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Enhancing Formazan Dissolution to Improve Accuracy in MTT-Based Cell Viability Assays

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Abstract

The 3-(4,-5-*dimethylthiazo*-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is a method that used to measure cell viability. It is based on the conversion of MTT by metabolically active cells succinic dehydrogenase enzyme into water insoluble formazan. Dissolution of formazan by using proper solvent is the most important step of MTT assay to obtain valid and reliable data. In this study, various solvents were assessed to facilitate faster, less labor-intensive, and more reliable dissolution of formazan. The solvents tested included dimethyl sulfoxide (DMSO), DMSO supplemented with 0.005 N hydrochloric acid (HCl), 10% sodium dodecyl sulfate (SDS) in water containing 0.005 N HCl, 5% SDS with 0.005 N HCl in DMSO, and 10% SDS with 0.005 N HCl in DMSO. Formazan solubility was assessed using BALB/3T3 cells. Following the addition of MTT, cells were incubated for 4 h at 37˚C, after which formazan crystals were dissolved either with or without medium, and absorbance was measured at 570 nm. Among the solvents compared, DMSO was the most effective for formazan dissolution in the absence of medium, whereas 10% SDS with 0.005 N HCl in DMSO yielded the highest optical density (OD) in the presence of medium. The lowest solubility was observed with 10% SDS and 0.005 N HCl in water. Additionally, the improved MTT assay eliminates the requirement for medium removal, hence enabling its employment under both conditions—with or without medium. This experimental improvement simplifies the adaptability of the MTT assay for adherent cell lines and streamlines its application in suspension cell lines, eventually improving its overall utility while simplifying the experimental procedure.

Keywords

MTT, Formazan Solubility, Cell Culture, Solvents

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1. Introduction

The MTT assay is one of the most widely used colorimetric techniques for measuring cell proliferation because it is safe, straightforward, affordable, and appropriate for a variety of cell lines [1] [2]. The methodology was developed on the bioreduction of yellow water-soluble MTT by mitochondrial [1], nonmitochondrial [3], and plasma membrane reductase [4] of metabolically active cells into the water insoluble formazan crystals. However, the amount of formazan produced is directly proportional to the number of viable, metabolically active cells. Multiple research investigations have examined the effects of different parameters on the conversion of MTT to formazan [5] [6], but the solvent's function in dissolving the byproduct formazan has received far less attention. Formazan is a precipitate that is insoluble in water, so a suitable solvent is needed to completely dissolve this precipitate into a consistent solution. The amount of formazan present can be determined by spectrophotometrically measuring the OD of this colored solution at a particular wavelength, which in turn indicates cell viability. In addition to producing the water-insoluble product formazan, an appropriate solvent is essential to fully dissolve the crystals and obtain a clear solution [7].

Although the MTT assay is widely employed in numerous studies, it continues to present technical challenges. These include incomplete dissolution of formazan crystals, limited stability of the formazan solution, and protein precipitation within the culture medium, all of which diminish the sensitivity and accuracy of bioassays [8]. The formazan crystals are dissolved in the culture media by adding acidified isopropanol in the first MTT testing procedure [1]. In MTT experiments, formazan is dissolved using a variety of solvents, such as 10% SDS with 0.01 N HCl [9], and DMSO [10], ammonia containing DMSO [11], 20% sodium dodecyl sulfate (SDS) in 50% dimethylformamide (DMF) [12] [13], 10% and 20% SDS containing 0.01 N HCl [14] and dimethylformamide (DMF) [9] [10]. The best solvent for dissolving formazan in adherent cell lines is undiluted DMSO, which is made easier by removing the culture media before use [10]. Each solvent possesses distinct properties that affect its ability to dissolve formazan. When organic solvents such as DMSO or isopropanol are added to the medium of suspension cells, proteins from lysed cells or supplemented serum may precipitate [8]. In contrast, solvents such as SDS and DMF do not require medium removal, but they require extended incubation to achieve complete dissolution of formazan [9]. However, if the medium for culture was not removed, the absorbance could be impacted by the presence of phenol red, which is frequently used as an indicator of pH in media for cell culture [15]. For these reasons, to dissolve formazan in the MTT experiment, the culture media must be removed before the solvent is applied. However, it is suggested that adherent cell lines are better suited for the MTT experiment than suspension cells. Therefore, to broaden its application in cell quantification, the MTT assay requires further optimization. The effectiveness of SDS lysis solution in promoting suspension cell lysis and formazan dissolution

has been investigated [9]. However, the mechanism by which formazan dissolves in cell culture media, whether through DMSO alone or SDS in combination with DMSO, remains unclear. Despite significant progress in the development of viability assays, the MTT assay remains extensively utilized and interpreted. Numerous investigations have examined confounding factors such as cell seeding density, MTT concentration, incubation time, culture medium composition, supernatant removal, optical density measurement, and treatment conditions. However, issues related to formazan solubility and the ongoing demand for improved solubilization strategies remain unresolved.

The aim of this investigation was to optimize the water-insoluble formazan's solubilization in the MTT assay for evaluating cell viability. To achieve faster, less labor-intensive, and more accurate measurements, five solvent systems were evaluated for their capacity to dissolve formazan, which includes DMSO, DMSO containing 0.005 N HCl, 10% SDS in water with 0.005 N HCl, 5% SDS with 0.005 N HCl in DMSO, and 10% SDS with 0.005 N HCl in DMSO. The efficacy of these solvents was examined using formazan generated by BALB/3T3 mouse fibroblast cells, both in the presence and absence of culture medium.

2. Materials and Methods

2.1. Materials

The mouse fibroblast cell line BALB/3T3 was obtained from the American Type Culture Collection (Manassas, VA, USA). Dulbecco's Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute (RPMI) 1640 medium were purchased from Life Technologies Corporation (Grand Island, NY, USA). Sodium dodecyl sulfate (SDS), dimethyl sulfoxide (DMSO), hydrochloric acid (HCl), Fetal Bovine Serum (FBS) and 0.25% trypsin-EDTA solution were obtained from Thermo Fisher Scientific (Cranbury, NJ, USA). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit was purchased from Millipore Sigma (Rockville, MD, USA).

2.2. Cell Culture Conditions

BALB/3T3 cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 0.01 mg/mL streptomycin sulphate. Prior to experimentation, BALB/3T3 cells were subcultured to account for their adherent growth properties. The cells were rinsed with about 10 mL of phosphate-buffered saline (PBS, pH 7.4) after the unused medium was removed. The cells were then allowed to separate from the monolayer for a few minutes at room temperature after about 3 mL of 0.25% trypsin-EDTA solution in Hanks' balanced salt solution (without calcium or magnesium) were added. The detached cells were then resuspended in complete medium, and cell viability was determined using the dye exclusion method [16]. The cells were maintained at greater than 90 percent viability throughout the study.

2.3. MTT Assay

The MTT assay was employed to evaluate the metabolic activity of BALB/3T3 cells and to assess the effects of different solvents on formazan dissolution. The basis of this colorimetric analysis is the reduction of the yellow tetrazolium salt of MTT in metabolically active cells by the mitochondrial enzyme succinate dehydrogenase to a water insoluble formazan crystal. The amount of formazan produced is directly proportional to the number of viable cells and can be quantified spectrophotometrically at 570 nm.

2.4. Effect of Different Cell Density on Formazan Solubility in Different Solubilizing Solvents

To evaluate the influence of cell density and incubation time on formazan solubilization, BALB/3T3 cells were seeded in 100 µL volumes at densities of 2500, 5000, and 10,000 cells per well. The cells were cultured for overnight at 37˚C in a humidified incubator containing 5% CO_2. Following incubation, 10 µL of MTT stock solution (5 mg/mL) was added to each well of two separate 96 well plates, and the plates were incubated for an additional 4 h under the same conditions to allow MTT reduction and formazan crystal formation.

After incubation, the culture medium was removed from the wells of the first plate without disturbing the adherent cells and the formazan crystals, whereas the medium in the wells of the second plate was left undisturbed. Subsequently, 200 µL of various solubilization solvents such as DMSO, DMSO containing 0.005 N HCl, 10% SDS in water with 0.005 N HCl, 5% SDS with 0.005 N HCl in DMSO, or 10% SDS with 0.005 N HCl in DMSO was added separately to each well. The plates were then incubated under the same conditions at 37˚C for 1 h to ensure complete dissolution of the formazan crystals and the formation of homogeneous solutions. Absorbance of the resulting-colored solutions was measured at 570 nm. Background absorbance for medium-containing wells was corrected using blank wells filled with 100 µL of complete medium, while background absorbance for medium-free wells was adjusted using 100 µL of each solubilization solvent alone.

2.5. Different Formazan Solubility Solvents Test

A suspension containing 5×10^3 BALB/3T3 cells in 100 µL was dispensed into each well of two separate 96-well plates. In parallel, two cell-free control plates were prepared to evaluate potential non-enzymatic reduction of MTT by culture medium components, FBS, and the solubilizing solvents [6]. In the first control plate, each well was added with 100 µL of a different solubilization solvent: DMSO, DMSO containing 0.005 N HCl, 10% SDS in water with 0.005 N HCl, 5% SDS with 0.005 N HCl in DMSO, or 10% SDS with 0.005 N HCl in DMSO. In the second control plate, each well added 100 µL of complete medium, but without cells.

The test and control plates were incubated overnight at 37˚C in a humidified atmosphere containing 5% CO_2. Following incubation, 10 µL of MTT stock solution (5 mg/mL) was added to each well, and the plates were further incubated for

4 h under the same conditions. At the end of this period, the culture medium in the wells of the first test plate was carefully aspirated to ensure complete removal without disturbing either the adherent cells or the formazan crystals formed on the plastic surface. However, the medium in the wells of the second test plate had remained unchanged. For the control plates, both the medium and the solvents were retained without removal. Subsequently, 200 µL of different solubilization solvents such as DMSO, DMSO containing 0.005 N HCl, 10% SDS in water with 0.005 N HCl, 5% SDS with 0.005 N HCl in DMSO, and 10% SDS with 0.005 N HCl in DMSO were added individually to each test and control plates wells. The plates were then incubated at 37˚C for periods ranging from 5 min to 24 h to allow dissolution of the formazan crystals and formation of a homogeneous solution in the respective solvents. The absorbance of the colored solutions resulting from the different solubilization solvents was measured at 570 nm at each test time point. The background absorbance in medium-containing wells was adjusted using blank wells that contained 100 µL of complete medium. Similarly, background absorbance in medium-free wells was subtracted by using 100 µL of each solubilization solvent independently. These experiments used data from two or three replicate wells to calculate each point, and three independent assays were used to evaluate each test condition.

2.6. Effect of HCl Concentration in Different Solvents

To examine the impact of HCl concentration in formazan solubilization solvents, BALB/3T3 cells were seeded into two separate 96-well plates at a density of 5000 cells per well in 100 µL of suspension. The plates were incubated for overnight at 37˚C in a humidified atmosphere containing 5% CO_2. After incubation, 10 µL of a 5 mg/mL MTT stock solution was added to each well, and the plates were incubated for an additional 4 h under the same conditions. Following incubation, the culture medium was carefully removed from the first plate's wells without disturbing the cells or the formazan crystals that had developed, while medium had remained in the second plate's wells. Then, 200 µL of the respective solubilization solvents were individually added to each well: DMSO, DMSO containing 0.01 N HCl, 10% SDS in water with 0.01 N HCl, 5% SDS in DMSO with 0.01 N HCl, and 10% SDS in DMSO with 0.01 N HCl. Afterward, the plates were incubated at 37˚C for varying time intervals ranging from 10 min to 24 h to ensure complete dissolution of the formazan crystals and the formation of homogeneous solutions under each solvent condition. The absorbance of the colored solutions resulting from the different solubilization solvents was measured at 570 nm at each test time point. Blank wells containing 100 µL of complete medium were used to adjust the background absorbance in medium-containing wells. Similarly, 100 µL of each solubilization solvent was used separately to subtract background absorbance in medium-free wells. Each point in these experiments was calculated using data from two or three replicate wells, and each test condition was assessed using two separate assays.

3. Results

3.1. Determined the Effect of Varying Cell Numbers on Formazan Solubility in Different Solvents

The assay is dependent on the ability of viable cells to metabolize a water-soluble tetrazolium salt into a water-insoluble formazan product. To examine the effect of cell density and incubation conditions on formazan solubilization, BALB/3T3 cells were seeded in 96-well plates at densities of 2500, 5000, and 10,000 cells per well in 100 µL volumes. Following incubation with MTT, two approaches were used: in one plate, the medium was completely aspirated without disturbing the cells or formazan crystals (**Figure 1(A)**), while in the other plate the medium was retained (**Figure 1(B)**). Subsequently, 200 µL of various formazan-solubilizing solvents such as DMSO, DMSO with 0.005 N HCl, 5% SDS and 0.005 N HCl in DMSO, 10% SDS and 0.005 N HCl in DMSO, and 10% SDS and 0.005 N HCl in water were added to each well, and the plates were incubated for 1 h at 37˚C. In theory, the intensity of the formazan color directly reflects the number of viable cells. The resulting OD values (**Figure 1(A)** and **Figure 1(B)**) demonstrated that OD increased with cell seeding density, reflecting the greater amount of formazan produced by larger cell populations. This result is consistent with earlier studies demonstrating that increasing cell counts lead to proportionally stronger MTT signals [17], while OD values begin to decline once cell numbers surpass a critical threshold [17]. When formazan solubilizing solvents are added directly to the culture medium, an increase in OD at 570 nm is typically observed (**Figure 1(B)**). In the present experiment, however, no saturation in OD was detected with rising cell density. This result is unsurprising, as the maximum seeding density employed (10,000 cells per well) corresponded to only ~70% - 80% confluence at the time of measurement (following overnight incubation), as confirmed by light microscopy observations.

To understand how assay parameters affect the relationship between OD and cell number, these results show that thorough control experiments are necessary prior to using the MTT assay to assess cell viability. Importantly, because formazan production depends on more than just the number of viable cells, OD values may still be biased. MTT uptake [18] and metabolic activity [19] can be changed by experimental conditions or treatments, which can impact the result. Since such, MTT reduction, formazan formation, and absorbance are all influenced by a variety of factors rather than just one, which is ultimately reflected in the measured OD. Results are means from three independent experiments. In subsequent assays, a seeding density of 5,000 cells per well was selected based on this prior cell density evaluations.

3.2. Formazan Was Solubilized Using Different Solvents

The present investigation tested whether the combination of DMSO, SDS, and HCl provides a synergistic impact on the dissolving of water-insoluble formazan,

considering that DMSO and SDS lysis solutions are acknowledged as among the most effective solvents for formazan [9] [10]. The choice of solubilizing solvents was guided by preliminary test results as well as evidence from the literature [20]. The suitability of SDS and the organic solvent DMSO for use in the MTT assay for BALB/3T3 cells was tested using a variety of formazan solubilization solvents, such as DMSO, DMSO with 0.005 N HCl, 5% SDS and 0.005 N HCl in DMSO, 10% SDS and 0.005 N HCl in DMSO, and 10% SDS and 0.005 N HCl in water. Since phenol red is frequently used as a pH indicator in cell culture medium. It might have an impact on formazan's absorption spectrum in MTT colorimetric tests [15]. To mitigate this effect, 0.005 N HCl was added to the solubilizing solvents. At this acidic pH, all the phenol red is protonated and evenly yellow, reducing its spectral interference. This kind of the dye has little to no impact on absorbance measurements at an appropriate wavelength. The aim of this investigation was to examine the solubility of formazan without medium and in medium with different solvents. The OD of the colored formazan solution, dissolved in different solubilization solvents, was measured at a wavelength of 570 nm.

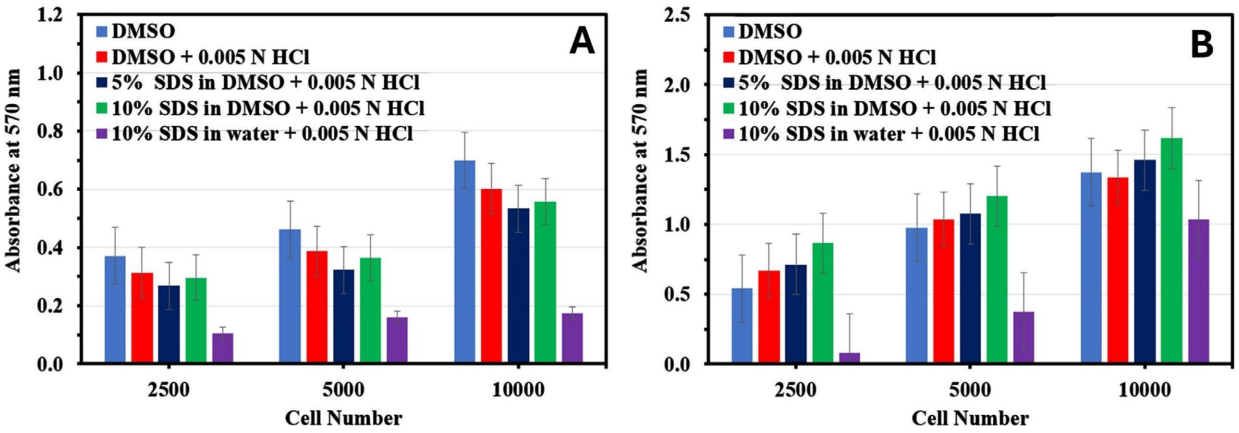

Figure 1. Formazan solubility depends on cell seeding number: As described in the experimental section, the BALB/3T3 cells were seeded at densities of 2500, 5000, and 10,000 cells in 100 µL per well in separate 96 well plates. After incubated with MTT. (A) The medium was completely removed from each well and (B) Medium was retained in the wells of the plate. Subsequently, 200 µL of different solubilization solvents (DMSO, DMSO containing 0.005 N HCl, 10% SDS in water with 0.005 N HCl, 5% SDS with 0.005 N HCl in DMSO, 10% SDS with 0.005 N HCl in DMSO) was added to each well. Both plates were then incubated at 37˚C for 1 h. The absorbance of was measured at 570 nm. Wells with medium and different solubilizing solvents were used for background correction. Data represent the mean of duplicate wells, and results are shown as the average of two independent experiments.

BALB/3T3 cells were incubated with MTT in two independent 96-well plates for 4 hours at 37˚C to assess the efficiency of various solvents in dissolving formazan. In the first plate, the culture medium was carefully aspirated, leaving the cells and formazan crystals undisturbed, whereas in the second plate the medium was retained. Subsequently, 200 µL of the respective solvents was added to each well of both plates. The plates were maintained at 37˚C, and formazan dissolution was monitored at predetermined time points (5 min, 10 min, 30 min, 1, 2, 3, 4, 5, and 24 h). The most effective solvent for dissolving formazan was determined by

comparing DMSO, DMSO with 0.005 N HCl, 5% SDS and 0.005 N HCl in DMSO, 10% SDS and 0.005 N HCl in DMSO, and 10% SDS and 0.005 N HCl in water, both in without and with a medium (Figure 2(A) and Figure 2(B)). The results showed that after discarding the medium, DMSO accelerated the rapid dissolution of formazan (Figure 2(A)), which is consistent with previously reported observations [10]. In the absence of culture medium, DMSO effectively dissolved formazan, resulting in a bright purple solution with high OD (Figure 2(A)). In contrast, formazan dissolution in DMSO with 0.005 N HCl was incomplete (Figure 2(A)), resulting in lower optical density compared to DMSO alone. These findings suggest that formazan is probably unstable under acidic conditions, resulting in slower degradation and reduced solubility in acidified solvents.

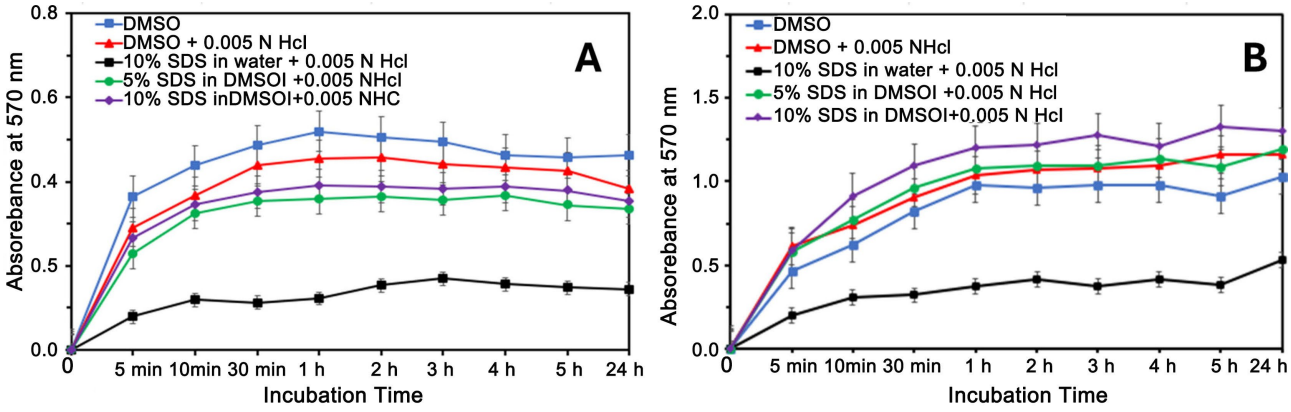

Figure 2. The solubility of water-insoluble formazan was evaluated using the MTT assay. Procedure as described in the experimental section. Followed by the incubation of MTT (A) Complete removal of culture medium without disturbing adherent cells or formazan crystals, and (B) Retention of culture medium in the wells. Subsequently, separately 200 µL of different solubilization solvents was added to each well: DMSO, DMSO containing 0.005 N HCl, 10% SDS in water with 0.005 N HCl, 5% SDS in DMSO with 0.005 N HCl, or 10% SDS in DMSO with 0.005 N HCl. Plates were incubated at 37˚C for 5 min to 24 h. Absorbance was measured at 570 nm at each time point. Background correction was performed using blank wells containing complete medium, while wells without medium were corrected using the corresponding solubilization solvent. All values represent the mean of duplicate wells, and data are presented as the average of three independent experiments.

Figure 2(A) demonstrates that 5% and 10% SDS in DMSO containing 0.005 N HCl exhibited similar dissolution patterns. Although these SDS-based solvents produced a purple solution, the OD was lower than that achieved with DMSO alone, indicating reduced efficiency. Furthermore, formazan dissolved more slowly and often incompletely in acidified DMSO with both 5% and 10% SDS (Figure 2(A)). These findings suggest that, while acidified SDS in DMSO is capable of dissolving formazan, the combination is suboptimal. A water-based SDS solution is therefore recommended for consistent dissolution, as acidified DMSO may promote SDS precipitation and diminish its effectiveness. To further evaluate this conclusion, formazan solubility was tested in water containing 10% SDS and 0.005 N HCl. OD measurements revealed that acidified SDS in water produced a lower OD (Figure 2(A)). These results indicate that the hydrophobic formazan crystals dissolve inefficiently in water-based SDS solutions. This reduced solubility in acidified aqueous SDS likely promotes the formation of precipitates or leaves crystals undis-

solved. Collectively, the data demonstrate that DMSO is the most effective solvent for formazan dissolution, particularly in the MTT assay when the culture medium is completely removed prior to solvent addition.

Similarly, formazan was dissolved by adding various solubilizing solvents directly to wells containing culture medium, including DMSO, DMSO with 0.005 N HCl, 5% SDS with 0.005 N HCl in DMSO, 10% SDS with 0.005 N HCl in DMSO, and 10% SDS with 0.005 N HCl in water. Among these, DMSO supplemented with 10% SDS and 0.005 N HCl produced the highest OD (**Figure 2(B)**), indicating that this combination most efficiently and rapidly solubilizes formazan crystals and cells. Collectively, these findings demonstrate that 10% SDS in DMSO with 0.005 N HCl is the optimal solvent for rapid formazan dissolution when the culture medium is retained. However, implementing this mixture of solvent has larger benefits since it may be introduced directly to formazan crystals in a medium and does not require overnight incubation [14]. Similarly, the results demonstrated that solvents such as 5% SDS with 0.005 N HCl in DMSO, DMSO containing 0.005 N HCl, and DMSO alone dissolved formazan crystals more slowly. Moreover, the OD values obtained with these solvents were lower than those measured with 10% SDS and 0.005 N HCl in DMSO, indicating that formazan was not fully solubilized under these conditions.

Complete dissolution of water insoluble formazan crystals in culture medium typically requires a solubilization solution containing high concentrations of SDS [9], most commonly at 10%. The OD increases proportionally with the degree of formazan dissolution, indicating the efficiency of the solubilization process. Furthermore, 10% SDS with 0.005 N HCl in water was evaluated, and this condition produced the lowest OD, indicating that formazan crystals were least soluble in this aqueous SDS solution when culture medium was present. These findings show that, when the culture medium is present during the MTT experiment, 5% SDS with 0.005 N HCl in DMSO, 10% SDS with 0.005 N HCl in DMSO, or DMSO alone are the most effective solvents for dissolving formazan when compared to 10% SDS and 0.005 N HCl in water solution. Furthermore, because the media did not need to be removed, the modified MTT assay can be used on adherent cells both with and without medium. It is also likely applicable to suspension types of cells. The mean of three separate experiments is used to report all results.

The results suggest that incubation of 5000 BALB/3T3 cells per well in a 96-well plate with 0.5 mg/mL MTT for four hours gave the optimal level of MTT reduction. However, both cell density and MTT concentration were observed to influence the saturation time point [6]. The results demonstrated that wells in which the medium was retained yielded the highest OD values, whereas wells with medium removal showed the lowest OD. This difference can be attributed to the contribution of the medium itself to the absorbance signal, thereby elevating the total OD. Moreover, once formazan is dissolved in DMSO or other DMSO-based solvents, the OD values remain stable for several hours. Overall, the results show that

formazan dissolves quickly in the presence of medium when a mixture of 10% SDS and 0.005 N HCl in DMSO is used, while formazan dissolves effectively in DMSO alone after the medium has been completely removed. In addition, this result can be related to the chemical properties of DMSO, a polar organosulfur molecule that is miscible with water and a variety of organic solvents, allowing it to dissolve polar and nonpolar chemical compounds.

3.3. Components of the Cell-Free Medium That Impact MTT Reduction

Some medium components can reduce MTT, leading to misleading results in formazan solubility assays. To mitigate this, blanks containing the complete medium, but no cells were prepared and analyzed in parallel with experimental samples. In the absence of metabolically active cells, medium components such as FBS, amino acids, reducing sugars, vitamins, and other reducing agents may react with MTT to generate formazan crystals. This abiotic reduction produces artificially elevated OD values, resulting in inaccurate results. Therefore, the possibility of non-enzymatic reduction of MTT by culture medium containing FBS and various solubilizing solvents was examined in two independent 96-well plates. Found that none of the five tested solvents such as DMSO, DMSO with 0.005 N HCl, 10% SDS in water with 0.005 N HCl, 5% SDS with 0.005 N HCl in DMSO, or 10% SDS with 0.005 N HCl in DMSO were capable of reducing MTT to formazan (Figure 3(A)). In all cases, the OD values obtained from the solvent mixtures were nearly identical to those of the blank control, confirming the absence of MTT reduction. These results clearly indicate that solvent-based solubilizing mixtures do not contribute to non-enzymatic MTT reduction.

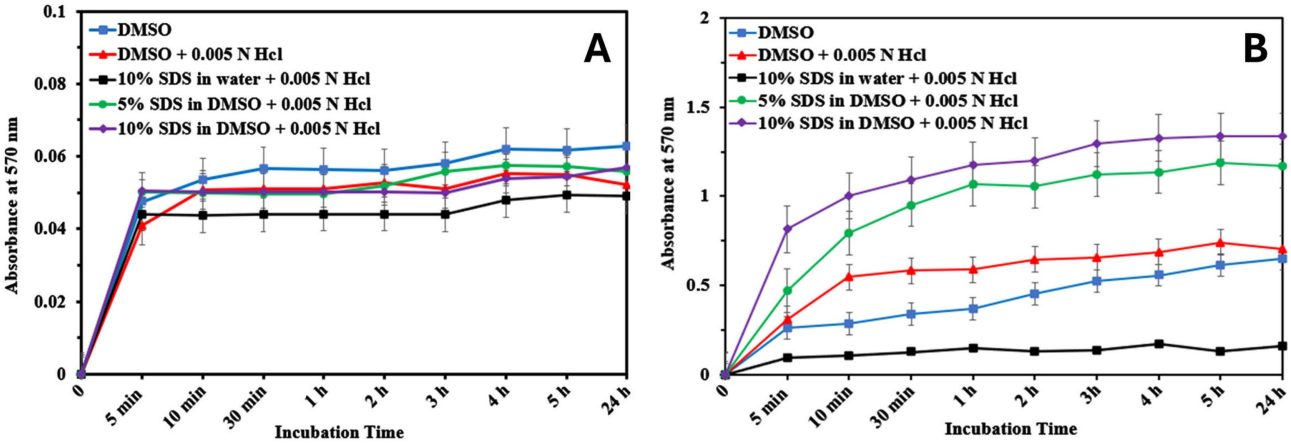

Figure 3. Non-enzymatic reduction of MTT by culture medium components and solubilizing solvents was assessed using cell-free controls, as described in the experimental section. Two sets of controls were prepared: (A) 100 µL of each formazan-solubilizing solvent incubated with MTT, and (B) 100 µL of culture medium incubated with MTT. Following incubation, 200 µL of the respective solubilization solvent (DMSO, DMSO containing 0.005 N HCl, 10% SDS in water with 0.005 N HCl, 5% SDS in DMSO with 0.005 N HCl, or 10% SDS in DMSO with 0.005 N HCl) was added to each well. Plates were further incubated at 37˚C for 5 min to 24 h. Then absorbance was measured at 570 nm at each time point. Data represent the mean of duplicate wells, and results are presented as the average of two independent experiments.

When different solvents were individually added to the culture medium, the plate containing medium (**Figure 3(B)**) revealed that 10% SDS with 0.005 N HCl in DMSO produced the highest OD, followed by 5% SDS with 0.005 N HCl in DMSO. DMSO alone and DMSO with 0.005 N HCl also generated measurable OD values, though these were lower than those obtained with SDS-containing solvents. These results suggest that components of the medium may contribute to non-enzymatic reduction of MTT, while 10% SDS with 0.005 N HCl in DMSO provides the most efficient formazan solubilization, yielding the highest OD. In comparison, 5% SDS with 0.005 N HCl in DMSO yielded lower OD values, suggesting that a higher SDS concentration (10%) is required for complete dissolution of formazan in medium. The markedly lower OD values observed with DMSO and DMSO containing 0.005 N HCl further emphasize the essential role of SDS in effective solubilization. The lowest OD was recorded with 10% SDS in water containing 0.005 N HCl (**Figure 3(B)**), confirming that dissolution of water-insoluble formazan requires the presence of an organic solvent such as DMSO in combination with SDS for complete solubilization.

3.4. Study on the HCl Concentration in Formazan Solubilizing Solvents

The concentration of HCl used in formazan solutions varies across protocols. Commonly reported values include 0.01 M, 0.025 M [9]-[14], and 0.04 N HCl [21]. In assays such as MTT, formazan crystals are dissolved in these acidic solutions, and both the stability and solubility of the resulting solution can be affected by the specific HCl concentration employed. The influence of HCl concentration in formazan-solubilizing solvents was examined using 0.01 N HCl to minimize interference from phenol red in the growth medium. Because the acid converted phenol red to yellow, its contribution to OD at the measured wavelength was negligible. After incubation, the culture medium was carefully removed without disturbing the cells and formazan crystals (**Figure 4(A)**) or medium retained in the wells (**Figure 4(B)**). Formazan crystals were dissolved in five different solvents: DMSO, DMSO with 0.01 N HCl, 10% SDS in water with 0.01 N HCl, 5% SDS in DMSO with 0.01 N HCl, and 10% SDS in DMSO with 0.01 N HCl. Compared with formazan dissolved in DMSO, DMSO with 0.005 N HCl, 10% SDS in water with 0.005 N HCl, 5% SDS in DMSO with 0.005 N HCl, and 10% SDS in DMSO with 0.005 N HCl (**Figure 2(A)** and **Figure 2(B)**), the OD values obtained with the higher-acid solvents were consistently lower.

Formazan, the chromogenic product of the MTT assay, displays a characteristic purple color when fully solubilized. However, at highly acidic pH levels, formazan becomes unstable and may degrade or undergo shifts in its absorption spectrum, leading to yellow discoloration or complete loss of color. These spectral changes reduce absorbance at the standard quantification wavelength (570 nm), thereby lowering OD values even when the actual amount of formazan generated by the cells remains constant. Consequently, the concentration of HCl in the solvent sys-

tem is a critical parameter, as excessive acidity disrupts the colorimetric properties of formazan and can result in underestimation of cell viability or metabolic activity. In this study, SDS- or DMSO-based solvents containing 0.005 N HCl produced higher OD values (Figure 2(A) and Figure 2(B)), whereas increasing the HCl concentration to 0.01 N resulted in reduced OD values (Figure 4(A) and Figure 4(B)). These findings demonstrate the importance of optimizing HCl concentration to ensure reliable and reproducible outcomes in formazan-based assays.

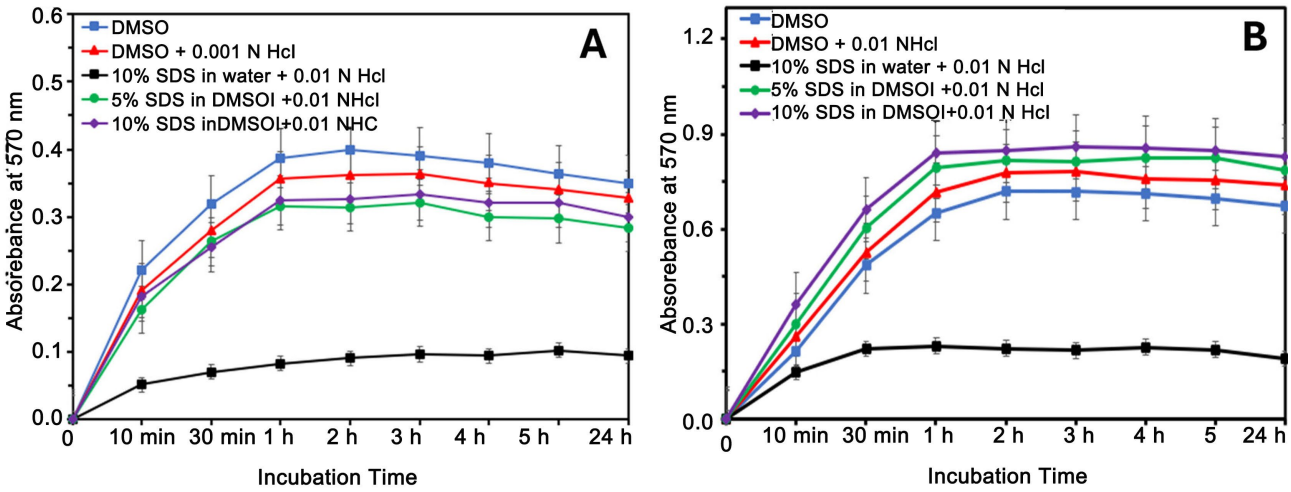

Figure 4. The effect of HCl concentration in formazan-solubilizing solvents was evaluated by monitoring changes in optical density. As described in experimental section after incubated with MTT. (A) complete removal of culture medium without disturbing adherent cells or formazan crystals, and (B) Retention of culture medium in the wells. Subsequently, 200 µL of one of the following solubilization solvents was added to each well: DMSO, DMSO containing 0.01 N HCl, 10% SDS in water with 0.01 N HCl, 5% SDS in DMSO with 0.01 N HCl, or 10% SDS in DMSO with 0.01 N HCl. Plates were incubated at 37˚C for periods ranging from 10 min to 24 h. Then absorbance was measured at 570 nm at each time point. Background correction was performed using wells containing complete medium and the respective solvents. Data represent the mean of duplicate wells, and results are presented as the average of two independent experiments.

4. Discussion

The MTT assay is widely employed due to its simplicity and applicability across diverse cell types. However, it presents certain limitations, including precipitation formation and instability of the water-insoluble formazan product, which can contribute to high background signals and variability in results. Because formazan crystals form within the culture medium, the use of a suitable solvent is essential to ensure complete dissolution and accurate absorbance measurements [10]. Formazan has been dissolved using various solvents, including DMSO, isopropanol, SDS, and DMF. Among these, DMSO is the most effective solvent, but only when used at absolute concentrations. To maximize its dissolving capacity, the culture medium must be removed prior to adding DMSO [10]. However, this approach has limitations: formazan crystals may be lost because MTT reduction occurs not only in mitochondria and cytoplasm but also at the cell surface [3]. Furthermore, if DMSO is added directly to the culture medium without removal, precipitates can form [1]-[10]. Based on its chemical properties, DMSO is a polar

organosulfur compound that is miscible with water and numerous organic solvents, enabling dissolution of both polar and nonpolar chemical substances. Formazan formation is influenced by both cell density and the amount of MTT applied. In theory, the intensity of the formazan color directly reflects the number of viable cells.

In this preliminary study, the effects of varying BALB/3T3 cell numbers were assessed using two experimental approaches. In the first, the culture medium was completely aspirated without disturbing the cells or formazan crystals (**Figure 1(A)**), whereas in the second, the medium was retained (**Figure 1(B)**). After incubation with MTT, the resulting formazan was solubilized using different solvents, including DMSO, DMSO with 0.005 N HCl, 5% SDS with 0.005 N HCl in DMSO, 10% SDS with 0.005 N HCl in DMSO, and 10% SDS with 0.005 N HCl in water. A linear correlation between OD and cell number was observed. In both approaches, increasing cell seeding density produced higher OD values at the tested MTT concentration and incubation time, consistent with previous findings that greater cell numbers yield more formazan [17]. When solubilizing solvents were added directly to the culture medium, OD values were generally higher (**Figure 1(B)**), and no saturation was observed with increasing cell density. This outcome is expected, as the maximum seeding density of 10,000 cells corresponded to approximately 70% - 80% confluence at the time of measurement, based on light microscopy observations. Collectively, MTT reduction, formazan formation, and absorbance are influenced by multiple factors rather than a single variable, which is reflected in the measured OD. These effects, however, may vary depending on cell type, experimental conditions, and treatment parameters.

Furthermore, the accuracy of the assay may be affected by the non-enzymatic reduction of MTT within the culture medium. In the absence of viable cellular metabolism, various constituents of the culture medium including FBS, amino acids, reducing sugars, vitamins, and other redox-active molecules are capable of chemically reducing MTT, resulting in the non-enzymatic formation of formazan crystals. However, this abiotic reduction causes misleading OD readings and affects the accuracy of the results. In this study, the non-enzymatic reduction of MTT was assessed in five different solvents. None of the tested solvents such as DMSO, DMSO with 0.005 N HCl, 10% SDS in water with 0.005 N HCl, 5% SDS with 0.005 N HCl in DMSO, or 10% SDS with 0.005 N HCl in DMSO were able to convert MTT into formazan. The OD values from the solvent mixtures were almost the same as the blank in all of them, indicating that MTT was not reduced. These results suggest that solvent-based solubilizing mixtures have no effect on MTT's non-enzymatic reduction. In a similar manner, addition of the five different solvents directly to the culture medium enhanced MTT reduction with 10% SDS in DMSO containing 0.005 N HCl and 5% SDS in DMSO containing 0.005 N HCl, likely due to interactions between MTT and medium components. Although DMSO alone and DMSO with 0.005 N HCl yielded measurable OD values, these were lower than those observed with SDS-containing solvents. However, combin-

ing 10% SDS with 0.005 N HCl in water did not promote non-enzymatic reduction of MTT in culture medium.

In addition to direct optical interference, culture media components may contribute to assay variability through unexpected chemical interactions with MTT, such as reducing MTT or catalyzing its reduction. These reactions can generate products like formazan, which alter the measured OD. Consequently, any change in OD observed after adding MTT to the culture medium may indicate abiotic reduction of MTT or other chemical reactions involving media constituents. To evaluate this possibility, we compared OD values in cell-free conditions both with and without culture medium. The results of this study show that although MTT reduction is commonly associated with mitochondrial activity, it may also occur in lysosomes, endosomes, and through other non-mitochondrial components [18].

Since DMSO and SDS lysis solutions are known to efficiently dissolve formazan [9] [10], this study investigated whether combining DMSO, SDS, and HCl would improve the dissolution of water-insoluble formazan. The MTT assay was performed using BALB/3T3 cells to investigate the efficiency of different solubilization solvents, with and without culture medium. Solvents examined included DMSO, DMSO supplemented with 0.005 N HCl, 5% SDS with 0.005 N HCl in DMSO, 10% SDS with 0.005 N HCl in DMSO, and 10% SDS with 0.005 N HCl in water. After removal of the medium, formazan crystals were dissolved using different solvents. Rapid dissolution occurred in DMSO, yielding higher OD values than DMSO supplemented with HCl. In contrast, 5% and 10% SDS in DMSO containing HCl dissolved formazan less efficiently, and OD values were particularly low with 10% SDS and HCl in water.

Similarly, formazan was dissolved by directly adding solubilizing solvents to medium. Among the tested solvents, DMSO combined with 10% SDS and 0.005 N HCl produced the highest OD, indicating that this mixture was most effective for rapidly solubilizing both formazan crystals and cells in medium (**Figure 2(B)**). In contrast, 5% SDS with 0.005 N HCl in DMSO, DMSO with 0.005 N HCl, and DMSO alone dissolved the crystals more slowly and yielded lower OD values, reflecting incomplete solubilization. Notably, 10% SDS with 0.005 N HCl in water was particularly ineffective, giving the lowest OD among all tested conditions. Overall, the results demonstrated that DMSO was the most effective solvent for dissolving formazan once the medium was completely removed, supporting its use as a universal solvent system across different cell types. Therefore, when the medium was retained, the optimal solvent for rapid formazan crystal solubilization was 10% SDS in DMSO supplemented with 0.005 N HCl. This mixture is especially useful because it dissolves formazan crystals directly in the growth medium and avoids the need for overnight incubation [20].

To minimize interference from phenol red in the culture medium during absorbance measurements at 570 nm, HCl was incorporated into the formazan-solubilizing solvents. This modification enabled the MTT assay to be applied effec-

tively under conditions with or without medium. It has been reported that the color of formazan solutions varies with pH [13], and that SDS solutions supplemented with HCl can attenuate the MTT reaction. Specifically, a decrease in pH induces a shift of the formazan solution toward yellow, resulting in reduced OD measurements [13]. In this investigation, two concentrations of HCl (0.005 N and 0.01 N) were evaluated. The findings are consistent with previously published observations, demonstrating that solvents containing 0.01 N HCl produced lower OD values than those containing 0.005 N HCl. The solvents tested included DMSO with 0.01 N HCl, 5% SDS with 0.01 N HCl in DMSO, 10% SDS with 0.01 N HCl in DMSO, and 10% SDS with 0.01 N HCl in water. These results suggest that the yellow shift induced by higher HCl concentrations interferes with OD measurements at 570 nm. In contrast, when solvents contained 0.005 N HCl, formazan crystals were effectively dissolved and yielded higher OD in both the presence and absence of medium.

The reproducibility of the current study depends on by several factors, including the type of assay used, the composition of the culture medium, experimental conditions, cell density, incubation time, MTT concentration, the proportion of metabolically active cells, and the presence of non-enzymatic components in the medium. Careful analysis of these parameters is required to ensure consistency and dependability of results.

5. Conclusions

In the present study, among the solvents tested, DMSO proved to be the most suitable solvent for dissolving formazan in the MTT assay when the culture medium was completely removed without disturbing the cells and formazan crystals. Under these conditions, DMSO achieved rapid solubilization of formazan. In contrast, 10% SDS with 0.005 N HCl in DMSO was identified as a highly effective solvent for direct addition into the medium. Both approaches enabled complete solubilization of formazan within one hour, regardless of the presence or absence of medium, eliminating the need for overnight incubation.

Similarly, 5% SDS with 0.005 N HCl in DMSO and 0.005 N HCl in DMSO alone demonstrated comparable solubilization efficiency. However, increasing the HCl concentration to 0.01 N resulted in reduced OD values, indicating that the optimal HCl concentration in SDS-DMSO mixtures requires further validation. Notably, in both the presence and absence of medium, 10% SDS with 0.005 N HCl in water produced very low OD readings, suggesting poor solubility of formazan under these conditions. This observation warrants additional investigation to elucidate the underlying mechanism of reduced solubility.

Authors Contributions

A. S. Prakasha Gowda conceptualized, designed, executed the experiments, carried out data analysis and wrote the manuscript. A.D. Schaefer oversaw laboratory operations and provided overall management for the study and manuscript re-

view. All authors granted their approval for publication.

Conflicts of Interest

The authors confirm that this article content has no conflict of interest. This article does not contain any studies with human or animal subjects. The company had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results; in both Disclosure form and manuscript file.

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