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An improved MTT colorimetric method for rapid viable bacteria counting

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ABSTRACT

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay has been employed in the analysis of bacterial growth. In comparison to experiments conducted on mammalian cells, the MTT bacterial assay encounters a greater number of interfering factors and obstacles that impact the accuracy of results. In this study, we have elucidated an improved MTT assay protocol and put forth an equation that establishes a correlation between colony-forming units (CFU) and the amount of formazan converted by the bacteria, drawing upon the fundamental principle of the MTT assay. This equation is represented as CFU $= k$F. Furthermore, we have explicated a methodology to determine the scale factor "k" by employing *S. aureus* and *E. coli* as illustrative examples. The findings indicate that *S. aureus* and *E. coli* reduce MTT by a cyclic process, from which the optimal reduction time at room temperature was determined to be approximately 30 mins. Furthermore, individual *E. coli* exhibits an MTT reduction capacity approximately four times greater than that of *S. aureus*. HPLC analysis proves to be the most accurate method for mitigating interferences during the dissolution and quantification of formazan. Additionally, this study has identified a new constraint related to the narrow linear range (0–125 μg/mL) of formazan concentration-absorbance and has presented strategies to circumvent this limitation.

1. Introduction

Rapid enzymatic tetrazolium salts reduction has been widely used to assess microbial viability and estimate growth. This method offers great advantages over conventional methods such as the time-consuming steps of counting colony-forming units (CFU) (Gabrielson et al., 2002; Rahman et al., 2004; Tunney et al., 2004; Wang et al., 2010). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is the most used compound (Benov, 2021; Bernas and Dobrucki, 2000; Foongladda et al., 2002; Grela et al., 2015; Houdkova et al., 2017; Hundie et al., 2016; Hut et al., 2021; Li and Song, 2007; Oh and Hong, 2022; Wang et al., 2010). The MTT assay is based on the enzymatic reduction of water-soluble yellow dye to a water-insoluble purple crystal called formazan. The amount of formazan is therefore positively correlated with cell viability (Carmichael et al., 1987; Morgan, 1998; Mosmann, 1983). The whole assay can be divided into two stages: the reduction of MTT into formazan and the measurement of the amount of formazan. Tested bacteria are often many in number and have quite high MTT reduction efficiency and suspension characteristics which can block light and reduce the efficiency of spectrometer readings moreover each stage still in the process has some technical limitations. These limitations include uncertain reaction times of MTT, incomplete dissolution of formazan, high turbidity of bacterial suspension, and the effect of pH on the conversion of formazan (Grela et al., 2018; Grela et al., 2015; Wang et al., 2010; Wang et al., 2012). However, several modified protocols are available, but they only focus on improving the dissolution of formazan and neglect the other interfering factors. Furthermore, there is interference caused by the choice of solubilizer used (Benov, 2021; Oh and Hong, 2022; Wang et al., 2010; Wang et al., 2012).

The objective of this study was to enhance the MTT bacterial assay protocol to eliminate interference and achieve precise quantification of formazan. Additionally, the study aimed to establish a correlation equation between colony-forming units and formazan production for selected bacteria, thereby offering an alternative approach to the conventional colony plate counting method. *Staphylococcus aureus* and *Escherichia coli*, representative species of gram-positive and gram-negative bacteria, were chosen for the MTT assay and subsequent creation of equations.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Gram-positive *Staphylococcus aureus* (ATCC 25923) and Gram-

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negative *Escherichia coli* (ATCC 25922) strains were used as illustrative examples in this study. The strains used were obtained from the Microbiology Department of Massey University, and stored in brain heart infusion (BHI, Difco) agar slants at 4 °C. For each experiment, a single colony of each strain was picked and cultured in BHI broth at 37 °C for 24 h. The current experiment refers to this culture condition as the standard condition.

2.2. Preparation of MTT and MTT-formazan standard solutions

A concentration of 5 mg/mL MTT (99% HPLC grade, AK Scientific, USA) was prepared in sterile water and stored at 4 °C in the dark. MTT-formazan (99% HPLC grade, Sigma-Aldrich, USA) with a concentration of 1000 μg/mL was prepared in dimethyl sulfoxide (DMSO, AR grade, Merck) and stored at room temperature (22 °C) in the dark.

2.3. MTT reduction time determination and formazan quantification

The MTT reduction time was determined by a continuous time-yield study. The volume of 1 mL (V1) of *S. aureus* and *E. coli* suspensions were accurately taken and centrifuged (2400*g, 2 min) to remove the supernatant. Twenty-four samples were prepared for each bacterium and divided into 8 groups. Then all samples were resuspended in 1 mL of sterile water or phosphate buffered saline (We found in multiple replicate experiments that changing PBS to sterile water did not affect the bacterial ability to reduce MTT, data not shown), mixed with 100 μL 0.5% MTT (V: V = 10:1) and stirred. Groups 1–8 were left to stand for 5, 15, 30, 45, 60, 75, 90, and 105 min, respectively, at room temperature (22 °C). After each MTT reduction time point, the samples were centrifuged (11,337*g, 2 mins), the supernatant was removed, and 1 mL (V2) of isopropanol (AR grade, Merck) was added for *S. aureus* and 2 mL (V2) of isopropanol for *E. coli* to dissolve the formazan. The volume V2 is a variable depending on the type of bacteria tested, and will be described in detail in 3.4. Ultrasonic equipment (Bandelin DT 52) was used to accelerate the dissolution.

The obtained formazan solution was centrifuged (11,337*g, 10 mins) and filtered through a 0.22 μm syringe filter (13 mm diameter, Whatman Uniflo) to remove bacteria and precipitates, and then measured by high-performance liquid chromatography (HPLC, Thermofisher Ultimate 3000) with a diode array detector (DAD, Dionex Ultimate 3000) at 570 nm under the following conditions: Thermofisher Hypersil GOLD C18 Column 100 × 2.1 mm, particle size 1.9 μm and column guard Accucore C18 10 × 2.1 mm were used. Running method: Eluent A-MilliQ water, eluent B-Acetonitrile; 0–2 min 5% B, 2–3 min 5%–100% B, 3–10 min 100% B, 10–10.5 min 100%–5% B, 10.5–15 min 5% B. Column oven temperature: 25 °C; inject volume: 10 μL; flow rate: 0.3 mL/min. Each sample was repeated two-fold, followed by a blank run for washing accumulated salt in the column. The column was washed using the following running method: 0–15 min 5% B. Column oven temperature: 25 °C; flow rate: 0.2 mL/min.

2.4. MTT colorimetric assay on bacteria

Newly cultured *S. aureus* and *E. coli* suspensions (BHI broth, 37 °C, 24 h) were serially diluted into seven concentration gradients (10%, 25%, 40%, 55%, 70%, 85% and 100% of the initial concentration) with BHI broth respectively, and the optical density (OD) at 600 nm (OD_{600}, referred to as y_1) of each concentration was measured using a microplate reader (96 well plate, 200 μL /well, Thermofisher Varioskan LUX). For both bacteria, 1 mL (V1) of each concentration gradient was accurately taken and an MTT assay with a reduction time determined in 2.3 was performed. The peak area (x) of formazan was measured by HPLC as described in Section 2.3. The values of y_1 and 'x' were used to calculate key eq. 1 mentioned in 3.1.

2.5. Enumeration of viable bacteria

The dilution plate-counting method was used to obtain the concentration of colony-forming units (referred to as y_2 CFU/mL) of each gradient. Each bacterial suspension was diluted 10^6 or 10^7 times with sterile water, and 400 μL was put into the plate and mixed well with liquefied BHI agar (45 °C) and repeated 4 times. All plates were incubated at 37 °C for 24 h–72 h. The values of y_2 and y_1 (measured in 2.4) were used to calculate the key eq. 2 described in Section 3.1.

2.6. MTT-formazan peak area-concentration standard curve

The formazan standard solution with the initial concentration of 1000 μg/mL was serial two-fold diluted seven times and used to test linear intervals and derive a standard curve using the same HPLC conditions mentioned in 2.3. Each concentration was repeated 6 times. Key eq. 3 mentioned in 3.1 was derived from formazan concentration (y_3) and peak areas (x) to calculate the amount of formazan produced by bacteria.

2.7. Statistical analysis

Chameleon 7.2.7 (Thermofisher) was used for HPLC results analysis. Linear equations were calculated by GraphPad Prism 9.3.1, and linear regression analysis and linear correlation coefficients were calculated. Each data point was averaged from two to six replicates and expressed as mean ± standard deviation.

3. Results and discussion

3.1. Establishment of the equation

Based on the principle of the MTT assay, within a certain range of time and number of viable bacteria, the number of bacteria is linearly related to the amount of formazan transformed by them (Oh and Hong, 2022; Wang et al., 2010).

Therefore, we proposed the following equation:

$$CFU = kF$$

CFU is the number of colony-forming units, "k" is a constant scale factor, and F is the mass of formazan produced by bacteria.

This study introduced a method to find "k" in this equation so that a relatively accurate number of viable bacteria can be quickly calculated by detecting the amount of formazan produced.

It is known that within a certain range, the concentration of bacteria is linearly related to their optical density, and that the concentration of formazan is also linearly related to its absorbance (Stevens and Olsen, 1993). Showing the linear relationship between the optical density of bacterial suspension and the absorbance of formazan produced by bacteria is the key step to calculating "k".

"k" was calculated using three key equations:

(1) The equation between the OD_{600} of bacterial suspension (referred to as "y_1") and the peak area of the formazan solution (referred to as "x"), is displayed as follows:

$$y_1 = ax \quad (1)$$

"a" is the value of the scale factor.

Tested bacteria were liquid-cultured under a standard condition described in 2.1. The OD_{600} of bacterial suspension (y_1) was measured by a microplate reader. To initiate formazan production, 1 mL (V1) of the bacterial suspension was utilized in a 30 min MTT assay. Subsequently, the supernatant was replaced with either 1 or 2 mL (V2) of isopropanol, depending on the specific type of bacteria used. Isopropanol serves two functions: it inactivates bacteria and dissolves

formazan. The formazan isopropanol solution was measured by HPLC, and the peak area "x" was then calculated. The whole process was conducted on 7 different concentrations of bacterial suspension to obtain the value of the scale factor (a).

(2) The equation between the concentration of bacteria (y2, CFU/mL) and the OD600 of the bacterial suspension (y1), is shown below:

$$y_2 = by_1 \tag{2}$$

"b" is the value of the scale factor.

The traditional plate count method was applied for enumerating bacteria. The newly cultured bacterial suspension was mixed with different amounts of broth to prepare multiple concentrations. Followed by multiple plate counts were immediately performed to obtain the bacteria concentration (y_2, CFU/mL) and the OD_{600} of the bacterial suspension (y_1) under each concentration gradient was measured. The linear relationship equation was therefore obtained.

(3) The equation between the concentration of MTT-formazan standard (y3) and its peak area (x):

$$y_3 = cx \tag{3}$$

"c" is the value of the scale factor.

The MTT-formazan standard was accurately prepared at 1000 μg/mL, the peak area was measured by HPLC after multiple two-fold dilutions.

Combine the above equations:

$$\begin{cases} y_1 = ax \\ y_2 = by_1 \\ y_3 = cx \end{cases} \rightarrow y_2 = \frac{ab}{c} y_3$$

$\because y_2$ is the bacteria concentration.

$\therefore y_2 = \frac{CFU}{V1}$, CFU is the number of colony-forming units, and V1 is the volume of bacteria suspension used for formazan production.

$\because y_3$ is formazan concentration.

$\therefore y_3 = \frac{F}{V2}$, F is the mass of formazan produced by bacteria, and V2 is the volume of solvent used to dissolve formazan.

$\therefore \frac{CFU}{V1} = \frac{ab}{c} \cdot \frac{F}{V2}$

$\therefore CFU = \frac{abV1}{cV2} \cdot F$

$\therefore k = \frac{abV1}{cV2}$

For a single species, this equation describes the relationship between a certain number of CFUs and the mass of formazan produced by them at room temperature for 30 min.

3.2. Optimization of the MTT reduction procedure

MTT reduction time of 2–4 h is commonly used in most protocols and is likely selected by following the protocols used for mammalian cells (Brambilla et al., 2014; Corrado and Rodrigues, 2004; Foongladda et al., 2002; Li and Song, 2007; Mshana et al., 1998; Shi et al., 2007; Walencka et al., 2008; Wang et al., 2007; Wu et al., 2010). Continuous MTT assays at 37 °C have already been done on several kinds of bacteria, such as Gram-negative bacteria *E. coli* (Oh and Hong, 2022; Wang et al., 2010) and *P. mirabilis* (Grela et al., 2015), and Gram-positive bacteria L. *mesenteroides* (Oh and Hong, 2022) and *S. aureus* (Stevens et al., 1991). For all the bacteria we tested, MTT quickly reduced in the first 10–20 min, and then the reduction rate decreased rapidly over the next 40–100 min. This phenomenon may be caused by limited space in the bacteria for formazan to accumulate and the high efficiency of bacterial transformation of MTT. Cell injury caused by the transportation and accumulation of MTT and formazan crystals may also have affected the MTT reducing efficiency (Lü et al., 2012). Unlike mammalian cells, bacterial formazan tends to form cell-bound complexes. Some researchers also suggest that formazan can form complexes with biofilms, rather than forming needle-like crystals on the surface (Liu et al., 1997; Wang et al., 2010). To optimize the MTT reduction time at room temperature and to observe the time point of possible cell damage, we performed a continuous MTT assay, and took microscopic observations.

In the current study, to simplify the process and to avoid potential interference caused by changing temperature, as well as to slow down the efficiency of bacterial transformation of MTT, experiments were performed at room temperature after bacteria were cultured overnight. Bacteria have a high conversion efficiency of MTT; we found that at 37 °C, the bacteria produced formazan quantities beyond the linear range in a short period of time, thus performing the MTT assay at 37 °C is likely to lead to inaccurate results. Fig. 1 shows the results of this continuous MTT assay for 105 mins at room temperature. Compared with the production efficiency of formazan at 37 °C reported in articles (Grela et al., 2015; Oh and Hong, 2022; Wang et al., 2010), at room temperature, the efficiency slowed down and peaked at 30–40 min, and then showed a significant downward trend.

It is worth noting that in many parallel experiments, we found that both curves formed dips at specific reduction time points: 60 min for *S. aureus*, and 45 min and 75 min for *E. coli*, respectively. We compared the state and behavior of bacteria across time periods by microscopic observation and found that during 0–40 min, both tested bacteria began to rapidly form formazan in the cells and excreted formazan complexes at a specific time so that free formazan began to appear in the medium. This phenomenon has also been found in *P. mirabilis* (Grela et al., 2015). As shown in Figs. 2-1 and 2-3, formazan complexes can be observed inside the cell, and in Figs. 2-2 and 2-4, free formazan complexes can be found in the medium. To fully dissolve the formazan in the cells, the medium was removed prior to the formazan measurement. Thus, formazan excretion is an explanation for the dips formed on the curves since formazan excreted via exocytosis was not measured in this experiment. It can also be seen from the curve that after the first exocytosis, the bacteria continued to accumulate formazan in the cells, but the production efficiency dropped significantly, which we think is affected by the cell damage caused by the exocytosis of formazan (Lü et al., 2012).

Overall, in continuous MTT assays, the bacteria exhibit recurring patterns of MTT reduction and formazan excretion, as evidenced by the current findings indicating a cycle duration of approximately one hour, and this cyclic process potentially leads to continuous cellular impairment.

To ensure a more accurate representation of cell viability, it is recommended to measure the amount of formazan prior to the initiation of the first exocytosis phase, before cellular damage occurs. Consequently, a reduction time of 30 min at room temperature was selected as the

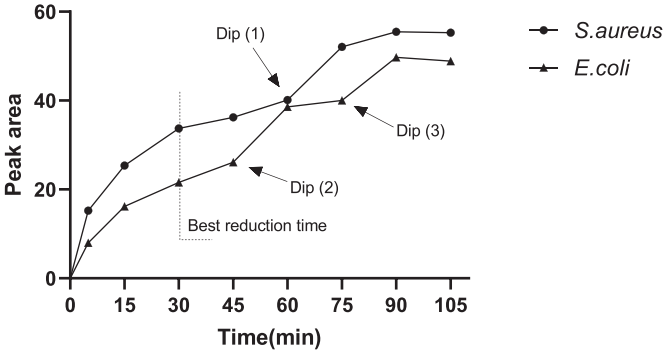

Fig. 1. Time-course profiles of formazan yield by *S. aureus* and *E. coli*. Tested bacteria were cultured in fresh BHI broth for 24 h and their capacity to reduce MTT at 5, 15, 30, 45, 60, 75, 90, and 105 mins were measured respectively. The formazan produced was dissolved with isopropanol and measured by HPLC at 570 nm.

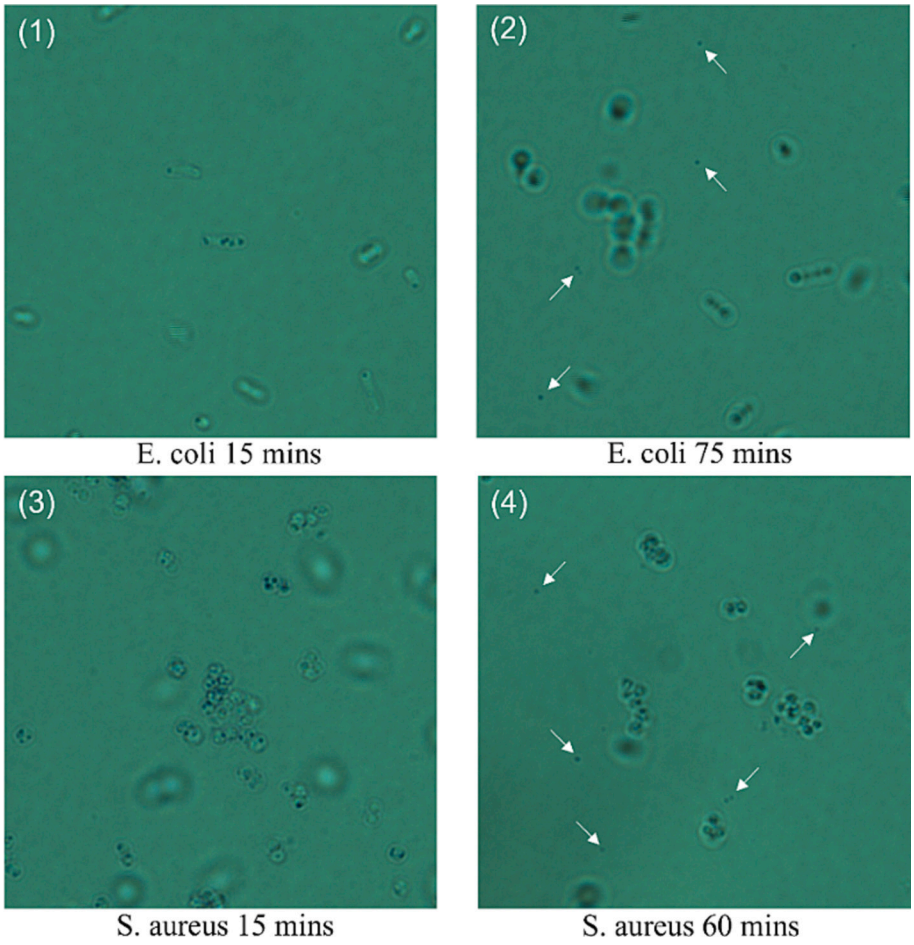

Fig. 2. Oil immersion microscope observation of *E. coli* and *S. aureus* during the MTT assay. In pictures 1 and 3, the presence of formazan is exclusively observed within the bacterial cells. The identification of free formazan complexes is denoted by arrows in pictures 2 and 4, corresponding to dip 3 and dip 1 in Fig. 1, respectively.

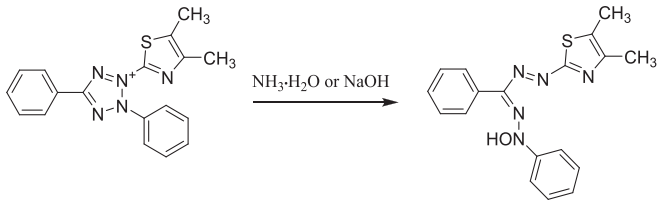

Fig. 3. The spontaneous conversion of MTT to formazan in $NH_3{\cdot}H_2O$ (1–10 M) and NaOH (0.1–1 M) solution.

optimal duration for MTT reduction in *S. aureus* and *E. coli.*

3.3. Optimization of the dissolution and quantification procedures of formazan

To measure the formazan accurately, adequate dissolution of formazan and suppression of interferences is essential. Some protocols advise removing the culture medium prior to adding the organic solvent, to ensure full dissolution of the formazan crystals and to avoid errors lead by the medium, such as unreduced MTT and chemical components that can disturb the formazan dissolution or the absorbance of formazan (Li and Song, 2007; Shi et al., 2007; Wang et al., 2010; Young et al., 2005). Removing the culture medium requires an additional step, and different amounts of culture medium residues also cause errors. Other researchers aim to avoid this step by using potent solubilizers such as ammonia and NaOH (Benov, 2021; Niks, 1990; Oh and Hong, 2022; Perri et al., 2016; Wang et al., 2012). However, we observed the following tetrazolium ring-opening reaction under room and higher temperatures in 30 mins, shown in Fig. 3. This property of MTT has been suggested for antioxidant ability testing (Liu and Nair, 2010). But in cell viability testing, it will give false positive results, leading to a read higher than the actual value.

It is also worth noting that the commonly used absorption wavelengths of formazan (490–630 nm) (Broughton and Jahans, 1997; Deb and Vimala, 2018; Mosmann, 1983; Shi et al., 2007; Stevens and Olsen, 1993; Stowe et al., 1995; Wang et al., 2007; Wang et al., 2012) overlaps with the wavelength range used for most microorganisms (400–770 nm) (Wang et al., 2011), which means neither method mentioned above can avoid the error caused by the bacteria itself; a clarified solution is difficult to obtain even with potent solubilizers. In addition, some other interferences cannot be ignored. Prolonged reduction time possibly causes the conversion of formazan to colorless derivatives (Stowe et al., 1995). Using a low pH buffer or acidic organic solvent causes absorbance to shift, leading to a lower reading (Plumb et al., 1989; Wang et al., 2012).

To eliminate all the above interferences, we suggest that the best path is to isolate formazan from the cell suspension and measure it individually. Structurally speaking, MTT formazan is a non-polar lipophilic compound; it will accumulate inside the cells before being actively transported by bacteria. In this experiment, we optimized the MTT reduction time (30 mins); we then completed the MTT assay (taking advantage of the property that MTT formazan is insoluble in water and is deposited inside the cells), separated the bacteria from

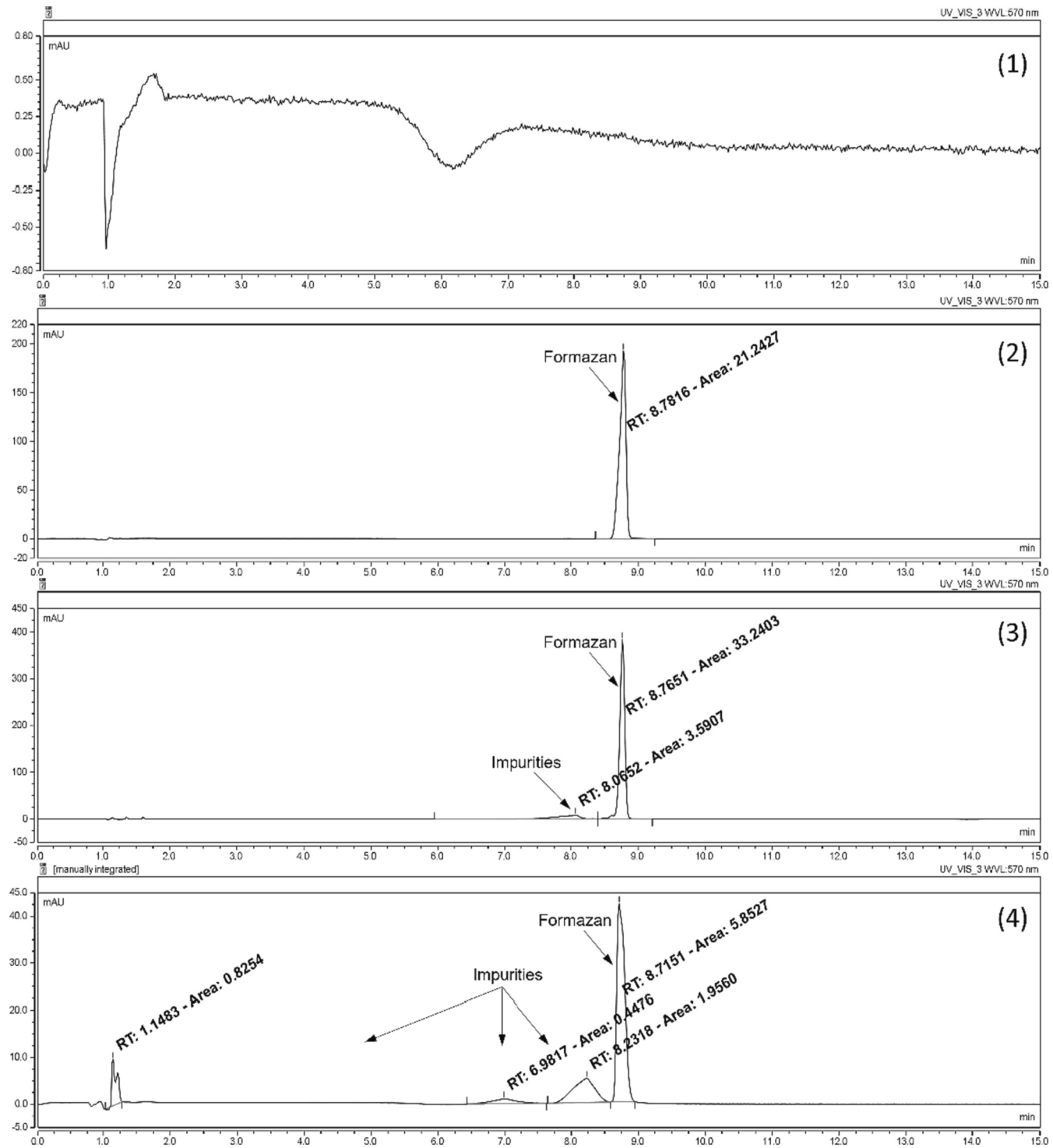

Fig. 4. Examples of HPLC measurement of formazan at 570 nm. 1) Pure water (Blank); 2) Formazan standard. No significant impurity signal peaks were observed; 3) Formazan produced by a high concentration of bacteria. The peak area of formazan accounts for 90% of the total peak area; 4) Formazan produced by the low concentration of bacteria. The peak area of formazan accounts for 64% of the total peak area.

aqueous solution by centrifugation, followed by pure organic solvent immersion and ultrasound-assisted dissolution. Next, the formazan was isolated by HPLC to achieve precise quantification. In this step, the solution loss during HPLC sample preparation did not affect the results because the concentration and injection volume of formazan solution was constant. Besides, during the analysis, a column guard was used to filter possible cell debris, and blank runs were applied after each sample for washing accumulated salt and monitoring the column pressure. The present study did not observe an obvious pressure increase after hundreds of runs, indicating that the HPLC sample preparation steps described in Section 2.3 were effective to remove debris. For the smooth entry of organic solvents into cells and subsequent reversed-phase HPLC analysis, we recommend acetone, isopropanol and DMSO, which are less polar ($\leq$ 50% water polarity) and miscible with water. In this experiment, the low-cost solvent isopropanol was used.

3.4. Linear equation of bacteria optical density and formazan peak area

The disadvantage of the microplate reader is that there is no selectivity for the target compound. That is, components that interfere with absorption will be read as well. HPLC results are shown in Fig. 4. Compared with the blank sample (1) and formazan standard (2), the impurity detected in the high-yield group (3) caused an error of around 10%, and the impurity detected in the low yield group (4) caused an error of over 30%. This result indicates that despite removing the bacteria and precipitates during sample preparation, there are still non-negligible impurities that can be read.

Therefore, in this experiment, HPLC was chosen as the best formazan measurement method for eliminating errors to the greatest extent. The peak area of formazan can be selectively and accurately obtained, demonstrating strong reproducibility in experiments.

To compare the reducing ability of the two tested bacteria, we first prepared *S. aureus* and *E. coli* suspensions into seven concentration gradients respectively and measured the OD_{600} with a microplate reader before the MTT assay. Thus, the OD_{600} of each concentration gradient was *S. aureus*: 0.0795, 0.1448, 0.2212, 0.3089, 0.4612, 0.6387, and 0.7709; *E. coli*: 0.0813, 0.1465, 0.2266, 0.3131, 0.5061, 0.6393, and 0.7640.

During the assay, we noticed that under culture conditions, the amount of formazan (peak area) produced by *S. aureus* had a good linear relationship with the concentration of the selected bacterial suspension, while *E. coli* lost the linear relationship when the OD_{600} of the bacterial suspension exceeded 0.5 and the measured value was lower than the theoretical value. At first, we thought that the increase in bacterial density reduced the overall viability of *E. coli*. However, when we double-diluted the formazan produced by *E. coli* and measured it again, a good linear equation was obtained. This result shows that for *E. coli*, when the OD_{600} of the bacterial solution is >0.5, the amount of formazan produced at room temperature for 30 mins still can exceed the linear range of the formazan absorbance, resulting in a lower reading than the actual value.

In the current protocol, for the *S. aureus* assay, we replaced the medium with the same volume of organic solvent to dissolve the formazan (V2 = V1 = 1 mL) and used twice the volume of organic solvent for the *E. coli* assay (V2 = 2 V1 = 2 mL) to ensure that subsequent testing results were all within the linear interval. The regression curves of bacterial OD_{600} and formazan peak area are shown in Fig. 5.

This step measured the scale factor "a" of both selected bacterium in key eq. 1: $y_1(\mathrm{OD600}) = ax(\mathrm{Peak\ area})$

S. aureus: $y_1 = 0.009840x$ (Intercept set to 0, $R^2 = 0.9962$, 1/slop = 101.6, $P < 0.0001$).

E. coli: $y_1 = 0.01071x$ (Intercept set to 0, $R^2 = 0.9918$, 1/slop = 93.33, $P < 0.0001$).

Interestingly, we obtained two similar equations after a double dilution of the formazan produced by *E. coli*. This result suggests that at the same volume and OD_{600}, the capacity of *E. coli* to reduce MTT is roughly twice that of *S. aureus*.

3.5. Linear equation of bacteria optical density and the number of CFUs

The pour plate technique was used in this experiment to reduce cell aggregation and cell adhesion caused by using the spreader. In our comparative pre-experiments, the higher CFU counting results were obtained by the pour plate technique, which agreed with the results in Pyar and Peh (2014).

Like the bacterial suspension preparation process mentioned in Section 2.4, the OD_{600} of each bacterial suspension was measured using a microplate reader before 10-fold dilution. We repeated the whole viable counting experiment three times, and each plate was counted twice after 24 h and 72 h, respectively. The linear relationship, with outliers removed, is shown in Fig. 6.

This step measured the scale factor "b" of both selected bacterium in key eq. 2: $y_2(\mathrm{CFU/mL}) = by_1(\mathrm{OD600})$

S. aureus: $y_2 = 488.3 \times 10^6 y_1$ (Intercept set to 0, $R^2 = 0.9961$, 1/slop = 0.002048, $P < 0.0001$).

E. coli: $y_2 = 251.3 \times 10^6 y_1$ (Intercept set to 0, $R^2 = 0.9959$, 1/slop = 0.003979. $P < 0.0001$).

The equations suggest that, under the current culture conditions, at the same volume and OD_{600}, the CFUs of *S. aureus* are roughly twice that of *E. coli*. In Section 3.4 we concluded that the capacity of *E. coli* to reduce MTT at the same volume and OD_{600} was twice that of *S. aureus*. Therefore, we conclude that under current culture conditions, individual *E. coli* to demonstrates a roughly four times greater capacity for MTT reduction compared to *S. aureus*.

3.6. Linear equation of formazan peak area and formazan concentration

Formazan has a long history as a dye compound (Aljamali et al., 2019). According to the color wheel, a dye normally absorbs light that is complementary to the reflected light; the deeper the color, the stronger the absorption (Kumar et al., 2021; Parkhurst and Feller, 1982). Due to this property, the absorbance-concentration linear interval of dyes tends to be much narrower than colorless substances since they may have stronger light absorption properties at very low concentrations.

In the linear interval measuring experiment, the real deviation was observed at formazan concentration higher than 125 μg/mL, as shown in Fig. 7. This result indicates that the linear range of the formazan dye as an indicator is indeed very narrow. It also further supports our inference in Section 3.4 that bacterium could produce formazan beyond the linear range in a short period of time. Moreover, the MTT reduction time should be controlled in a short period of time or the applicable concentration of MTT should be prepared much lower than that commonly used for mammalian cell experiments.

The linear equation within the linear interval of Fig. 7 was calculated. This step measured the scale factor "c" in key eq. 3: $y_3(\mu\mathrm{g/mL}) = cx(\mathrm{Peak\ area})$

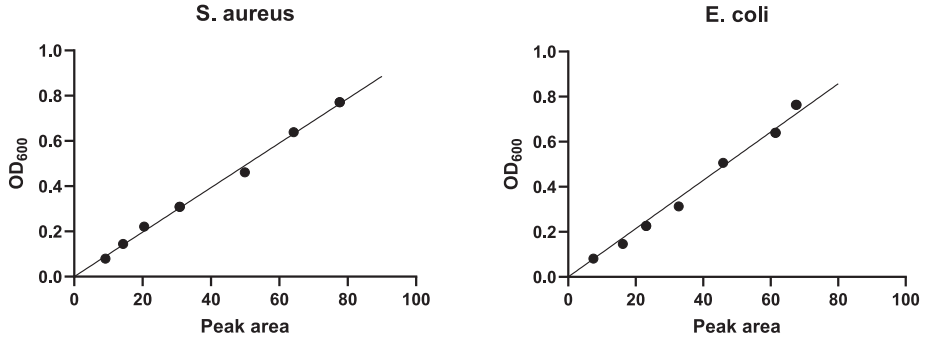

Fig. 5. The linear relationship between the optical density (OD) of bacterial suspension measured by microplate reader at 600 nm and the amount of formazan yield during MTT assay measured by HPLC at 570 nm.

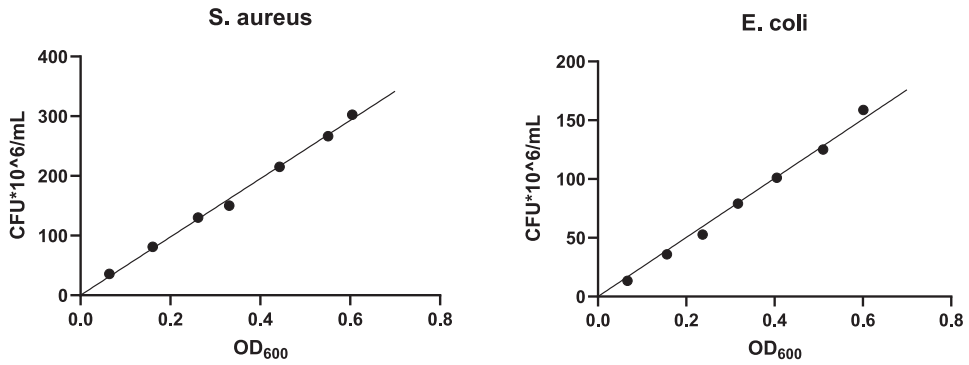

Fig. 6. The linear relationship between the optical density (OD) of bacterial suspension measured by microplate reader at 600 nm and CFU number obtained by the pour plate counting method.

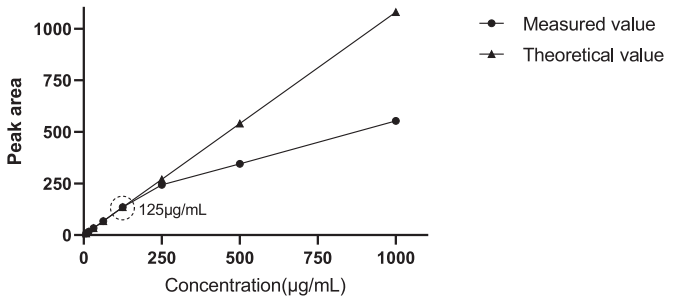

Fig. 7. Concentration-absorbance relationship curve of formazan standard measured by HPLC at 570 nm. The real deviation was observed at concentrations above 125 μg/mL.

$y_3 = 0.9278x$ (Intercept set to 0, $R^2 = 1.0$, 1/slop = 1.078, $P < 0.0001$).

3.7. Linear equation of the number of CFUs and the mass of formazan produced

Back to the proposed equation $CFU = kF = \frac{abV1}{cV2}.F$

'CFU' is the number of colony-forming units, and 'F' is the mass of formazan produced by bacteria. 'V1' is the volume of bacterial suspension to be tested, and 'V2' is the volume of organic solvent used to dissolve formazan. Both are artificially chosen constants. 'a', 'b' and 'c' are the constants associated with the bacterial culture conditions that have been calculated in the three key equations described above.

Therefore, the CFU number and the amount of formazan relationship equations are:

S. aureus: $CFU = \frac{abV1}{cV2}.F = \frac{0.00984 \cdot 488.3 \times 10^6 \cdot 1}{0.9278 \cdot 1}.F = 5.1788 \times 10^6 \cdot F$

E. coli: $CFU = \frac{abV1}{cV2}.F = \frac{0.01071 \cdot 251.3 \times 10^6 \cdot 1}{0.9278 \cdot 2}.F = 1.4504 \times 10^6 \cdot F$

Using these equations, the number of CFUs in the bacterial suspension (cultured under standard conditions) can be calculated directly by measuring the amount of formazan produced at room temperature for 30 mins. Moreover, the CFU calculated by these equations can also be used as a unit for the overall viability of bacteria, representing the equivalent of bacteria cultured under other conditions relative to standard conditions.

This method also has the potential to improve the current evaluation methods such as minimum inhibition concentration (MIC) testing. For the conventional broth dilution method, by performing an MTT assay, the inhibition rate at each concentration of tested compound can be evaluated by calculating the initial and final CFU number, thereby obtaining the growth inhibition curve to evaluate the amount-effect relationship. Furthermore, this method greatly reduces the errors caused by colored compounds (colored solution) as well as non-water-soluble compounds (turbid liquid).

4. Conclusion

The present study provided an improved protocol that aims to eliminate interferences in both the reduction and measurement stages of the bacterial MTT assay, achieving an accurate quantification of formazan. We described a method to establish an equation for a given bacterium to calculate its CFUs by measuring the amount of formazan. Providing a possible alternative method for microplate counting greatly saves time for subsequent experiments. This equation is based on the fundamental principle of the MTT assay. Theoretically, the establishment method of the equation is applicable to most microorganisms. In the current study, *S. aureus* and *E. coli* were used, and the reduction time was determined according to their cycle of production and excretion of formazan. It may be necessary to re-determine the reduction time by using the continuous MTT assay described in Section 2.3 when it is applied to other strains.

Our experiment also identified a new limitation of using the MTT assay on bacteria, which is the narrow linear interval of formazan concentration-absorbance and provided the strategies to solve it. The experiment was conducted under controlled conditions at room temperature to reduce formazan production. Additionally, the concentration of the resulting formazan solution was regulated within the linear range using solvent dilution. The current findings provide evidence that *S. aureus* and *E. coli* reduce MTT by a cyclic process of formazan accumulation, exocytosis, and re-accumulation. The first cycle takes about 40–60 min and causes cell damage and formazan leakage. The optimal reduction time for both bacterial strains tested is 30 mins. The capacity of individual *E. coli* to reduce MTT is roughly four times greater than that of *S. aureus*. To avoid interferences in the dissolution and quantification of formazan, HPLC was employed to separate the formazan from the mixture, enabling precise measurement of the peak area. This method is different from conventional MTT assay protocols for bacteria and achieved a significant improvement.

In summary, this enhanced MTT bacterial assay serves as a means of data standardization through HPLC analysis. The precise quantification of formazan facilitates the accurate determination of CFUs, potentially obviating the necessity for protracted and repetitive plate-counting procedures in extended experiments. Furthermore, the refinement of this methodology may yield valuable insights for other assays associated with MTT.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

Data availability

All raw data and experimental details are available upon request.

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Appendix. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2023.106830.

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