Development of Lab-on-a-Disc Integrated with Resazurin-based Assay for Total Bacterial Counting in Raw Milk

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Abstract. The quality of milk and dairy products depends largely on micro-organisms contamination which can pose serious health risks. Therefore, the total viable count (TVC) in raw milk needs to be daily checked to guarantee high quality. A resazurin is used as a dye indicator for the TVC quality in raw milk. However, this method is relatively complicated and inconvenient for on-site measurement. During the wait time, changes in physical and chemical properties of the raw milk were observed and they might affect the accuracy of the test results. Therefore, lab-on-a-disc (LOAD), a low-cost, user-friendly, and fast detection centrifugal microfluidic platform for the TVC test, was proposed in this study. The LOAD device (φ: 9.0 cm) was fabricated by laser machining of polymethyl methacrylate (PMMA, acrylic) and assembled with pressure sensitive adhesive (PSA) films. The 10\%(v/v) of resazurin-based assay that incubated at 25 °C was used to detect three different bacterial concentration levels of 4.0 log CFU/mL (low), 6.0 log CFU/mL (medium) and 8.0 log CFU/mL (high) in raw milk. The reduction process changed the color from purple (resazurin) to pink (resorufin) and to white (dihydroresorufin) subsequently. The resazurin and resorufin showed the maximum absorbance at the wavelength of 600 and 570 nm, respectively. After the LOAD was rotated at 5000 rpm for 5 minutes at 25 °C, the color of resazurin in the raw milk with medium and high levels of bacterial concentration started to change from purple to pink at 60 and 15 min, respectively. However, the color of resazurin in raw milk with low level of bacterial concentration remained dark mauve after 120 min. Overall, the LOAD integrated with resazurin-based assay is an alternative method to determine TVC in raw milk and various food stuffs.

1. Introduction
Raw milk is high nutritious content for humans which mainly consists of proteins and fat globules. It also contains minerals with the pH close to neutral [1]. However, raw milk can be contaminated with some bacteria apart from its endogenous bacteria from various sources. Bacterial contamination can lead to low milk quality, resulting in the short shelf-life of dairy products. Psychro-tolerant bacteria are able to proliferate during storage at 4°C and lead to the production of extracellular lipases and proteases, resulting in milk spoilage. Bacteria in raw milk is generally a normal flora such as lactic acid bacteria, other spoilage microbes, e.g., \textit{Pseudomonas}, \textit{Clostridium}, \textit{Bacillus} and pathogens, e.g., \textit{Listeria monocytogenes}, \textit{Salmonella}, \textit{Escherichia coli} (\textit{E. coli}), \textit{Campylobacter} [2]. Therefore, in the current practice for the dairy industry, it is necessary to detect total viable count (TVC) in raw milk for pricing evaluation before sending to the dairy processing. Typically, the methods for the detection of...
TVC in raw milk are conventional methods involving the growth of bacteria in culture media by the protocol of National and International Accreditation Boards [3]. However, this method takes a long period of time for testing (up to 24 h) and is relatively laborious. In addition, there is limited access to the milk quality testing laboratory at the milk collection centers due to insufficient equipment. These limitations lead to the issue linked to the changes in physical and chemical properties of raw milk can be occurred, resulting in lower quality and the pricing for milk sold.

Therefore, this study aims to develop a rapid method and high-throughput tests by utilizing a dye reduction assay for the detection of TVC. A resazurin dye, a phenoxazine dye that is redox reaction sensitive is considered appropriate for testing viable cells. This dye has been regularly used to estimate the active growth of live organisms and measure metabolic activity of microorganisms [4]. The highly pink compound of resorufin in the presence of high viable cells will turn to the dihydroresorufin (white) during dye reduction [5]. This resazurin-based assay is a good indicator as it can be visualized to classify raw milk quality for accepting or rejecting in a short time.

The developed resazurin-based reaction for the detection TVC in raw milk will be applied into the LOAD platform which will allow testing to be easier for handling and operating at ambient temperature (25 °C). This integration platform is an innovative alternative approach for testing the raw milk quality due to testing time and immediate result generated. Another highlight of LOAD platform is that miniaturization of LOAD platform is portable and easy to transport at different locations/regions for milk testing [6]. Typically, the LOAD platform employs rotation of the unique disc can deliver small quantity of samples to be reacted with resazurin dye solution. The change in colors will indicate the reaction completion which is expected to occur within 1 h at ambient temperature (25 °C). The raw milk quality monitoring can be performed in the same disc which can save time and cost for milk collection centers and dairy cooperatives. This developed method does not require complicated instruments, thus allowing easy access for all centers and laboratories.

2. Methodology
2.1. Preparation of inoculated raw milk
In this study, raw milk samples were obtained from the Faculty of Natural Resources, Prince of Songkla University, Hat Yai, Songkhla and the dairy center of Kasetsart University, Bangkok, Thailand. Raw milk was spiked with E. coli and Staphylococcus aureus (S. aureus) by inoculating a single colony of a given bacteria from tryptone soy agar (TSA; Himedia) plate grown overnight into 5 mL of raw milk. Inoculated raw milk was incubated at 37 °C for 6 and 18 h to achieve the concentration of approximately 6.0 and 8.0 log CFU/mL, respectively. The corresponding concentrations were confirmed by a plating method on TSA. The resulting inoculated raw milk was used in subsequent assays.

2.2. Evaluation of cytotoxicity effects of resazurin on viable cells in raw milk
Inoculated raw milk (approximately 8 log CFU/mL) was transferred into a 96-well plate. Each inoculated raw milk was mixed with three different concentrations: 10, 20 and 30 % (v/v) of resazurin (BDH chemicals; UK) prepared and diluted following the manufacturer’s instructions. Each reaction with a total volume of 200 μL was incubated at 25 °C. Monitoring of the cell concentration in each reaction was performed every 15 min for 2 h by a plating method on TSA. The cell recovery was calculated according to equation (1).

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\text{% cell recovery} = \left( \frac{\text{concentration (log CFU/mL) final}}{\text{concentration (log CFU/mL) initial}} \right) \times 100 \quad (1)
\]

2.3. Detection of the optical density and color change (RGB system) of resazurin reaction in inoculated raw milk at different cell concentrations
Inoculated raw milk was prepared with three different concentrations: low level of bacterial at 4.0 log CFU/mL, medium level at 6.0 log CFU/mL and high level at 8.0 log CFU/mL. Inoculated raw milk (180 μL) was transferred into a 96-well plate and the LOAD (see detail below). Each reaction was
incubated with optimum concentration of resazurin (20 μL/well). Each reaction with a total volume of 200 μL was incubated at 25 °C. The LOAD was rotated at 5000 rpm for 5 min at 25 °C. Reactions on both platforms were measured the optical density (OD). The maximum absorbance was measured using the FLUOstar Omega microplate reader (BMG LABTECH, Germany) at the wavelengths for the detection of resazurin and resorufin at 600 and 570 nm, respectively. Each reaction was calculated resazurin reduction (%) or resorufin formation (%) according to equation (2). Each reaction was monitored every 15 min for 2 h. Color change of each reaction was monitored by photograph analysis with the image acquisition system of a camera phone with resolution of 12 MegaPixels. The camera was placed in a white plastic box and images were taken at maximum resolution (3024 x 4032 pixels) and connected to the USB port of a computer to transfer data.

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\% \text{ resazurin reduction or resorufin formation} = \frac{(\text{OD}_{\text{control}} - \text{OD}_{\text{sample}})_{\text{initial}} - (\text{OD}_{\text{control}} - \text{OD}_{\text{sample}})_{\text{final}}}{(\text{OD}_{\text{control}} - \text{OD}_{\text{sample}})_{\text{initial}}} \times 100
\]

2.4. LOAD design and fabrication

The LOAD for testing TVC in raw milk was designed with a diameter of 9.0 cm. The length from center of the disc to bottom of the channel was 42.0 mm. The LOAD consists of ten sets of channels, chambers, inlet holes, and outlet air vents. Each channel has an approximately maximum capacity of 200 μL (Figure 1a). The LOAD contained five layers. The first and third layers made of polymethyl methacrylate (PMMA, Sumiplex Thailand) are for adding solution, while the second and fourth layers made of polyester and attached with an adhesive tape are for inlet holes (Figure 1b). The LOAD was fabricated by a laser cutting method for creating a pattern on PMMA. Each disc was cleaned with an ultrasonic cleaner with deionized water mixed with detergent and subsequently assembled with pressure sensitive adhesive (PSA, 3M Thailand) by alignment and assembly tools in a class 10k clean room.

![Figure 1. (a) Design of LOAD for testing TVC in raw milk; (b) Schematic diagram of LOAD components](image_url)

3. Results and Discussion

3.1. Evaluation of cytotoxicity effects of resazurin on viable cells in raw milk

Recovery of bacterial cells in raw milk with different resazurin concentrations at 25 °C was evaluated. The decrease of viable cells was significantly different \( (p < 0.05) \) after 90 and 105 min of incubation time with 30 %/(v/v) when compared to 10 %/(v/v) and 20 %/(v/v) of resazurin (Figure 2). For a longer period of incubation time (120 min), the decrease of viable cells was significantly different \( (p < 0.05) \) with 20 %/(v/v) and 30 %/(v/v) when compared to 10 %/(v/v) of resazurin. This result is consistent with a previous study which reported that high concentration of resazurin and longer incubation time could have an effect on a decrease of viable cells of bacteria [4]. The resazurin concentration of 44 μM was recommended due to the absence of cytotoxicity effect on various viable cells of bacterial microflora in raw milk including \( P. \ aeruginosa \) 1244, \( E. \ coli \), and \( S. \ aureus \) [7].
Overall, results obtained in this study suggest the appropriate resazurin concentrations between 10 % (v/v) and 30 % (v/v) with 120 min of incubation time to maintain high percentage of cell recovery in raw milk.

3.2. Detection of the optical density (OD) and color change (RGB system) of resazurin reaction in inoculated raw milk at different cell concentrations

Reduction of resazurin (%) was evaluated based on the relationship of the change in OD$_{600 \text{nm}}$ in 96-well microplate and LOAD platform using different levels of bacterial cells in raw milk. At 10 % (v/v) of resazurin in raw milk with medium cell numbers (6.0 log CFU/mL), no significantly difference percentage of reduction of resazurin was detected ($p > 0.05$) during 15 to 45 min and 60 to 120 min of incubation time in 96-well microplate, and 15 to 120 min in LOAD platform (Figure 3a). The resorufin formation (%) was not significantly different ($p < 0.05$) during 15 to 45 min of incubation time in 96-well microplate (Figure 3b). Color remained purple in both platforms.

Higher bacterial level (6.0 log CFU/mL) rapidly reduced resazurin (Figure 4a) to resorufin (Figure 4b) at 15 min in both platforms resulting in the color change of purple to pink. The lowest resazurin reduction was observed at 45 min in both platforms while there was a significant difference ($p < 0.05$) between platforms. The change in color from purple to pink and subsequently changed into clear white (hydroresorufin) at 15 min of incubation was observed after a rapid reduction of resazurin with the highest bacterial level tested here (8.0 log CFU/mL) (Figure 5a).

**Figure 3.** (a) Resazurin reduction and (b) resorufin formation in raw milk with 10 % (v/v) of resazurin in the presence of low bacterial level (4.0 log CFU/mL) during 120-min incubation time at 25 °C. All values provided as mean ± standard deviation of triplicate. Different lowercase letters for each incubation time indicate a significant difference ($p < 0.05$), and the asterisk (*) indicates a significant difference ($p < 0.05$) between the 96-well plate and LOAD platform.
Figure 4. (a) Resazurin reduction and (b) resorufin formation in raw milk with 10 % (v/v) of resazurin in the presence of medium bacterial level (6.0 log CFU/mL) during 120-min incubation time at 25 °C. All values provided as mean ± standard deviation of triplicate. Different lowercase letters for each incubation time indicate a significant difference (p < 0.05), and the asterisk (*) indicates a significant difference (p < 0.05) between the 96-well plate and LOAD platform.

Figure 5. (a) Resazurin reduction and (b) resorufin formation in raw milk with 10 % (v/v) of resazurin in the presence of high bacterial level (8.0 log CFU/mL) during 120-min incubation time at 25 °C. All values provided as mean ± standard deviation of triplicate. Different lowercase letters for each incubation time indicate a significant difference (p < 0.05), and the asterisk (*) indicates a significant difference (p < 0.05) between the 96-well plate and LOAD platform.

A significant difference in the percentage of resazurin reduction was observed (p < 0.05) between platforms during 15 to 90 min of incubation. The formation of resorufin was correlated with the reduction of resazurin during 120 min of incubation (Figure 5b).

Color change (red, green, and blue) from the three primary colors presenting various proportions and intensities of colors in the RGB system of resazurin reactions after 60 min incubation was observed as shown in Table 1. Overall, the changes of each color in 96-well microplate and LOAD platform were significantly different (p < 0.05) since the LOAD platform could separate the milk contents (fat, pellet, and milk layer) after centrifugation which may leave different compositions to react with resazurin, yielding off-color detected. Advantage of LOAD platform can be used to separate bacterial cells from other milk contents [8], thus easy to react with resazurin and leading to identify color change. However, hydrogen ion required for bacterial propagation can accumulate on the surface of milk layers along with fat and white blood cells resulting in the transformation of resazurin to resorufin, and subsequently into hydrosresazurin [9]. Moreover, the reaction of resazurin depends on the number of bacteria presented in the raw milk.

Overall, color of raw milk at low bacterial level in the range of RGB system showed dark purple, indicating that it was suitable for raw milk acceptance. However, the color change was not comparable between results detected by the optical density and the RGB system because the optical density detected only resazurin and resorufin which showed the dark purple and pink color, respectively. For RGB system, the transmitted light was detected based on colors’ proportions and intensities of different colors...
while covering a wide color values for human perceives recognize colors in the retina [10].

Table 1. Color change in the RGB system of resazurin reactions in raw milk at different bacterial levels after 60-min incubation time at 25 °C

| Bacterial level (log CFU/mL) | Red | Green | Blue |
|-----------------------------|-----|-------|------|
| 96-well plate LOAD platform |     |       |      |
| Low                         | 135.0±2.6<sup>a</sup> | 141.7±3.5<sup>a</sup> | 156.7±4.9<sup>b</sup> |
| Medium                      | 186.7±2.9<sup>b</sup> | 138.3±3.1<sup>a</sup> | 142.7±4.5<sup>c</sup> |
| High                        | 250.7±3.2<sup>c</sup> | 218.3±7.6<sup>c</sup> | 222.7±7.1<sup>c</sup> |

All values provided as mean ± standard deviation of triplicate. Different lowercase letters for each platform indicate a significant difference (p < 0.05) between bacterial levels: 4.0 log CFU/mL (low); 6.0 log CFU/mL (medium); 8.0 log CFU/mL (high). The asterisk (*) indicates a significant difference (p < 0.05) between the 96-well plate and LOAD platform.

4. Conclusions

The newly developed assay in 96-well plate could be used to detect viable cells in raw milk using an optimal concentration of resazurin at 10 % (v/v) which has been evaluated without cytotoxicity effects. The color change from resazurin reactions was not comparable between results detected by the optical density and the RGB system. Data need to be separately analysed to evaluation the relationship of the color change from resazurin reactions in raw milk in the presence of various bacterial cell levels included in this study. However, the newly developed assay could be integrated with the LOAD platform which could generated reliable results from resazurin reactions in raw milk in the presence of various bacterial cell levels. This LOAD platform is useful for further development of a rapid detection for testing TVC in raw milk samples.

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