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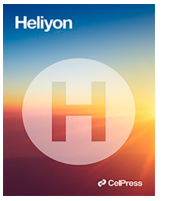

A spectroscopy-based proof-of-concept (POC) for developing loading of pathogen analyzer (LOPA) for dairy products

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ABSTRACT

Detection of bacterial contamination in dairy products of daily use is a challenge worldwide. We have utilized Methylene Blue Reduction Test (MBRT) for quantification of the microbial count in dairy products (milk) and developed a proof-of-concept (POC) based on this for in-filed applications. In this study, we have used pasteurized milk contaminated with model bacteria *Escherichia coli*, and *Staphylococcus aureus* for the calibration and validation of the developed POC. The conversion of MB to Leuco-MB i.e., the colorimetric change due to the reduction of MB to Leuco-MB in presence of microbes has been utilized as the tool to detect presence of microbes in milk. The absorbance peak for methylene blue (MB) at 664 nm decreases significantly in presence of microbes and the blue color becomes faded. In our study, we have employed methylene blue (MB) discolouration phenomenon to estimate the microbial count in milk samples using our developed spectroscopy based POC. The limit of detection (LOD) and the limit of quantitation (LOQ) of the POC were found to be 0.32 CFU/mL and 0.97 CFU/mL. The end users of the developed POC are primarily those involved in the production, processing, testing, regulation, and research of dairy products to ensure they meet safety standards and protect public health. These include retailers, dairy farmers, dairy processors, quality control laboratories, regulatory agencies and research institutions. In our experiment, we have observed a significant change in MB absorption in the milk contaminated with microbes. The indigenously developed sensor strips designed for the working of the POC turn to colorless Leuco-MB compared to milk without the microbes. The analysis of the strips has been measured in the developed device.

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1. Introduction

Dairy products are high in nutrients and promote good health [1–3]. It is well-known that consumption of dairy products improves bone mass, cardiovascular health, and gut microbiota [4]. However, dairy producers and manufacturers around the world are struggling to maintain a high standard of dairy product safety and quality [5]. Some dairy products, like milk and yoghurt, have a very short shelf-life and can easily get contaminated with various pathogenic agents, like bacteria and fungus during production and distribution [6–8]. Improper storage or exposure to heat [9] and light [10] during any part of the supply chain causes fermentation of lactose. Milk oxidation mainly occurs when exposed to natural or artificial light, including sunlight, fluorescent, and LED light. Activated photosensitizers in milk (such as riboflavin) excite oxygen to its singlet state, which actively reacts with other nutrients in milk such as unsaturated lipids, vitamins, and proteins and oxidises them. Light-induced oxidation then rapidly forms off-flavor in milk, which resulted in a profound negative effect on consumer acceptance of milk. Growth of micro-organisms reduces the nutritional value of milk by oxidation of fat which in turn increases the acid levels. Fresh milk pH level usually ranges between 6.6 and 6.8 and below pH level 6 the milk starts to spoil and below 5.5 pH it is unsuitable for human consumption [11]. If milk and its products contain hazardous microbes and their toxins, they might cause significant illnesses in consumers [12,13]. Effective dairy management requires rigorous regulation of the total amount of bacteria in raw milk, which works as a marker of basic hygienic indication [14]. The presence of pathological microorganisms in milk is mainly due to an underlying disease harboured by the cattle such as mastitis. *Staphylococcus aureus* in milk is important for identifying mastitis, which is one of the most common and costly infections in dairy cows, causing severe milk loss, poor milk quality, and an increased risk of cow death. Subclinical mastitis is difficult to detect in the early stages and can be indirectly identified by detecting the presence of *S. aureus* in the early stage of the disease [15]. On the other hand, *Escherichia coli* (*E. coli*) can be transmitted to raw milk and milk products through fecal contamination during the milking process, as well as poor hygiene practices [16]. In addition, mastitis in cows can also be caused by *E. coli* which can be also lead to their presence in milk.

In regulatory testing protocols, the plate count approach [17,18] to evaluate the growths of total aerobic bacteria, psychrotrophic bacteria and lactic acid bacteria is commonly used for assessment of the hygienic quality of raw milk. For detection of *Staphylococcus aureus* and to diagnose cow mastitis, the use of an electronic tongue using the technique of impedance spectroscopy was also explored [19]. However, the procedure was extremely time-consuming and laborious [20]. To identify and monitor the freshness index of milk, electrical conductivity test approach was carried out [21]. However, the method is temperature specific and works at room temperature around 20°C and unable to detect the quality of refrigerated milk [11]. Enzyme-based detection technique was also reported which uses a paper-based sensor to detect alkaline phosphatase enzyme, a marker of pasteurization [22]. Despite being very accurate, it requires costly instruments and skilled workers. Some other techniques are also available but due to their limitation they cannot be brought into the market.

In this regard, bacterial load quantification using colorimetric spectroscopic tool could be advantageous in terms of development towards low-cost field deployable strategy. Colorimetric indicator can be a potential agent for the direct quantification and monitoring of oxygen demand in a sample. Environmental conditions significantly impact the rate of bacterial proliferation. These factors include pH levels, temperature, water availability, nutrient composition, oxygen presence, and the existence of toxins. Oxygen requirements vary among bacteria and can be correlated with bacterial growth, specifically BOD (biochemical oxygen demand). The presence of pathogens was found to have a strong correlation with organic matter levels, particularly in terms of biochemical oxygen demand [23]. This correlation implies a probable rise in oxygen demand alongside intestinal pathogens. Methylene Blue (MB) is a well-known colorimetric indicator which can measure the amount of dissolved oxygen (DO) in solution through the formation of Luco-Methylene blue [24]. It has to be noted that use of MB for the detection of bacteria in milk is well-reported [25–27]. In presence of a suitable reducing/dissolved-oxygen consuming agent, it is converted to Leuco-MB revealing quantitative fading of its blue color. Using spectroscopic studies, the change in DO can be correlated with the amount of Leuco-MB produced in the system. It is worthwhile to mention that, the main limitation of Methylene Blue Reduction Test (MBRT) is the shelf life of methylene blue in solution phase which is approximately 1 month and also its photo degradation effect in response to ambient light.

In the present study, we have developed a simple and affordable technique for quantitative assessment of the microbiological quality in dairy products, where discoloration of methylene blue (MB) to leuco-MB has been used as a sensing tool. Here, we have correlated the discoloration of MB with oxygen demand and thereby with pathogen present in the medium. Further, we have developed a simple and affordable paper-based POC for quantitative assessment of the microbiological quality of dairy commodities, where the cellulose membrane used as substrate was dipped into methylene blue (MB) dye and used as a sensing strips. For the purpose of validation of the POC, two model aerobic gram negative and gram positive bacterial strains, *Escherichia coli* XL1-Blue and *Staphylococcus aureus* MTCC 3160 was used respectively.

2. Material and methods

2.1. Chemicals

All of the chemical reagents, such as Methylene Blue (MB), Ethanol, Ascorbic Acid, and Luria Broth, are of analytical grade and purchased either from Sigma Aldrich or Merck. The chemicals have been used without further processing. The used water is from Millipore system. Whatman filter paper Grade 4 was procured from Sigma-Aldrich. Pasteurized Milk (4 % Fat, 9 % SNF, total carbohydrate 4.9 g, protein 3.1 g, calcium 119 mg, 48 h of shelf life from packaging when kept at 8 °C) was purchased from Amul

Cooperative, Gujrat, India. All experiments were performed using fresh milk samples, within 24 h post pasteurization.

2.2. Characterization techniques

In this study, JASCO V-750 Spectrophotometer is used to collect the absorbance spectra and a Peltier attachment has been utilized for the temperature dependent study. During the liquid state experiments, a quartz cuvette with an optical path length of 1 cm has been utilized.

2.3. Degradation of MB by ascorbic acid

In order to evaluate the reduction of MB, ascorbic acid was used as the reducing agent. 10 μM of MB was used for this purpose. To this MB solution increasing concentration of ascorbic acid (28.4 μM, 56.7 μM and 113.5 μM) was added and the spectra was recorded after 10 min of incubation. In order to obtain the kinetics of degradation of MB to Leuco-MB, 10 μM of MB was subject to same concentrations of ascorbic acid (28.4 μM, 56.7 μM and 113.5 μM) and the kinetics was recorded.

In order to identify the role of dissolved oxygen, decolouration of 10 μM MB in the presence of the fixed concentration ascorbic acid (56.7 μM) at different temperatures (10 °C to 60 °C) was monitored at 664 nm.

2.4. Bacterial strain, culture conditions, and sample preparation

The glassware, suction nozzles, and culture medium were sterilized in an autoclave at a high pressure of 0.1 MPa and a temperature of 120 °C for 30 min before experiments. Bacterial cultures were cultivated in a sterilized LB broth (Himedia, India), and incubated at 37 °C with a shaking incubator for 24 h. The bacteriological assays were conducted by using the strain *E. coli*. (XL1-Blue) and *S. aureus* (MTCC 3160). The *E. coli* and *S. aureus* cells were cultured at 37 °C in liquid Luria–Bertani (LB) medium. Stock cultures of *E. coli*. and *S. aureus* was maintained at ~4×10^8 CFU/mL. For growth curve analysis, individual samples were prepared by adding 100 μl of bacterial samples from the stock cultures to 2 mL LB media and incubated at 37 °C to allow the bacterial growth. The absorbance at 600 nm was monitored at an interval of 1 h till 12 h to map the bacterial growth. 10 μM of MB was added to the bacterial suspension to estimate the decolourization of MB during the bacterial growth. The absorbance at 664 nm was also monitored to observe the decolouration of MB at an interval of 1 h till 12 h. Data points were taken after 1 h interval to ensure optimum duration to the bacteria for growth. Decolouration of MB i.e. production of Leuco-MB occurs due to the increase of bacteria which utilizes the dissolved oxygen from the system for their growth. Only bacterial culture without MB was considered as control. The absorbance of MB was plotted against time in presence of bacteria to estimate the biological oxygen demand (BOD) of bacteria in terms of decrement of DO in system. 10 μM MB in LB media without bacterial load was also prepared as LB control to check the decolourization of MB in LB. All bacterial assays were done in triplicates and are expressed as Mean ± Standard deviation, until and unless mentioned.

2.5. Design of paper-based sensor strips and procedure for the detection of bacteria using LOPA device (patent pending: TEMP/E-1/45713/2024-KOL)

Whatman Grade-4 filter paper is used as the cellulose membrane substrate for the paper-sensor. The cellulose membrane it is made of 98 % cellulose fiber (pore size: 20–25 μm, thickness: 205 μm, ash: ≤0.06 %, and basic weight: 92 g/m^2) which decreases the contamination possibility and the homogeneous and fast flow rate provides a uniform distribution of the analyte over it. Here, 0.7 cm × 4 cm filter paper has been used as bacterial growth monitoring bed. After casting the sample on it, the sensor strips were sealed in such a way so that no air can flow inside or outside both and kept at 37 °C for 24 h. After 24 h of incubation at 37 °C the paper sensor strip was covered in such a way so that it appears as a diagnostic sensor strip with a circular working area of around 5 mm diameter.

For the detection of bacteria, the model device (POC) has been calibrated using *E. coli*. bacteria as a model bacterium on the paper sensor strip. Overnight stock *E. coli* cultures were used for this purpose. The paper sensor strip was drop casted with 100 μl of LB broth containing 0.4 mM of methylene blue and different concentration of *E. coli* bacteria (2×10^6 CFU/ml to 16×10^6 CFU/mL). The paper sensor strips were sealed and kept for 24 h at 37 °C before further analysis. During validation of the technique with milk samples, the paper sensor strip was drop casted with 100 μl of milk containing 0.4 mM of methylene blue and 4×10^5 CFU/mL of *E. coli* bacteria. In the real samples like milk, the milk itself acts as the bacterial growth media. Therefore, no broth solution was added for the bacterial growth in milk. It is worthwhile to mention that for the validation of the POC was done using one type of pasteurized milk sample spiked with *E. coli* bacteria (4×10^5 CFU/mL) in addition to its original microbial count and the measurement was taken after 0 h and 24 h of incubation. It is worthwhile to mention that, the bacterial count in raw milk varies from ~1.29×10^6 CFU/mL which reduces to ~ to 1.2×10^4 CFU/mL on pasteurization [28,29]. Grade A milk has a count less than 1×10^5 CFU/mL and grade B milk has count with less than 3×10^5 CFU/mL, however, a bacterial count ranging between 9×10^5–9×10^6 CFU/ml as detected by the mean standard plate count method is acceptable [30]. The concentration ranges chosen for the calibration and validation of the study is below the permissible limit to successfully identify the milk samples unfit for consumption.

In order to detect the bacterial load in milk, we have used the methylene blue degradation in the presence of bacterially contaminated milk in terms of the intensity (I) absorbed at red wavelength band (~664 nm). Whereas, as a pre-condition, the reference intensity (I_0) is also noted with time and the corresponding absorbance has been calculated using (I/I_0). Thereby, turbidity of the milk samples automatically absorbed in the detection process through the reference intensity. On the other hand, as the sensor strips remains in its wet form throughout the measurement process thus provide easy light transmission through the sensor strip. Thus, any pre-

treatment of the milk is not required.

3. Results and discussion

3.1. Reduction of methylene blue (spectroscopic probe) in presence of reducing agent

Methylene blue, a well-known spectroscopic probe (blue colour) gets reduced by reducing agent and gets transformed into its colourless Leuco-methylene blue (Leuco-MB) form. In this work, ascorbic acid (AA) has been used as the reducing agent. In Fig. 1, absorption of Methylene blue (MB) is shown in presence of difference concentration of AA. Addition of AA to MB results in decrease of absorbance of MB (Fig. 1a). In the reaction medium it promptly donates an electron to MB and converts it to its reduced colourless form Leuco-methylene blue (Leuco-MB) [31]. The generated Leuco-MB then reacts with the dissolved oxygen and passes its extra electron to the dissolved oxygen for regenerating its oxidized form MB [32]. As these two reactions are always occurring in the medium simultaneously, a specific time have been determined and denoted as t_{equ} (time equilibrium), where, these forward and backward reaction reaches the equilibrium state. So, all the absorbance of MB in Fig. 1a, in the presence of AA were recorded after equilibrium time (t_{equ} = 10 min). Hence from Fig. 1a we can conclude that some part of MB that has been reduced by AA cannot be converted again to MB due to the shortage of dissolved oxygen. Fig. 1b describes the absorbance correlation functions (CF) with respect to time, where the reaction rate is controlled by the concentration of AA and is represented by following equation (1).

$$CF = \frac{C(t) - C(\infty)}{C(0) - C(\infty)} \tag{1}$$

where (t) , $C(\infty)$ and $C(0)$ represent absorbance of MB at 664 nm at arbitrary time 't', equilibrium time and time $t = 0$ respectively. It shows that with increasing concentration of AA, the absorbance of MB decays out faster as the number of reducing agent in the reaction medium gets increased in comparison to the oxidizing agent (dissolved oxygen) and the single exponential fitting component provides the time constant (0.440 min) is highest for the highest AA concentration (113.5 μM).

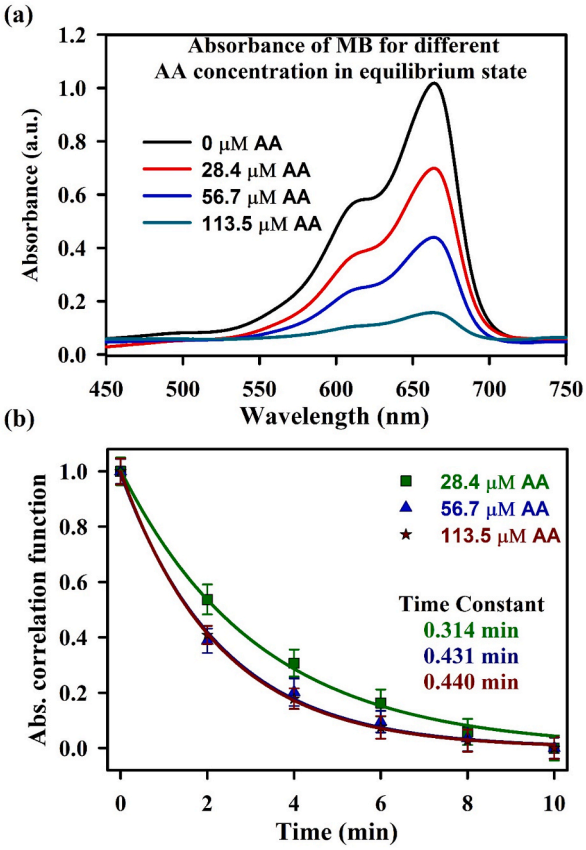

Fig. 1. (a) Absorbance of Methylene Blue (MB) in presence of different ascorbic acid (AA) concentrations. (b) Decay of the MB absorbance in presence of AA.

3.2. Calibration of dissolved oxygen in a system and the differential absorbance of the probe methylene blue

Dissolved oxygen has significant role in the extent of decolouration of MB in the presence of the fixed concentration AA. The role of dissolved oxygen in this regard has been explored. The amount of dissolved oxygen has been regulated by heating the reaction medium to specific temperatures and the absorbance has been recorded after the equivalent time. Herein Fig. 2a represent the differential absorbance of MB (ΔOD = absorbance of MB in absence of AA at the corresponding temperature after t_{equ} - absorbance of MB in presence of 56.7 μM AA at the corresponding temperature after t_{equ}). It is evident from Fig. 2a that with increasing temperature the absorbance of MB decreases, as a result of which the differential OD increases. With increasing temperature, the amount of dissolved oxygen decreases. Hence, the conversion of Leuco-MB to MB i.e., the regeneration of blue-colour declined. This experimental set-up can be treated as a mimic of the reduction of MB in some solution medium (Luria broth or milk) during the bacterial growth. In the

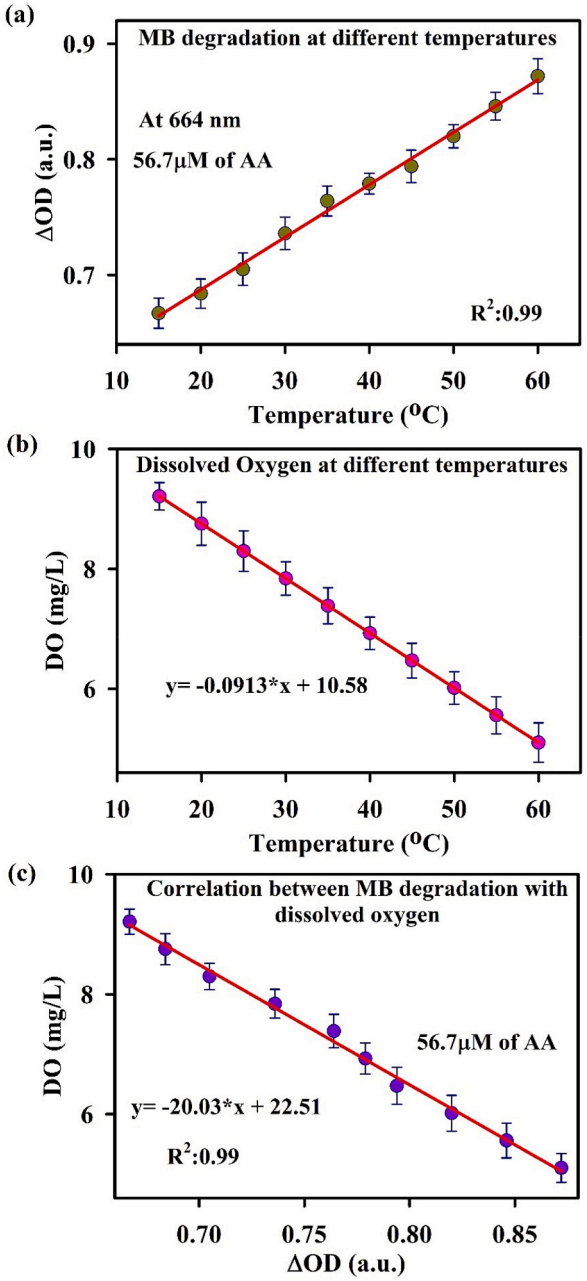

Fig. 2. (a) Shows temperature dependent differential absorbance (ΔOD) of methylene blue (MB) in presence of ascorbic acid (AA), monitored at 664 nm (absorbance peak of MB). (b) Depicts the relation between dissolved oxygen (DO) in water with temperature. (c) Represents the calibration curve of DO with respect absorbance change in MB (ΔOD).

aforementioned set-up the bacteria present in milk reduces MB with the help of a membrane bound reductase enzyme and converted it to Leuco-MB. This colourless reduced Leuco-MB re-oxidized to MB with help of dissolved oxygen. As the bacterial load increases the amount of dissolved oxygen in the medium declined proportionally and the amount of regeneration of MB also decreases [33–38]. Fig. 2b shows the correlation curve of dissolved oxygen with respect to temperature replotted from a previous study [39,40] using the following equation

$$y = -0.0913x + 10.58 \quad (2)$$

which gives the amount of dissolved oxygen at a given value of temperature. Fig. 2c shows the calibration curve of dissolved oxygen with respect to Δ OD of MB in presence of AA. Here, the following equation, obtained from fitting parameters (from graph) gives the amount of dissolved oxygen at a given value of Δ OD.

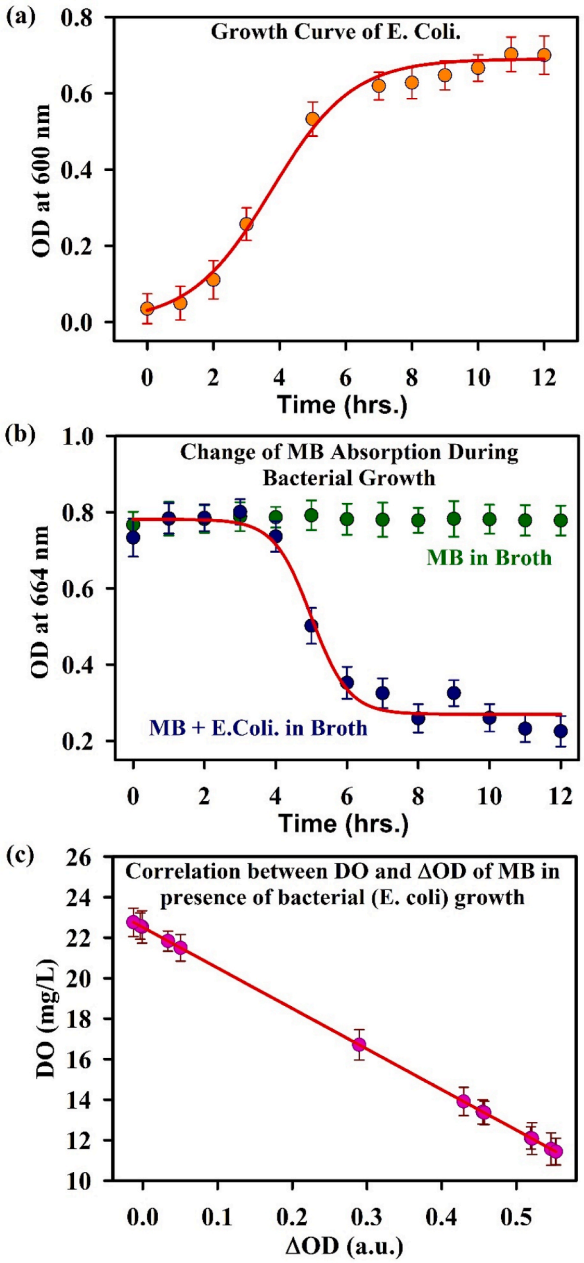

Fig. 3. (a) Growth curve of *E. coli* in Luria broth medium (b) Spectroscopic change of MB during bacterial (*E. coli*) growth. MB in LB media without bacterial load was considered as LB control (c) Change of DO in solution with respect to ΔOD during bacterial growth.

3.3. Correlation between the biochemical oxygen demand (BOD) during the pathogenic bacterial growth with the reduction of methylene blue dye (probe)

Due to the biochemical oxygen demand (BOD) of the growing bacteria, the amount of oxygen decreases in the bacteria containing media. This BOD of bacteria could reduce the MB by snatching the oxygen from it and produce Leuco-MB. Fig. 3a represents the growth curve of *E coli* bacteria. The absorption experiment was conducted in UV–vis region and the 600 nm result was plotted to measure the bacterial growth with respect to time. The *E-coli* bacteria were cultured in broth medium, and the OD of the medium was recorded in 1-h interval for 12 h. The growth curve shows characteristic 'S' shape, which is basically sigmoidal in nature. The lag phase and log phase of bacterial growth were clearly visible from Fig. 3a. Fig. 3b shows the bacteria sensing characteristics of MB at 664 nm (absorbance peak of MB). The variation of optical density of MB in broth medium was first recorded when the medium was bacteria free. It shows no variation in OD with time (green dots). Next, the *E. coli* bacteria infested broth medium was added to the MB containing broth medium.

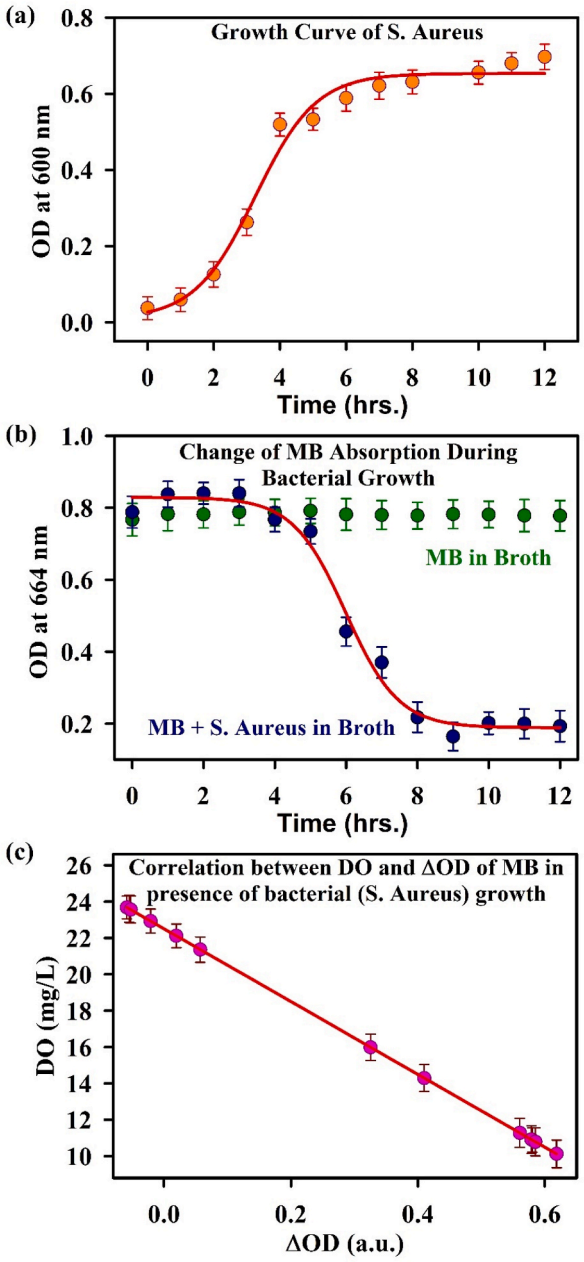

Fig. 4. (a) Growth curve of *S. aureus* in Luria broth medium (b) Spectroscopic change of MB during *S. aureus* growth. MB in LB media without bacterial load was considered as LB control (c) Change in DO in solution with respect to ΔOD during bacterial growth.

It shows completely complimentary characteristics of bacterial growth curve, as shown in Fig. 3a. Initially, when the bacterial growth was low, the MB was retaining its colour and no apparent change in OD was observed. With time, as the bacterial growth increases, the amount of dissolved oxygen from the solution decreases, which was also indicated by the production of leuco-MB, hence the decrement in absorbance of MB. Fig. 3c shows the in DO level in the system with respect to the measure Δ OD of the MB in presence of *E. coli* bacteria during its growth (observed for 12 h).

The growth curve of *S. aureus* bacteria is depicted in Fig. 4a. Like Fig. 3a, the absorption experiment was also carried out in the UV–vis region, and the 600 nm result was plotted to quantify bacterial growth over time. The *S. aureus* bacteria were grown in broth medium, and the OD of the medium was measured every hour for 12 h. The growth curve also exhibits 'S' shape characteristics and is sigmoidal in nature. Fig. 4b depicts bacteria sensing characteristics of MB at 664 nm. When the medium was bacteria-free, the fluctuation in optical density of MB in broth medium was measured which demonstrates no change in OD over time (green dots). The MB-containing broth medium was then mixed with the *S. aureus* bacteria-infested broth medium. As demonstrated in Fig. 4a, it exhibits totally complementary properties of the bacterial growth curve. When bacterial growth was low, the MB retained its colour and no discernible change in OD was observed. As bacterial growth progresses, the amount of dissolved oxygen in the solution decreases, as evidenced by the synthesis of Leuco-MB, and hence the drop in OD. Fig. 4c depicts the in-system DO level in relation to the measured Δ OD of the MB in the presence of *S. aureus* bacteria during its growth, observed for 12 h. It is worthwhile to mention that as both the bacterial growth curves attained saturation phase within 12 h, the experimental time window for MB de colorization was monitored for 12 h, to gain a complete insight in to the oxygen demand of the bacterial growth.

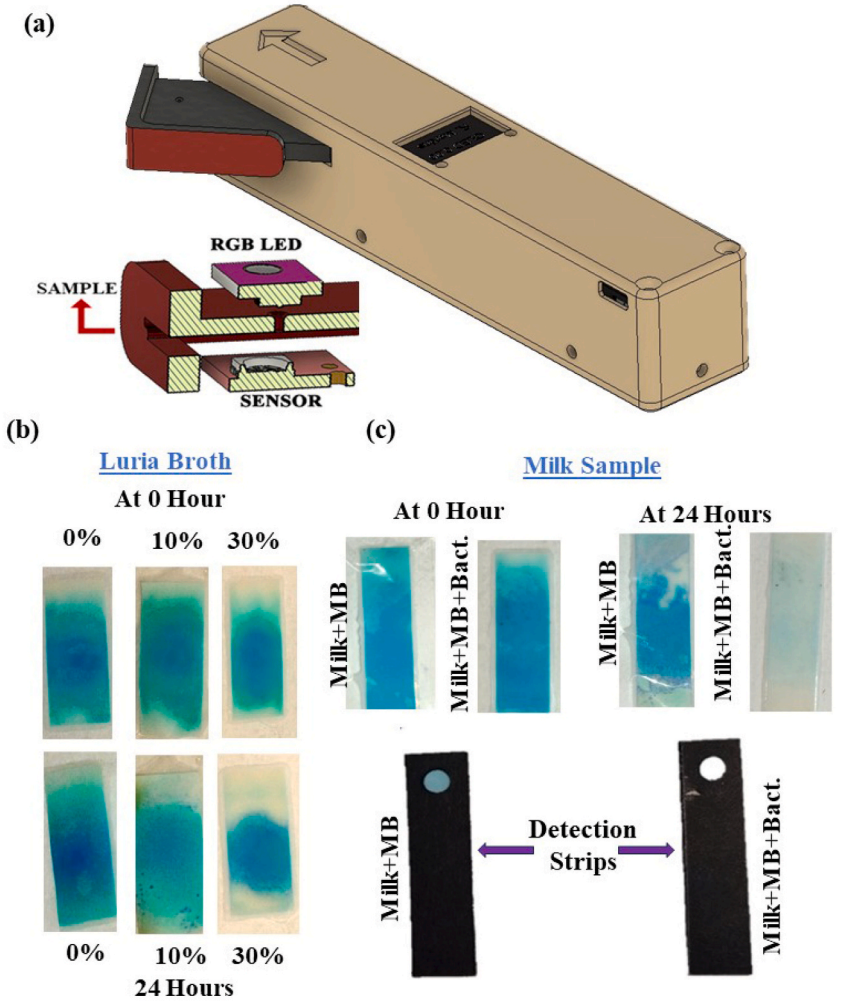

Fig. 5. (a) The schematic diagram of the loading of pathogen analyzer (LOPA) POC device (inset: components of the developed model device (POC) LOPA, the device containing colour sensors and associated IoT electronics) (b) The paper membranes containing bacterial contaminated Luria broth with different concentration of bacteria (bact.) (0 %, 10 %, and 30 %) and methylene blue (MB) at 0 h and 24 h. (c) Detection of contaminated milk and MB at 0 h and 24 h using paper sensor. Black envelope covered paper sensors for the feasible use in bacterial contamination detection.

3.4. Development of LOPA model device (POC) and microfluidic paper-based colorimetric sensing of pathogens

Fig. 5a shows the schematic of our POC. It was designed in such a way, that it can detect the change in colour of MB in presence of gram-positive and gram-negative bacteria. The inset of Fig. 5a shows the cross-sectional view of the POC device to depict the inner electronics arrangement. It contains a RGB LED for absorption studies, along with a sample holder slot and a sensor which collects the intensity of the RGB LED after transmitting through the sample. Beside these components the model device (POC) has a display screen to depict the data and a microcontroller to process the acquired raw data. Functioning of the developed POC device is being controlled by indigenously made software interface developed on the Arduino platform. Dimension of the developed device is 17.6 cm $\times$ 3.8 cm $\times$ 3.5 cm and it has a 5 V rechargeable power supply and enabled IoT functioning. Fig. 5b shows how the sensor changes its colour in presence of bacteria in broth medium and the colour change is very prominent after 24 h of bacterial loading. The colour change of methylene blue in milk-system has also been studied which is depicted in Fig. 5c along with the customised sensor strip used in our POC. The strip was studied first using a drop of milk with methylene blue and its change with time was observed with addition of bacteria (after 24 h). The de-colouration of MB in the strips inoculated with only the milk samples without the bacterial contaminants could be due to the presence of any inherent bacteria in the pasteurized milk present in the sample. However, the milk contaminated with bacteria was compared to milk without bacteria giving information about MB degradation as a function of bacterial growth, as reflected by the clear colour change in our sensor strips. As depicted in Fig. 5, compared to control sample strips a faded blue colour can be seen in bacterial-loaded samples. The change in sensor strip colour from blue to colourless shows the key feature of our detection mechanism. It has to be noted that the colour change in the control set was taken as the baseline and considered for all the concentration ranges.

Fig. 6a shows the intensity change of MB in broth medium in red channel of the model device (POC). The data was recorded for 24 h. The MB absorbance is at 664 nm, which lies at red region of the colour spectrum. Hence the red channel data of the protype was considered for calibration of the POC. It was seen that almost linear increase in the red channel (fading of blue colour) with the growth of the bacteria is evident. We have estimated the slope (s) and sigma (σ) of the growth curve to be 5.75×10^{-5} and 56.0×10^{-5} respectively. The limit of detection (LOD) and the limit of quantitation (LOQ) are found to be 0.32 CFU/mL and 0.97 CFU/mL respectively as depicted by following equations [41].

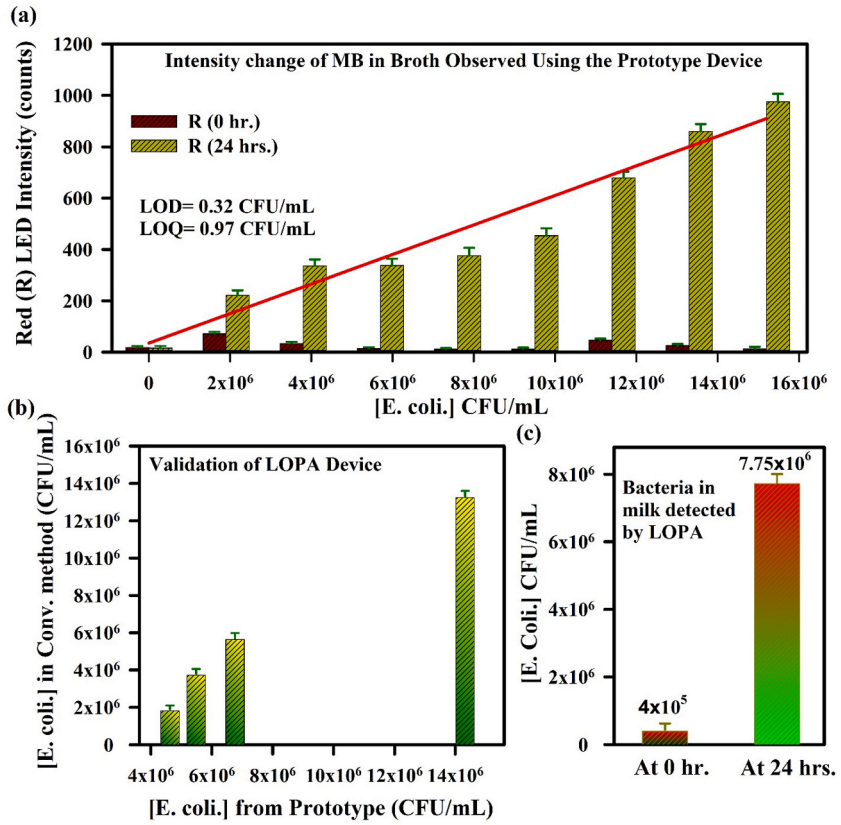

Fig. 6. (a) The intensity changes of MB in broth medium in presence of *E. coli* bacteria using LOPA device. The straight-line curve represents the calibration curve of the device. Validation of LOPA device in (b) Luria broth medium (c) milk.

$$LOD = \frac{3.3\,\sigma}{s} \tag{3a}$$

$$LOQ = \frac{10\,\sigma}{s} \tag{3b}$$

Fig. 6b depicts the validation of LOPA device in broth medium. Fig. 6c represents the change in red channel intensity of the POC device during the growth of bacteria in milk.

Overall, we have provided a table with references therein (Table 1) that summarizes the available techniques with their limitations for milk quality check and a column "limitation" has been dedicated specifically to showcase the advantages and uniqueness of our proposed method. The comparison between our proposed method and available ones clearly the proposed technique is one step ahead in terms of cost, mobility, point-of-care technology, operational process, and limit of detection. Our indigenously developed handheld POC device is not only able to detect the microorganism related toxic activities in milk, but also its IoT enabled module can monitor the quality of milk and related products at any part of the production line. Furthermore, as the decolourization of MB is solely dependent on the biological oxygen demand of the bacteria present in the milk, the method will be applicable for high fat milk samples also. Fat content of the milk will not be confounding factor for the quantification of bacteria in the sample. The limitation of the study is that the current study is a proof-of concept application of the MBRT assay and has not been evaluated in real field scenarios, however, in progress in our laboratory. Thus, in situations the strip might go colourless after a certain bacterial concentration, irrespective of the concentration of the bacteria in the sample making it qualitative, rather than quantitative. However, the current working range of the POC is ~1 CFU/mL to 16 $\times$ 10^6 CFU/ml which is suitable to identify microbial contamination in milk samples.

4. Conclusions

Detecting bacterial contamination in dairy products is a worldwide challenge. The present study investigated the spectroscopy of MB dye in the absence and presence of bacteria in aqueous solution in order to detect bacterial contamination in dairy products through the consumption of dissolved oxygen in the medium. The growth of *Escherichia coli*, *Staphylococcus aureus* was studied in pasteurized milk as model contaminating bacteria. The colorimetric shift of MB dye from deep blue colour to colourless solution in the presence of microorganisms has been used as a method to identify the presence of bacteria in milk. An IoT-based model device (POC) was developed to monitor the quality of milk in real field. Our POC may also be extended to any biologically relevant samples, like fruit juice and soil.

Table 1
Various techniques available for the milk quality check.

Method/Technique Applied	Detection medium	LOD/LOQ	Limitations	Reference
Spectroscopy-based colorimetric sensing	Pathogen contaminated milk	0.32 CFU/mL and 0.97 CFU/mL	Shelf-life of methylene blue	This work
Electrochemical sensing was developed based on dual amplification strategy of polymethylene blue nanoparticles (pMB NPs) and dumbbell hybridization chain reaction (DHCR).	Pathogenic bacteria assay	1 CFU/mL/ 10–10^8 CFU/mL	High cost and time consuming	[42]
Colorimetric sensing based on the intensity of the developed Prussian Blue-Based Dipstick Sensor	Bacterial and Biofilm Detection	10^1 CFU/mL	LOD to 10^1 CFU/mL and time-consuming synthesis	[43]
MBRT-based technique for yeast detection in samples like milk	Yeast detection	Not determined	Point-of-care technology translation	[44]
Colorimetric sensing using rapid culture detection nanowell device fabricated using polydimethylsiloxane (PDMS) and oxygen-sensitive redox indicator Methylene Blue	Bacteria detection in human milk of	10^2 CFU/mL	LOD and Rigorous synthesis process	[45]
Colorimetric sensing using the developed paper-based sensor with smartphone detection device and bromothymol blue-cetyltrimethylammonium bromide-alginate complex	Detection of penicillinase in milk	2.67 $\times$ 10^{-3} mU/ μL	Rigorous method	[46]
Rapid sensing based on SERS method using polyethylenimine-modified Au-coated magnetic microspheres and Au@Ag nanoparticles	Detection of bacteria in milk and water	10^3 cells per mL	Rigorous synthesis process	[47]
Sensing based on Combining Magnetic Relaxation Switching and Magnetic Separation	Detection of *S. enterica* in milk	10^2 CFU/mL	LOD	[48]
Electrochemical sensing using methylene blue-loaded nanocomposites	Detection of bacteria in milk	32 CFU/mL	Rigorous synthesis process	[49]
Impedimetric detection paper-based electrodes conjugated using tungsten disulfide nanostructure and aptamer	Detection of listeria monocytogenes in milk	10 CFU/mL/4.5 CFU/mL	Rigorous synthesis process	[50]
Au-coated magnetic nanoparticles (AuMNPs) conjugated with *Staphylococcus aureus* (S.aureus) antibody 190 *Staphylococcus aureus* SERS detection method	Detection of *Staphylococcus aureus* (S.aureus) in milk	10 CFU/mL	LOD	[51]
Electrochemical sensing using amperometric immunosensor based on covalent immobilization of synthesized methylene blue and penicillin polyclonal antibody	Detection of Penicillin G bacteria in milk	1.82 nmol L^{-1}	Rigorous synthesis process	[52]

Data availability

Data will be made available on request.

CRediT authorship contribution statement

Lopamudra Roy: Writing – review & editing, Writing – original draft, Validation, Methodology, Formal analysis, Data curation. **Amrita Banerjee:** Writing – review & editing, Formal analysis. **Nivedita Pan:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Ria Ghosh:** Writing – review & editing, Formal analysis, Data curation. **Susmita Mondal:** Writing – review & editing, Formal analysis. **Monojit Das:** Writing – review & editing, Formal analysis. **Md Nur Hasan:** Writing – review & editing, Formal analysis. **Soumendra Singh:** Writing – review & editing, Methodology, Formal analysis. **Arpita Chattopadhyay:** Writing – review & editing, Validation, Formal analysis. **Kallol Bhattacharyya:** Writing – review & editing, Supervision, Formal analysis. **Soumen Mondal:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Formal analysis. **Samir Kumar Pal:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Soumen Mondal and Samir Kumar Pal has patent pending to S. N. Bose National Centre for Basic Sciences. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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