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Microfluidic electrochemical device for real-time culturing and interference-free detection of *Escherichia coli*

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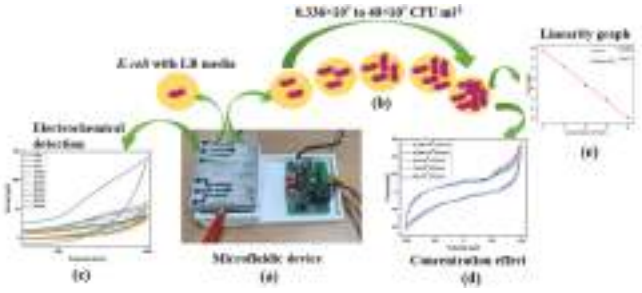
HIGHLIGHTS

- A novel microfluidic electrochemical device for *E. Coli* detection is presented.
- Incubator-free bacteria culturing and monitoring of the growth were achieved.
- A linear bacterial concentration ranges of 0.336–40 CFU/fL was attained.
- A detection limit of 0.35 CFU mL^{-1} was achieved.
- The platform continuously monitors the bacterial growth even in real sample.

GRAPHICAL ABSTRACT



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ABSTRACT

Bacterial contamination and infection is a major health concern today leading to the significance of its detection. Being lab-based bacterial culturing processes, the present approaches are time consuming and require trained skillset. An economical, and miniaturized lab-on-chip device, capable of simultaneous detection of bacterial growth, could be a benchmarking tool for monitoring the bacterial contamination. Herein, the microfluidic-based electrochemical device for a fast, susceptible, detection of *Escherichia coli* was developed. The device could aid incubator free bacteria culturing in the ambient atmosphere and simultaneously monitor and detect the growth electrochemically. A three-electrode system, integrated with a reservoir and a portable thermostat temperature controller was fabricated and assembled. To achieve this, three-electrodes were embedded on the microfluidic device by screen-printing carbon paste, and the working electrode was enhanced by graphitized mesoporous carbon. Cyclic voltammetry response was noted as the function of concentration and growth of *Escherichia Coli* in the reservoir. The device gave a linear bacterial concentration range of 0.336×10^{12} to 40×10^{12} CFU mL^{-1}, detection limit of 0.35 CFU mL^{-1} and the quantification limit of 1.05 CFU mL^{-1} which was less than the maximum allowable limit. The developed platform was further used to detect and continuously monitor the bacterial growth in the real sample (mango juice) for a period of 36 h. Finally, the

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interference from other common bacteria on the electrode selectivity was also investigated. Such approach in being further modified for specific sensing of bacteria in patients suffering from different diseases such as corneal ulcers, Diarrhea, tuberculosis, leprosy, and syphilis.

1. Introduction

Bacterial infection is a worldwide health concern. It causes significant morbidity and mortality, especially for those who are vulnerable [1]. With septic shocks, every hour of antibiotic treatment delay raises the risk of death of patient by 7.65% [2]. Failure to treat bacterial infections in their early and vital stages can be harmful due to their virulence factor, highlighting the need for new treatments to develop [3]. To evaluate the bacterial concentration, many approaches are utilized, such as plating, optical imaging, or biological alterations, which are costly and require expertise in handling. Moreover, their instruments are often bulky, expensive, and difficult to work with and they lack the ability to miniaturize for the point-of-care (POC) applications. The absence of protocol plus procedure uniformity also leads to differences in outcomes. Therefore, there is a pressing demand for diagnostic devices that are compatible with clinical microbiology lab workflow and competent for identifying bacteria concentration and growth in a rapid, real-time, high-throughput, and contact-free manner. [4,5].

Clinical assessment, medical examination, microbiology, biomedical techniques, and diagnostics have all been changed by the introduction of lab-on-chip microfluidic technology. Microfluidic technologies have recently been utilized a solid platform for collective therapeutic applications, together with clinical microbiology owing to inherent characteristics like transportability, minimum patient sample requirements, miniaturizing manual interference, profitable, and increased sensitivity, specificity, and greater output. Through the growing productivity of clinical practices, clinical assessment of serum and urine biochemical testing for resolving contagious diseases, drug development, microbiology, and pathophysiology studies are becoming totally automated [6–14]. After continuous work, microfluidic chip has been developed which can detect bacteria [15]. and its growth with high sensitivity. For microorganism detection, automated microfluidic based DNA extension/purification chip was designed but was less effective and had poor sensitivity. Another article produced a paper-based sensor for bacterial lipopolysaccharide detection, but because being a paper-based device, it is disposable and has limitations to use it for multiplexed analysis [16, 17]. Compare to this, more effective and sensitive as well as being portable device has been developed in this work.

Various bacterial species, like *Pseudomonas, Staphylococcus Aureus*, *Bacillus Cereus*, *Escherichia coli*, *Shewanella Putrefaciens*, and *Streptococcus Pneumoniae,* are the most common bacterial species that causes infection. Amongst these, *Escherichia coli* is the greatest prevalent microorganism with the maximum rate of infection and difficulties [18]. The bacterium *Escherichia coli* is a cylindrical-shaped pathogen that usually originates in the environment [19]. These pathogens can be found in contaminated food and untreated water, as well as in the open environment. Although the majority of *E. Coli* bacteria are considered symbiotic, a few have negative effects on human and animal health [20]. Diarrhea, fever, vomiting, and stomach cramps are all symptoms of *E. coli* infections [21]. *E. coli* survive without oxygen and is commonly present in the large intestine of humans. Their excess presence in the body can result in catastrophic illnesses such as meningitis, anemia, urinary tract infection, corneal ulcer, and kidney failure [22].

Food and drug administration regulatory authorities and manufacturers have undertaken numerous efforts to reduce the risk of foodborne diseases by adapting good manufacturing practices and safety hazards analysis, and risk assessment programs [23]. Nonetheless, minimizing the occurrence of microbial contamination is a difficult task. As a result, detection methods plays an important role in preventing and identifying foodborne infections.

Polymerase chain reaction (PCR) [24], Surface plasma resonance (SPR), [25] Enzyme-linked immunosorbent assay (ELISA) [26], Surface-enhanced Raman scattering (SERS) [27], Microarrays, and biosensor [28]. are some of the new laboratory techniques that have emerged in recent years. In contrast, while the present methods are precise and more prone, the high cost, schedule commitment, also specialized person requirements makes it difficult to put them to use in the field. To address these complications, new, simple, low cost, and effective technologies for detecting bacteria are required. Recent electrochemical approaches can be used in conjunction with bacterial cultivation. Electrochemical detection is becoming more common due to its benefits, which include faster analysis times, higher sensitivity, and fewer samples required. Hence, several reports are present in the literature for detection of *E. coli*. Table 1 gives the summary of some of the recent advances in this context.

This research presents a streamlined process of bacterial detection and growth monitoring in diverse conditions. The bacteria *E. Coli* of $DH_5\alpha$ strain with an optical density (OD-0.10) concentration of $0.336–40 \times 10^7$ were injected into the microfluidic reservoir and placed over the aluminum block. Aluminum block with heater and temperature sensor revealed significant potential for temperature monitoring. The present controller-based thermostat maintains a constant temperature of 37^oC. The developed microfluidic device was placed over the aluminum block whereby two holes at its ends were used as a heating medium. One of the ends was equipped with a heater, which constantly provides heat while the other has a temperature sensor, which senses the temperature. The system was equipped with a feedback system that cuts off the heater once the required temperature is attained. The alligator clips are connected to the electrode and cyclic voltammetry testing is performed [36,

Table 1
Several published publications describing electrochemical measurement of Escherichia coli utilising various electrodes and the electroanalytical methods were compared.

Method	Electrode Used	Concentration range (CFU mL^{-1})	LOD (CFU mL^{-1})	Analysis time (Minutes)	References
CV, CA	Ni foil	6.4×10^4 to 3.3×10^9	10^4	–	[29]
CV.CA	Au electrode array	1.5×10^5 to 1.7×10^6	10^3–10^4	8 min	[22]
EIS	3D gold Nano/ micro islands	1×10^2 to 1×10^4	2×10–1×10^5	–	[30]
EIS	Platinum electrode	10^2 to 10^5	10^2	10 min	[31]
CV	PI-5-CA/C-SWCNT	2.98×10^1–2.98×10^7	2.5	–	[32]
CV	ITO/PET	1×10^3 to 1×10^9	–	30 min	[33]
SWASV	PPCPE	1×10^3 to 1×10^7	8×10^2		[34]
CV, SWV	GMC	2.52×10^3 to 25.2×10^4	50.40	30 min	[35]
CV	GMC	0.336×10^{12} to 40×10^{12}	0.35	–	This work

CV: Cyclic voltammetry, CA: Chrono amperometry, EIS: Electrochemical impedance spectroscopy, PI-5-CA: Poly-5-carboxy acid, C-SWCNT: Carboxylated single-walled carbon nanotubes, ITO/PET: Indium tin oxide/polyethylene terephthalate, SWASV: Square wave anodic stripping voltammetry, PPCPE: Porous pseudo carbon paste electrode, SWV: Square Wave Voltammetry, GMC: Graphitized mesoporous carbon.

37].

Herein, an electrochemical detection was used to detect the growth and concentration of bacteria. The benefits of this study includes real-time, contactless, precise, and consistent detection of bacteria concentration in a range of microbial analyses. graphitized mesoporous carbon (GMC) was used to modify working electrode. GMC is a porous carbon nanomaterial. possessing characteristic physicochemical features and large surface area. The screen-printed carbon ink electrode is modified with GMC and utilized for the measurement of the growth of *Escherichia coli*. The GMC modified electrode has been validated to be a trustworthy and effective method for detecting *E. coli.* For the current study, several critical metrics like repeatability, reproducibility, sensitivity, and stability were perfectly attained. [35] Interaction of other bacteria such as *Shewanella putrefaciens* and *Streptococcus pneumoniae* were also investigated. A real sample analysis with pure mango juice was carried out. This research enables us to continue developing a quick, convenient, and diagnostic tool for the medical investigation of biofluids in microbial study, which may be used for both quick detections of bacterium and evaluation of the interference of bacteria, and the resistance of different antibiotics and the effectiveness of antibiotic using antibiotic susceptibility testing.

2. Methods and materials

2.1. Materials

Purified *Escherichia coli* was obtained from the Biological Science Department, BITS-Pilani, Hyderabad campus. Sodium phosphate monobasic dehydrate (NaH_2PO_4. $2H_2O$), and Sodium phosphate dibasic dehydrate (Na_2HPO_4. H_2O) for pH modification were obtained from S R Life sciences. The growth media like Luria Bertani (LB) Broth and LB broth with agar (Miller) were purchased from Sigma Aldrich. The polydimethylsiloxane (PDMS) and curing agent were acquired from Dow Corning, USA. PVC (polyvinyl chloride) and PMMA (polymethylmethacrylate) sheet were obtained from sigma Aldrich. Conductive carbon paste was procured from Engineered Materials system, Inc. Ag/AgCl was procured from ALS Co. Ltd., Japan, and Graphitized Mesoporous Carbon (GMC) was procured from Sigma Aldrich. Corning microscope slide 70 × 50 mm was kindly provided by Sigma Aldrich. A CO_2 laser (VLS 3.60) was purchased from Universal Laser Systems, AZ, USA.

2.2. Methodology

2.2.1. Fabrication of screen-printed electrodes

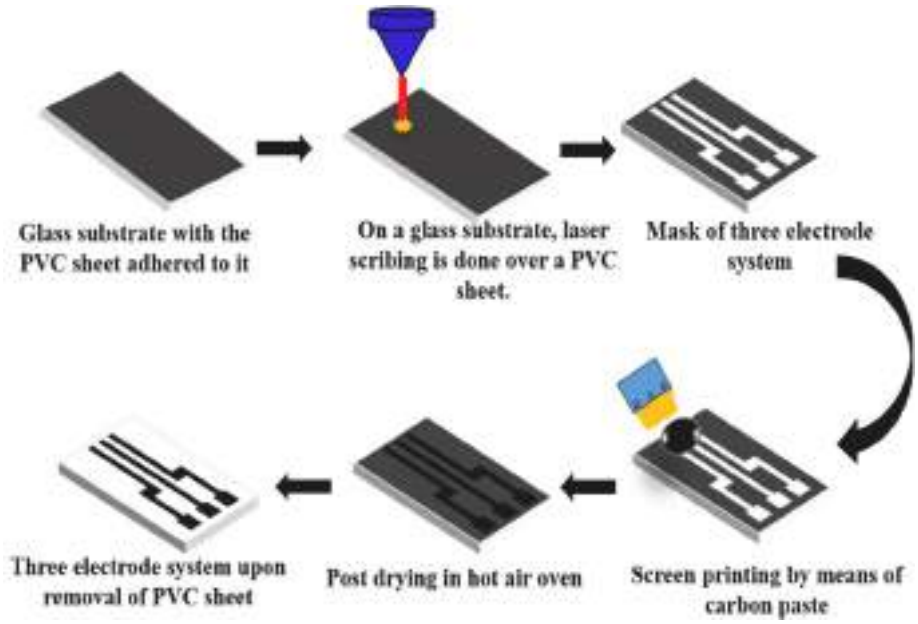
Screen printed technique has been used for the fabrication of a three-electrode system. Initially, a mask of the dimension 1000 μm in width with a spacing of 3000 μm between the two electrodes and thickness of 50 μm was prepared. The surface area of the electrode for the electrochemical reaction was around 0.2 mm^2. The mask was prepared on polyvinyl chloride (PVC) sheet adhered to a glass substrate using a CO_2 laser system. After that, using a squeegee, the carbon paste was applied over a mask. Subsequently, the substrate was kept in a hot air oven at 65° c for 60 min. After dying, the PVC sheet was removed leaving the three-electrode substrate on the glass slide. The conductivity of electrode was identified as 3.06×10^3 S/m. Scheme 1 displays the schematic of the fabrication method of screen-printed electrodes. In order to perform electrochemical analysis, from three electrodes one was modified with Ag/AgCl ink, to work as a reference electrode. Another electrode was modified with GMC as a working electrode and screen-printed carbon ink was a counter electrode. The electrodes were placed in the hot air oven at 65 °C for a period of 15 min for drying.

2.2.2. Microfluidic device fabrication

A microfluidic device was made of PDMS and glass substrate due to their durability and well-studied performance as a microfluidic device. The reservoir was 2 mm wide,17 mm in height, and 1.4 mm in length with a capacity of handling 476 μl of fluid. First, the design of the reservoir was prepared in CorelDraw software. The pattern was cut on a PMMA using a CO_2 laser. The microfluidic reservoir was prepared from a 10:1 ratio of PDMS to the curing agent. The unwanted gas was removed from mixture using desiccator. The PDMS polymer was transferred into the mould and heated at 65°C for 1 h. The reservoir was engraved out and 2 mm punches were drilled into the hardened polymer using a blunt needle and was bonded to the glass slide with screen-printed electrodes by treating the surfaces in the presence of oxygen plasma. The real image of the final microfluidic device with the integrated electrodes is shown in Fig. 1.

2.2.3. Fabrication of heater based on aluminium block

An aluminum block with two holes at its ends is used as a heating medium. One of the ends is equipped with a heater, while the other has a temperature sensor. The system is equipped with a feedback system that



Scheme 1. A step-wise process for fabrication of a screen-printed three-electrode system.

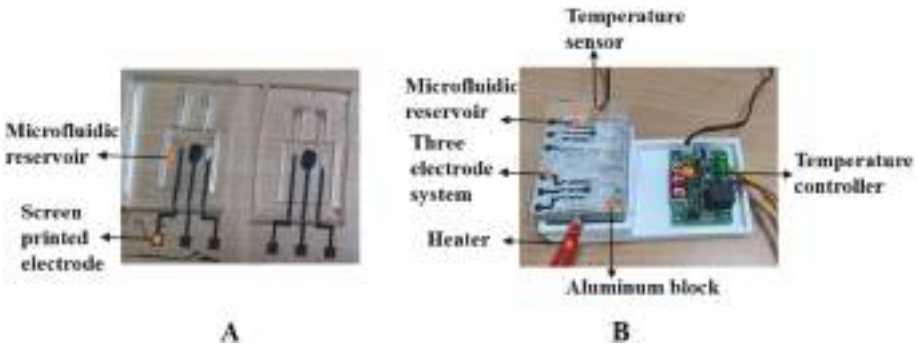


Fig. 1. (A) Microfluidic reservoir bonded to the modified screen-printed electrode (Three-electrode system, working electrode modified with GMC and reference electrode with Ag/AgCl) over the glass slide using plasma treatment (B) the microfluidic device integrated with screen-printed electrodes, aluminum block and thermal management system (Heater and temperature sensor) supported by the controller.

cuts off the heater once the required temperature is attained [36].

2.2.4. Experimental setup of the device

The microfluidic-based reservoir was secured onto the aluminum block with the help of an adhesive tape. The heater and temperature sensor were connected to the Aluminum block. The minimum voltage (12v) was provided through a direct current (DC) voltage booster. The temperature was maintained at 37°C for all sets of experiments. The entire setup can be seen in Fig. 1. The heat generated at this temperature was efficient to grow bacteria.

2.2.5. Bacterial sample preparation

The bacterium species utilized in this experiment was the *E. coli* $DH_5\alpha$ strain. During the preparation, 200 μl of *E. coli* $DH_5\alpha$ culture was inoculated into a 20 mL LB broth and incubated (37° C, using a continuous shaker at 180 Rotation Per minute) for about 36 h. Eventually, the optical density was determined by a smart digital photo colorimeter. Thereafter, the recently cultured microbial sample was diffused and submerged in a pH 7 phosphate buffer solution (PBS). Finally, the microbial cultured sample was diluted in a different concentration from 0.336×10^7 CFU mL^{-1} to 40×10^7 CFU mL^{-1}. CFU mL^{-1} is the most used unit for measuring *E. coli* bacterial concentration. A colony-forming unit is referred to as a CFU. The current changes on the surface of electrode was recorded as function of concentration.

Scheme 2 displays the diagrammatic representation of the serial dilution and plate count. The primary objective of the serial dilution method is to determine the unknown concentration the of a sample (number of bacterial colonies, organism, bacteria, etc.). A series of dilutions (10^8-10^{12}) were made from the original inoculum and to count the presence of microbes in the solution by a plating method. Six agar plates were prepared 100 μl of the sample was speeded over the agar plate from each diluted tube. The plates were kept in an incubator at 37°C for 12 h and formed colonies were counted by the naked eye (manually). The ideal number of colonies to count in a sample is between 30 and 300. If a plate has more than 300 CFU, colonies would become crowded and overlapping [38]. The CFU can be calculated by,

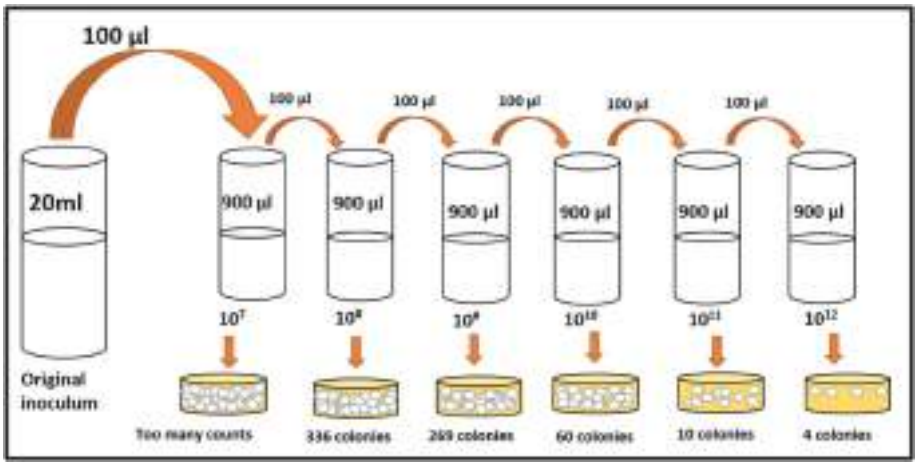
Number of microorganism(CFU mL^{-1}) = (no. of bacterial colonies × total dilution factor)/ volume of cultured plate in mL

2.3. Electrochemical analysis

A potentiostat (Origaflex 500 from Origalys, France) was employed for the electroanalytical measurement. A screen-printed three-electrode system, comprising of GMC electrode, Ag/AgCl as reference electrode, and carbon ink as a counter electrode, were used throughout the electrochemical analysis. GMC has a large surface area, higher void space, and good heat resistivity, which increases its activity manifold in performing functions [39]. The Phosphate buffered saline (PBS), containing a composition of 2.171 g of sodium phosphate dibasic dehydrate and 1.171 g of sodium phosphate monobasic dihydrate of concentration 0.1 M, was used for measurement. Electrolyte pH 7.4 was modified using 0.2 M sodium hydroxide.

2.4. Detection in the real sample

Food such as juices is often connected with foodborne outbreaks



Scheme 2. Diagrammatic picture of serial dilution and plate count.

[40]. Mango juice was procured from the supermarket. The pH of mango juice was adjusted to 6.94. *E. coli* colonies were scraped out from the plate and inoculated in mango juice. The prepared sample was injected into the microfluidic device. Then the electrochemical detection was carried out for the prepared samples.

2.4.1. Field emission scanning electron microscopy characterization of graphitized mesoporous carbon modified electrode

The GMC solution was prepared by the addition of 2 μg of GMC powder to 0.5 mL of 99% ethanol and sonicated for 20 min. Then 0.01 mL of the dispersed solvent was dropped over the screen-printed carbon ink electrode surface and dried in air for 60 min. As a result, sample was ready for the SEM analysis.

3. Results and discussion

3.1. Microscopic study of graphitized mesoporous carbon modified electrode

Fig. 2 illustrates the FESEM (field emission scanning electron microscopy) images of GMC modified electrode. The FESEM examination was carried out to evaluate the physicochemical characterization of diverse nanomaterials, as well as to compute the value of components quantitatively [41]. the image displays that the electrode surface was homogenous and covered with mesoporous carbon.

3.2. Electrochemical analysis of bacteria with graphitized mesoporous carbon

A syringe was used to transport the sample within the microfluidic reservoir. Before the trial, the reservoir was cleaned in 0.1 M PBS (pH 7.4) for 15 min. The prepared bacterial inoculated sample was injected into the microfluidic device and temperature (37 °C) was provided using the thermostat. An electrochemical measurement was directed by means of CV for a period of 36 h at an interval of 4 h to investigate the effect of growth in bacteria with respect to time. Fig. 3(A) shows a voltammogram performed for *E. coli* samples at different time intervals. With time and a constant supply of temperature, the concentration of the bacteria would be multiplying in the microfluidic device. In the graph, the peak

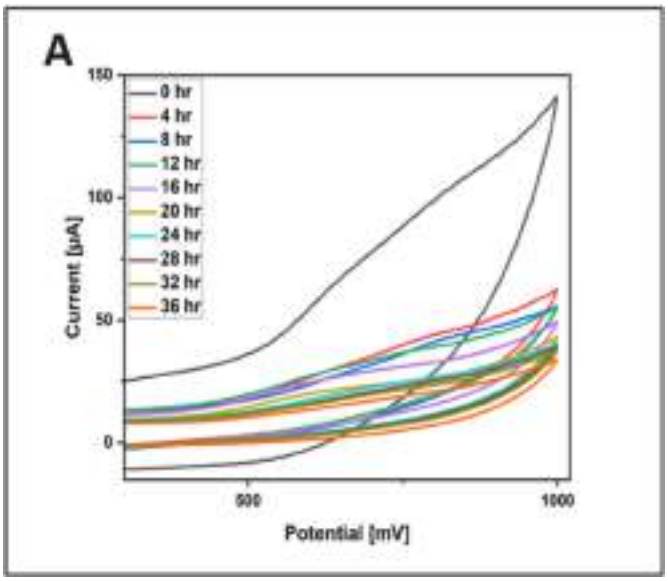


Fig. 3. (A) Cyclic voltammogram recorded for 36 h in microfluidic devices.

current values are gradually reduced with time [33]. Here, the peak current gradually decreases with an increase in time, which must be possible due to an increase in the bacterial concentration in LB media. The reduction of peak current in cyclic voltammetry correspondence to an increase in the incubation time of bacteria [35].

3.3. Concentration effect of Escherichia Coli

For detecting the bacteria in different concentrations, *E. coli* were prepared in PBS buffer (pH 7.44 as well as the Optical density score of 1.12) and inserted onto the microfluidic device. The concentrations of bacteria varied from 0.336×10^{12} to 40×10^{12} CFU mL^{-1} and the cyclic voltammetry was utilized to measure the peak current in the potential range +1 to −1 V, and scan rate of 50 mV s^{-1}. The selected range was sensitive to the GMC electrode below that range detection of bacteria was negligible and above that range, the electrode saturation was

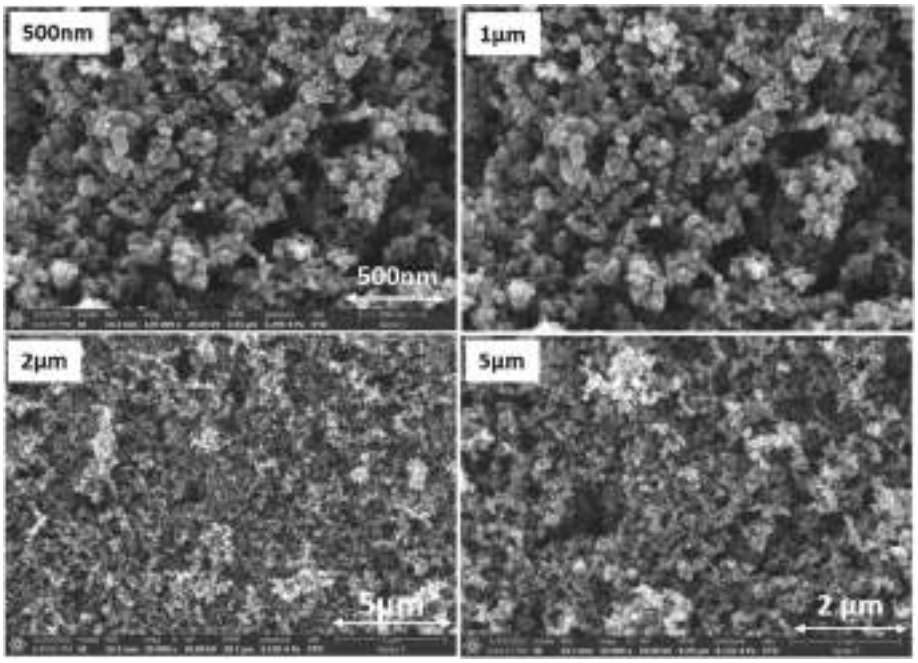


Fig. 2. Microscopic study of GMC modified electrode with different magnification.

observed. Fig. 3(B) shows the CV responses. Herein, the peak current increases as the *E. coli* bacterial concentration raises by *ex-situ* addition. In Fig. 4(A), the lowest concentration was the blue line has the lowest current value. Fig. 4(B) displays the corresponding linear plot relating to the peak current values (μA) and the concentration (CFU mL^{-1}). The correlation coefficient value was determined based on the calibration curve of E_{pa} vs. Conc. The value was observed as $R^2 = 0.997$. This signifies the number of the active site present on the electrode [42]. The limit of detection (LoD)and the limit of quantification (LoQ) were determined using, LoD = 3.3 × σ/slope, LoQ = 10 × σ/slope, and standard deviation represented by σ reported from the least concentration triplicated experiment. The calculated LoD and LoQ of the device are 0.35 and 1.05 CFU mL^{-1} respectively.

3.4. Reproducibility study

To check the consistency of the device the reproducibility study was carried out with different devices. The reproducibility of the device was examined from 6.0×10^7 CFU mL^{-1} of *E. coli*. Electrochemical detection of two devices yielded a reproducible peak current value and the relative standard deviation was 0.41871% (≤10%) presented in Fig. 5(A). The obtained relative standard deviation values redirect the reproducibility of the device.

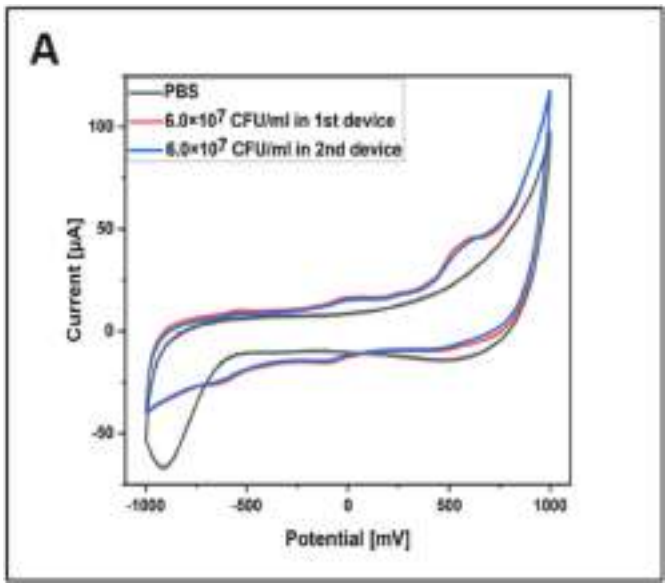


Fig. 5. (A) Cyclic voltammogram for reproducibility study for two different devices.

3.5. Interference study

Interference mitigation in a sensor platform is a vibrant topic for researchers to explore [43]. The specificity of the produced device in detecting *E. coli* was examined. Different variants of bacteria were tested with *E. coli* and in combination with 10^1 CFU mL^{-1} concentration of the selected variant. The same concentration of *Streptococcus Pneumonia* and *Shewanella putrefaciens* was injected into the device and respective peak current responses were evaluated. *Streptococcus Pneumonia* and *Shewanella putrefaciens* are other bacterial species which are quite often associated with infections and food poisoning. Hence, to identify the selectivity of the device in the real time scenario where these similar bacteria may be present, these specific species were chosen Fig. 6 (A and B) demonstrates the corresponding histogram against the *E. coli* and individual variants mixed with *E. coli*. As it can be observed that the bacteria *Streptococcus Pneumonia* and *Shewanella Putrefaciens* don't affect the specificity of the *E. coli* detection.

3.6. Real sample analysis

E. coli is a contagious food microorganism that can effect various organs in human body. A specific difficulty for food manufacturing industry's is that this bacterium may survive in acidic foodstuffs for example, fruit juices, and mayonnaise [44]. The CV measurement was done using real sample including mango juice. Fig. 7 (A&B) shows the signal obtained for the mango juice at different time intervals. As the incubation time increases, the oxidation peak current gradually decreases [32]. Because as incubation period increases, volume of juice reduces, the flow of ions diminishes, and the current value drops. This validates the viability of electrochemical detection in food beverages.

4. Conclusion

Intended for the sensitive measurement as well as culturing of *E. Coli*, herein a simple, easy to fabricate, and a cost-effective miniaturized electrochemical platform with thermal controller has been developed. The developed miniaturized platform was capable of culturing bacteria and detection at the same time. A three electrode system comprised of GMC-modified screen-printed working electrode, Ag/AgCl ink modified screen-printed reference electrode and screen printed carbon ink was the counter electrode. The width of electrode was 1000 μm with a spacing of 3000 μm between the two electrodes and thickness of 50 μm. The use of GMC-modified electrodes improved the sensor sensitivity and surface area. The cyclic voltammetry study was performed to detect bacterial growth. As the raising the concentration, the electrochemical response of *E. coli* bacteria raises. Over the sensing platform LoD, LoQ, reproducibility, and sensitivity were determined. The result demonstrates a linear detection range of *E. coli* concentration of 0.336×10^{12} to 40×10^{12} CFU mL^{-1} with LoD of 0.35 CFU mL^{-1} and LoQ of 1.05 CFU mL^{-1}.

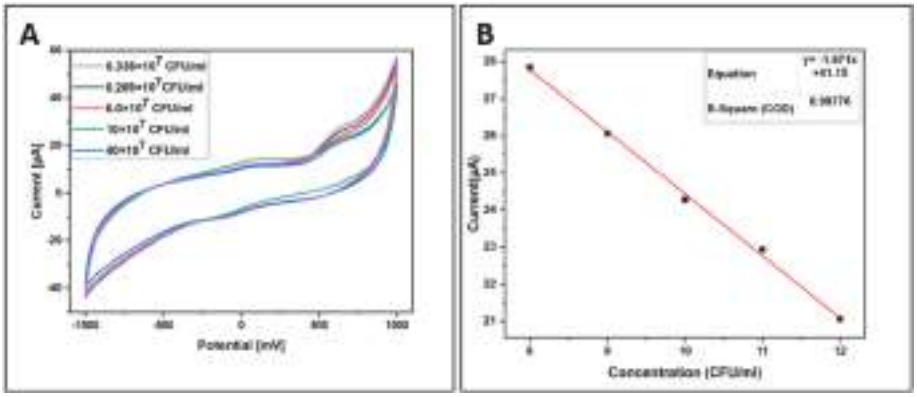


Fig. 4. (A) Cyclic voltammogram of GMC in numerous concentrations of *E. coli* in PBS. (B) Corresponding linear calibration plot for different concentrations.

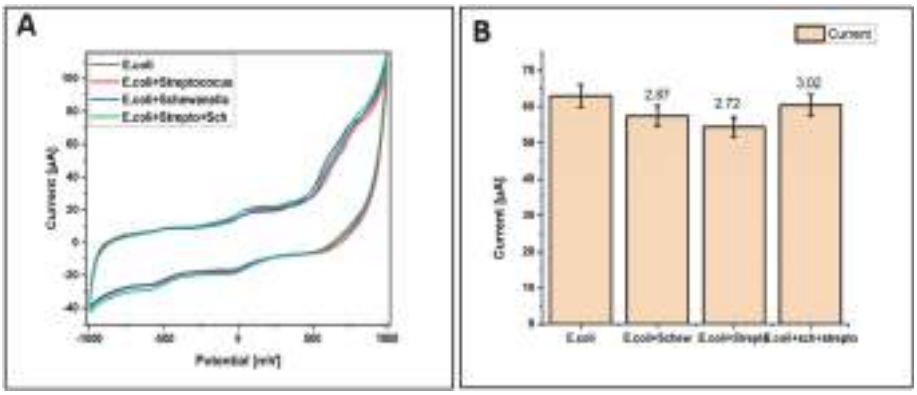


Fig. 6. (A) Cyclic voltammogram for interference study on the modified GMC electrode with two different bacteria (Streptococcus pneumoniae, Shewanella putrefaciens) (B) the Corresponding histogram showing the difference in peak current values associated with *E. coli* and the existence of another interferent of *Shewanella putrefaciens* and *Streptococcus Pneumoniae*.

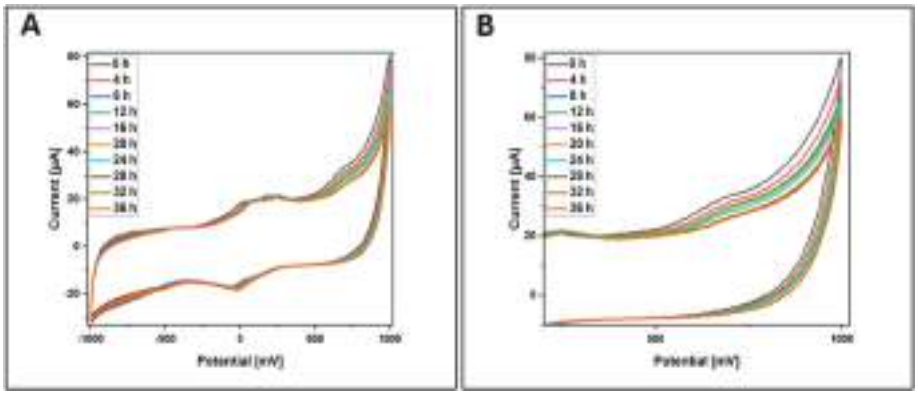


Fig. 7. (A) & (B) Cyclic voltammogram for real sample (fresh mango juice) analysis recorded for 36 h in a microfluidic device in the interval of 4 h. After every 4 h the CV response recorded.

The detected limit has been showed good sensitivity and specificity toward the GMC electrode. GMC significantly increases the overall performance of the sensor and increased the limit of detection 5-fold more than others. The interference study proved the developed sensing platform's high sensitivity toward *E. coli*. The real sample analysis using mango juice was performed showing good reliability.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgment

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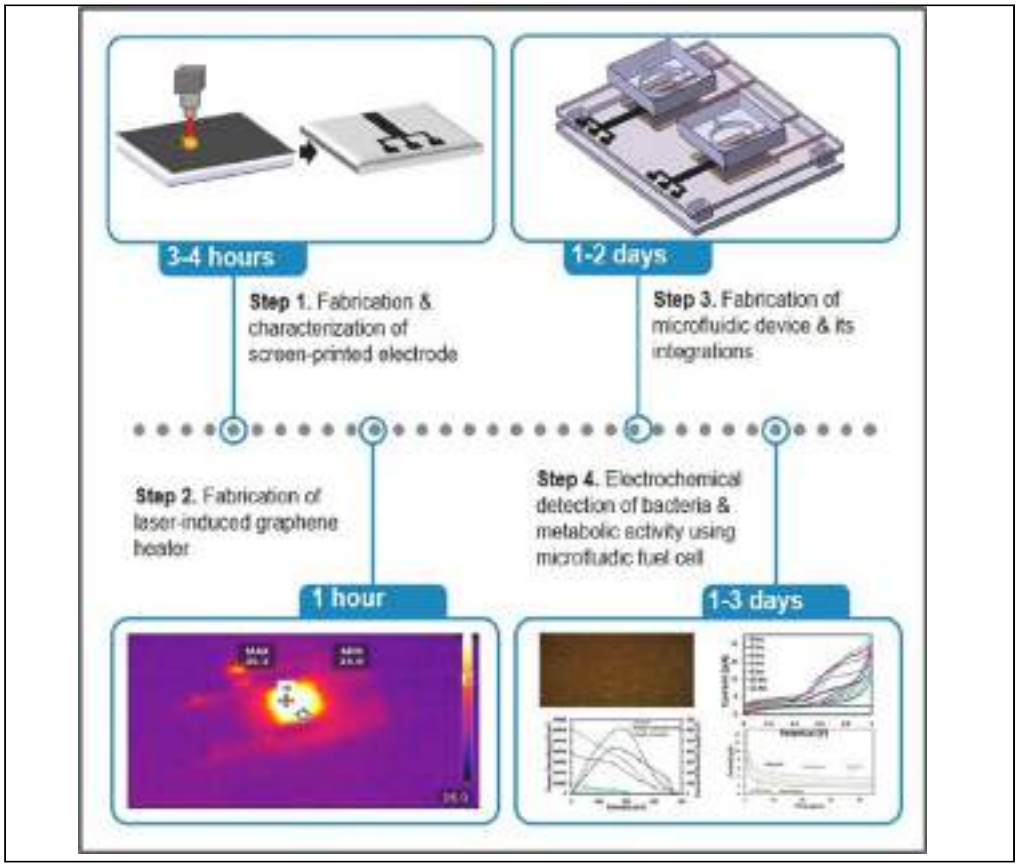
A protocol to execute a lab-on-chip platform for simultaneous culture and electrochemical detection of bacteria



Here, we present a protocol for a miniaturized microfluidic device that enables quantitative tracking of bacterial growth. We describe steps for fabricating a screen-printed electrode, a laser-induced graphene heater, and a microfluidic device with its integrations. We then detail the electrochemical detection of bacteria using a microfluidic fuel cell. The laser-induced graphene heater provides the temperature for the bacterial culture, and metabolic activity is recognized using a bacterial fuel cell.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Simultaneous bacterial culture and detection using a microfluidic device

Electrochemical analysis through screen-printed electrode modified with GMC

Obtain bacteria concentration in the range of 2×10^4 to 1.1×10^9 CFU/ml

Fabricate portable lab-on-a-chip platform by 3D packaging

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Protocol

A protocol to execute a lab-on-chip platform for simultaneous culture and electrochemical detection of bacteria

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SUMMARY

Here, we present a protocol for a miniaturized microfluidic device that enables quantitative tracking of bacterial growth. We describe steps for fabricating a screen-printed electrode, a laser-induced graphene heater, and a microfluidic device with its integrations. We then detail the electrochemical detection of bacteria using a microfluidic fuel cell. The laser-induced graphene heater provides the temperature for the bacterial culture, and metabolic activity is recognized using a bacterial fuel cell.

Please see Srikanth et al.[1] for comprehensive information on the application and execution of this protocol.

BEFORE YOU BEGIN

Microbial detection is crucial in numerous fields, for example, medical research, food technologies, and biochemical detection.[2] The traditional methods, such as colony counting, gram-staining, and another biochemical way like Enzyme linked immunoassay (ELISA), Polymerase chain reaction (PCR) are time-consuming,[3] show transfer errors, and require more labor to count microbial cells in a particular solution. Hence, a valid and sensitive approach to detecting bacteria is critical.[4] Here, a simple, economical, and competent way is used to detect bacteria. The electrochemical analysis[5] is carried out to check the growth of bacteria in intervals of every 2 h up to 12 h, which will be helpful for the early diagnosis of bacterial infection.

The protocol below describes a stepwise procedure for developing a microfluidic-based electrochemical platform for simultaneous cultivation and bacterial growth detection.[6] Here we use the electrochemical method to monitor the growth of bacteria. The bacterial culture with LB broth media is injected into the microfluidic chamber. The same is kept in the conventional incubator every 2 h, of the cyclic voltammetry response is recorded. And the result obtained is compared with a traditional incubator.

Materials

Timing: 1–2 h (for step 4)

Timing: 1–2 days (for step 5)

1. Multi-walled carbon nanotubes (MWCNT) are acquired from Sigma Aldrich, India, and conductive carbon ink is purchased from Engineered Materials System, Inc. The supplier of Ag/AgCl is ALS Co. Ltd. in Japan.
2. Poly (methyl methacrylate) is obtained from Dali Electronics, India. A CO_2 laser (VLS 3.60) is procured from Universal Laser Systems, Arizona, USA. The thermal camera is purchased from Fluke Technologies, India.
3. Potassium Ferricyanide is purchased from AVRA chemicals. Polydimethylsiloxane (PDMS) is purchased from Dow Corning, USA. Luria Bertani is purchased from SRL Chemicals.
4. Preparation of a three-electrode system
   a. Prepare a reference electrode by modifying it with Ag/AgCl.
   b. Prepare a working electrode by modifying it with MWCNT.
      i. Directly drop-cast MWCNT over the electrode surface.
      ii. Leave it to dry at room temperature (20°C–25°C) for 20 min.
      iii. Prepare the counter electrode with plane carbon ink (without any modification).
5. Preparation of bacterial culture medium

This step describes how to prepare a bacterial culture medium.

The Luria Bertani (LB) is the medium employed in this protocol. 5 g of LB powder and 250 mL of distilled water are mixed to prepare LB media.

6. To prepare the bacterium culture,[7] mix 0.2 mL of cultured E. coli bacteria with 20 mL LB media.
7. Keep the sample intact in a CO2 incubator with an orbital shaker, and adjust the temperature and speed to 36°C and 180 RPM, respectively.

   Note: The CO2 incubator provides a stable atmosphere with the following parameters: 37°C, and relative humidity of roughly 95%, which help microbial cell growth.

8. Check OD value every 2 h.

   Note: The culture can be utilized for the experiment once the desired OD (0.8–1) is reached.

9. Next, use the agar plating (Pour-plate) method to detect the colony-forming units. Prepare the agar gel by dissolving 8.7 gm of agar powder in 250 mL of water.
10. Autoclave the dissolved agar solution at 15 psi 120°c for 20 min.
11. Transfer the sterilized autoclaved agar solution into the Petri plates until the agar gel gets solidified.
12. Spread over the 10 μL of over the plate, and keep the containers in the incubator for 24 h at 37°c.

   Note: Sterile condition is required throughout the bacterial culture process.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial strains		
Luria Bertani	SRL Chemicals	29817
E. coli strain	Department of Biology, BITS Pilani Hyderabad campus	-
Agar medium	SRL Chemicals	L3147

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals		
Conductive carbon ink	Engineered Materials System	CI-2001
Ag/AgCl	ALS Co. Ltd., Japan	011464
Multi walled carbon nanotubes (MWCNT)	Sigma-Aldrich	308068-56-6
Polyimide	Dali Electronics	150FNB019
Potassium ferricyanide	AVRA Chemicals	13746-66-2
PDMS and curing agent	Dow Corning, USA	101697
Software and algorithms		
AutoCAD	Autodesk	https://getintopc.com/?s=AutoCad
Other		
CO_2 laser	Universal Laser Systems, AZ, USA	VLS 3.60
Digital photo colorimeter	Medizinn, India	-
CO_2 incubator with an orbital shaker	Eltek orbital shaker incubator	6161-LS
Polyvinyl chloride (PVC) sheet	Sigma-Aldrich	-
Copper tape	Robu Electronics	16045
CHI instrument	CH Instruments, Inc. 3700 Tennison Hill Drive Austin, USA	1030E
PMMA	Dali Electronics	-
Oxygen plasma	Femto Science, South Korea	-
Portable potentiostat (Sensit Smart)	Palmsens	-

STEP-BY-STEP METHOD DETAILS

Fabrication of screen printed electrodes

Timing: 3–4 h

This section describes the fabrication of a three-electrode system using the screen-printing method. The step-by-step procedure is mentioned below:

1. Adhere to the polyvinyl chloride (45 × 300 cm) sheet on the glass slide.
2. Build a mask of the requisite design (Width of 1000 μm, spacing of 350 μm, thickness of 50 μm) using a commercial CO_2 laser on a polyvinyl chloride (PVC) sheet. The design is shown in Figure 1.
3. Spread the carbon ink (The volume chosen here is 300 μL as the surface area of electrodes is 0.2 mm^2) with a squeegee.
4. Afterward, set the prepared mask aside in the laboratory hot air oven for 40 min at 70°C.
5. Remove the PVC film after the ink is dried, keeping the three-electrode system over the glass slide. The details of the fabrication steps are mentioned in Figure 2.
6. For electrochemical detection of bacterial growth, fabricate the three-electrode system.
   a. Treat one of the three electrodes with silver/silver chloride paste.
   b. Modify the working electrode with MWCNT, and dry it for 20 min at room temperature (20°C–25°C).

Note: The carbon ink should be spread uniformly over the electrode surface.

Characterization of a fabricated three-electrode system

Timing: 2 h

The initial electrochemical ferricyanide detection is performed on the developed electrodes to verify the effectiveness of the developed electrodes.

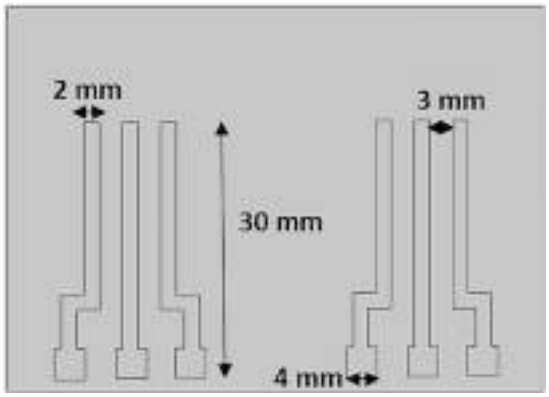

Figure 1. Pattern describing the design of the three-electrode system

7. Prepare 5mM potassium ferricyanide in 1mM potassium chloride.
8. Use a pipette to drop the prepared solution (20 μL) onto the electrode surface.
9. Perform cyclic Voltammetry in a CHI instrument to measure the transfer of electrons at a scan rate of 0.05 V/s.
   a. This indicates redox behavior within the potential range of -0.7 and +0.7 V.
   b. Cyclic voltammetry responses are shown in Figure 3.
10. Evaluate the electrodes for ordinary LB broth at potentials ranging from 0 to 1, and proper media peak is found at a potential of about 0.8 V.

Fabrication of laser-induced graphene heater

Timing: 1 h

The LIG film is developed as a heating material for bacterial culturing.

11. Stick a polyimide sheet of 10 mils × 920 mm thickness to the acrylic substrate using tape.
    a. Carved the polyimide sheet with a CO_2 laser to produce laser-induced graphene.
12. The LIG heater provides a temperature of 37°C, which helps bacteria grow. The temperature is optimized
    a. By giving different voltages
    b. Capturing the image in a thermal camera.

Figure 2. Schematic describing the step-by-step process showing how the three-electrode system is fabricated using screen printing

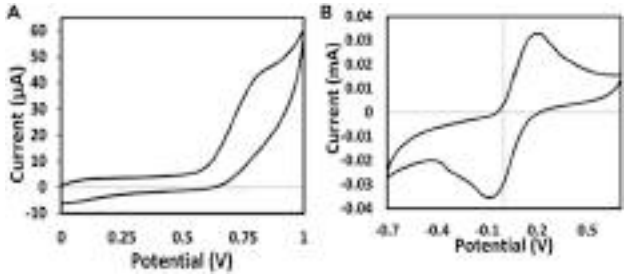

Figure 3. Cyclic voltammograms for plain LB media and potassium ferricyanide

Cyclic voltammograms representing (A) Plain LB media in the absence of bacteria within a potential of 0–1 and (B) Potassium Ferricyanide within a potential of -0.7 to + 0.7.

13. Once LIG pattern is formed over the polyamide sheet,
    a. apply the silver paste
    b. Stick the copper tape to provide electrical contact. The Schematic is shown in Figure 4.
14. Next, 2.3V of potential is applied to the buck-boost converter, which provides a temperature of 37°C.

Note: The temperature of the LIG heater should be maintained at 37°C. The fluctuation in temperature could cause cell death.

Fabrication of microfluidic device and its integration]

Timing: 5–8 h

This section describes the step-wise procedure for the fabrication of PDMS based microfluidic device[8] and its integration on the three-electrode system.

15. Fabricate a microfluidic device using soft lithography.[9]
16. Prepare a design of the desired pattern in AutoCAD software. The pattern of the PDMS device is shown in Figure 5.
17. Cut the pattern on a PMMA (polymethylmethacrylate) using a CO2 laser.
    a. Adhere it to a glass substrate.
    b. Place in a mold per the well-established protocol.
18. Prepare PDMS by mixing the silicone elastomer with the curing agent in a ratio of 10:1 and is thoroughly mixed.
19. Degases the mixture in a desiccator, pour over the mold, then place in an oven at 65°C for curing.
20. Punch the required holes on the inlet and outlet of the PDMS slab after Post curing
    a. Use blunt needles to make holes.
    b. Bond the glass slide with screen-printed electrodes by treating the surfaces in the presence of oxygen plasma.

Bacteria culture in the device

Timing: 1–2 days

Figure 4. Schematic representing the steps for fabrication of the LIG heater

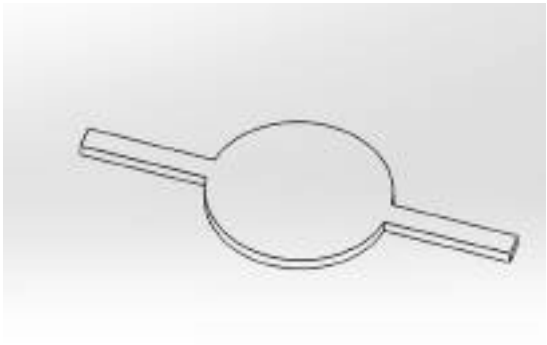

Figure 5. The pattern describing the design of the PDMS device

Note: Prepare Luria Bertani (LB) containing 10 grams of powdered LB broth in 250 mL of distilled water.

21. A culture of E. coli (DH5) is prepared in a Bacterial suspension of OD 1 by adding 0.2 mL of the bacteria to 20 mL of the media. The schematic is shown in Figure 6.
22. After the addition, the sample is well mixed (OD 0.8) and injected into a microfluidic reservoir using a syringe, and the temperature is kept at 36°C using a LIG heater. The response is also electrochemically detected.

Electrochemical detection of bacteria

Timing: 1–2 days

Electrochemical detection uses i) cyclic voltammetry (CV) and ii) chronoamperometry (CA) methods.

23. The inoculum of the *E. coli* is introduced into the microfluidic reservoir and the conventional incubator for 12 h, and the result is compared.[10]
24. The three electrodes are connected to the portable potentiostat (Sensit Smart, Current ranges 100 nA–5 mA, Potential range -1.7 to +2 V), and the CV technique is selected.
25. Next, the software sets parameters like potential 1 to -1, No. of cycle-4, and scan rate- 0.05V.
26. Then, the run starts by clicking on the play button.
27. After completing the four-cycle, the obtained data will be extracted, and the graphs will be plotted.
28. Similarly, the potential is fixed at 0.8V for 120 min for chronoamperometry, and chronoamperometry is performed for different concentrations.

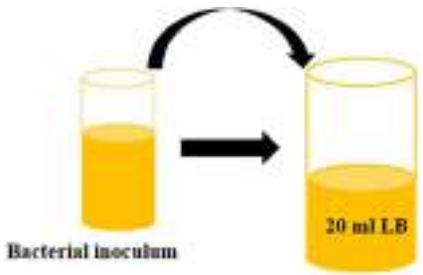

Figure 6. Schematic representing preparation procedure for bacterial culture

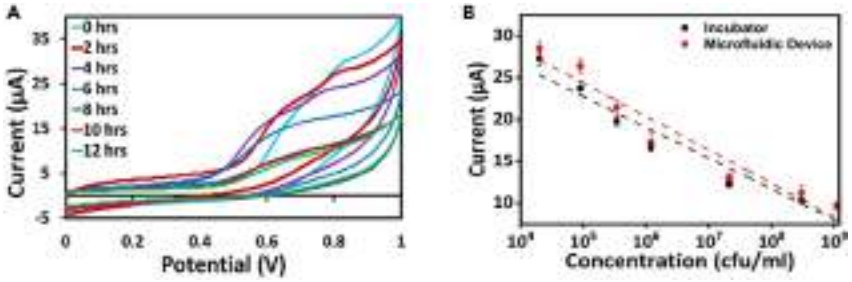

Figure 7. Cyclic voltammogram and calibration plots

(A) Cyclic voltammogram recorded for 12 h corresponding to different concentrations of bacteria ranging from 2 × 10^4 to 1.1 × 10^9 CFU/mL in a Microfluidic device.

(B) Calibration plots with error bars for samples incubated in a conventional and developed device.

29. The responses are recorded and compared with a conventional incubator.

EXPECTED OUTCOMES

Two electrochemical methods, Cyclic Voltammetry,[11] and Chrono Amperometry[12] are used to identify the presence of bacteria in the device.

Both approaches record the peak response, which detects the growth of bacteria in the device by plotting the calibration graph.

Initially, CV is performed for 12 h in the device and conventional incubator to check the growth of bacteria. And the current is measured at intervals of 2 h. The current value obtained is analyzed and compared with the conventional method. Figure 7 represents the plot for the bacteria culture carried out in the microfluidic device.[13] The concentration of bacteria increases over time in both the incubator and the microfluidic device.

In addition to a CV, the chronoamperometry approach is used in traditional and microfluidic devices to assess the characteristics of curves with a rise in bacterial concentration.[14]

Chronoamperometry generally records the current about the time at a fixed potential.[15] The peak is noted at approximately 0.8 V on the CV plots. One hundred 20 s are spent conducting each experiment at a constant potential voltage of 800 mV Figures 8A and 8B demonstrate that the current consistently decreases as concentration increases, similar to CV graphs.

LIMITATIONS

The vitality of the bacteria culture from the device can be further increased by changing the device to have a shaking module duplicating the condition of the conventional incubator. Different E. Coli species cannot be differentiated in the device.

TROUBLESHOOTING

Problem 1

Device fabrication & electrode modification is a manual process hence, the reproducibility for multiple devices is challenging and depends on manual skill (related to step 6).

Potential solution

Using an automated device fabrication approach, we can achieve excellent reproducibility.

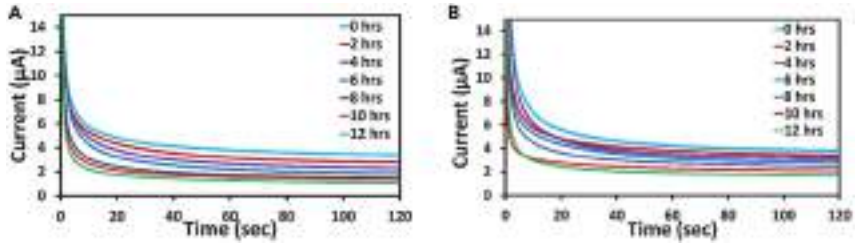

Figure 8. Chronoamperometric curves

Chronoamperometric curves recorded for 12 h corresponding to different concentrations of bacteria ranging from 2×10^4 to 1.1×10^9 CFU/mL in (A) Conventional incubator and (B) Microfluidic device.

Problem 2

How is the movement of the bacteria addressed? What if they move out of the range of the electrodes? (related to step 24).

Potential solution

The movement of bacteria can address by optimizing the design dimension. If they move the out of range, electrode saturation may be happening.

Problem 3

Are bacteria attracted to electrodes? How would this affect the results, or how could it be addressed?

Potential solution

Bacteria are not attracted to the electrode. However, adsorption may happen due to electrostatic forces.

Problem 4

Bonding the PDMS channel to the SPE electrode is difficult and leads to leakage during the experiment (related to step 20).

Potential solution

Minimizing the thickness of the electrode and exposing it for more time in plasma is helpful in perfect bonding. The optimized thickness of the three-electrode system is 50 μm, and the optimal time for plasma treatment is 1 min.

RESOURCE AVAILABILITY

Lead contact

The lead contact, Sanket Goel, can be reached at (sgoel@hyderbad.bits-pilani.ac.in) and will reply to any questions or requests for resources.

Materials availability

This protocol does not generate unique reagents or material.

Data and code availability

The lead contact will, upon request, provide the data provided in this research.

The lead contact will provide any further information needed to refocus the data given in this study.

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AUTHOR CONTRIBUTIONS

S.S., S.F., A.J., S.K.D., and S.G. created the study's design and conceptualized, designed, developed, and evaluated the gadget's performance. S.S. carried out the experiments. J.U.S. researched enzymatic biofuel cells. A.J., S.K.D., and S.G. performed the investigation, visualization, supervision, and document revision.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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