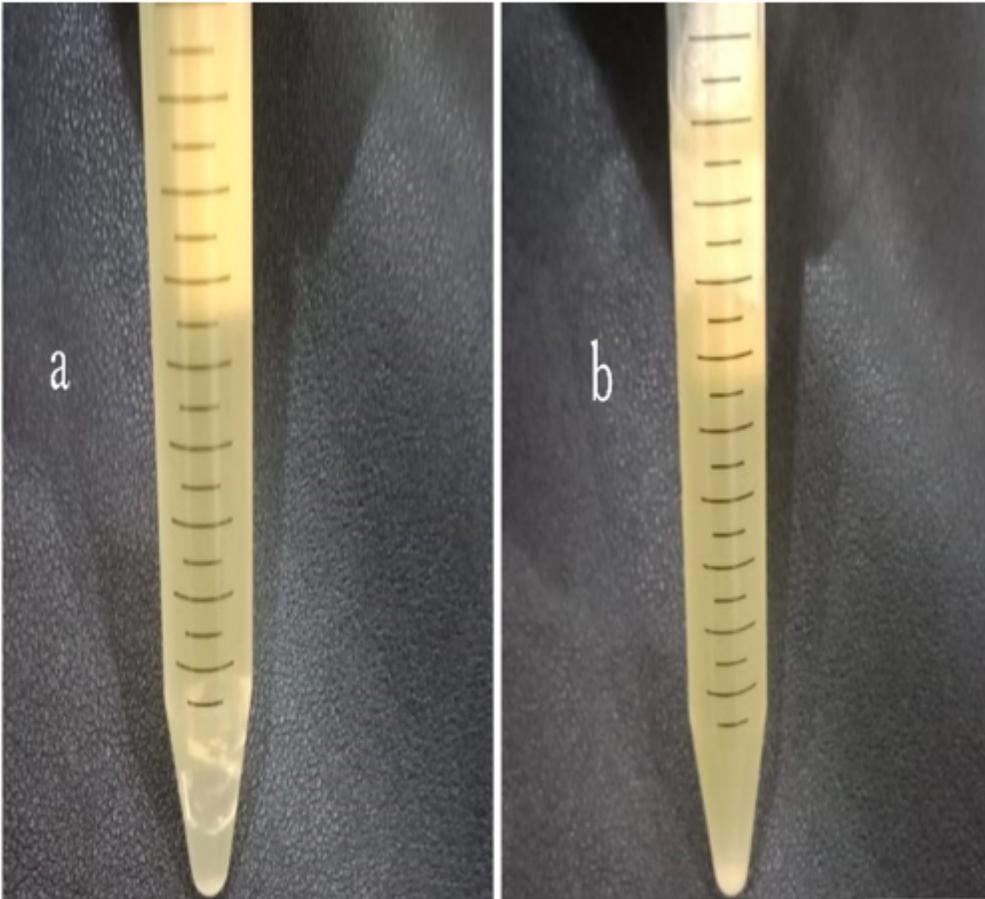
**Figure 5**

E.coli pellicle from immobilized cellulose paper discs



**Figure 6**

Turbidity from immobilized Silica 60



**Figure 7**

Turbidity in growth media (a) from immobilized Acrylic beads and (b) from immobilized polystyrene

## **Supplementary Files**

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