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Current methodology of MTT assay in bacteria – A review

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ABSTRACT

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium assay is a popular tool in estimating the metabolic activity of living cells. The test is based on enzymatic reduction of the lightly colored tetrazolium salt to its formazan of intense purple-blue color, which can be quantified spectrophotometrically. Under properly optimized conditions the obtained absorbance value is directly proportional to the number of living cells. Originally, the MTT assay was devised for use in eukaryotic cells lines and later applied for bacteria and fungi. As the mechanism of MTT reduction was studied in detail mostly considering eukaryotic cells, the lack of information resulted in generating a vast variety of MTT based protocols for bacterial enzymatic activity evaluation. In the presented article the main aspects of the MTT assay applicability in bacterial research were summarized, with special emphasis on sources of inaccuracies and misinterpretation of the test results.

1. Introduction

Microbiology practice utilizes a variety of indicators of bacterial physiological state, and therefore numerous definitions of the viability of cultured cells have been established. Most common techniques are based on reproductivity estimates, which assess viability of the population as the percentage of cells able to divide. The influence of factors that reduce microbial vitality but not necessarily affect multiplication can only be ascertained by detection of alterations in cells structure and function. These encompass observations of general morphology and specific membrane characteristics (membrane potential, integrity e.g. ability to exclude dyes, and cell motility) and biochemical profiling of specific molecules, including enzymes (Li and Song, 2007). In the context of this article, the prerequisite of culture viability is measurable enzymatic activity. Among numerous methods that associate the level of enzymatic activity with the condition of microbial population, assays based on biotransformations of tetrazolium salts have gained much popularity.

Over the years, tetrazolium salts have been incorporated in a variety of experimental protocols such as oxidoreductase activity measurements, subcellular localization of oxidoreductases, detection of superoxide radicals, *Mycoplasma* screening and – above all – microbial viability and growth estimation (Bernas and Dobrucki, 2000). Novel methods that utilize the reaction of tetrazolium salts reduction were invented and optimized in the late 20th and early 21st century. This research was accelerated due to growing knowledge of tetrazolium salts reduction mechanisms and synthesis of new compounds of this class.

One of the most common examples of tetrazolium salts used in bioassays is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). MTT-based procedures are widely applied to mammalian cell lines, bacteria and fungi. Notably, the reported protocols differ drastically in nearly all aspects of assay conditions (Young et al., 2005). Such incoherence is substantial especially when a novel set of experiments needs to be optimized. In the presented article, a summary of the most important aspects of the MTT assay of bacterial viability is reported.

2. General background

The term *tetrazolium* derives from the salts' chemical structure, as they are heterocyclic compounds with four atoms (*tetra*) of nitrogen (*aza*) in the tetrazole ring, defining this group of organic salts. The additional 1*H* or 2*H* in the nomenclature of tetrazolium salt describes the formal location of hydrogen atom (Fig. 1).

In general, tetrazolium salts in solutions are colorless or lightly colored compounds. The tetrazole ring (Fig. 2) can udergo enzymatic

Abbreviations: DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; MDR, multidrug-resistant (bacterial strain); MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium inner salt; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; SDS, sodium dodecyl sulfate; TTC, 2,3,5-triphenyl-2*H*-tetrazolium chloride; TV, tetrazolium violet, 2,5-diphenyl-3-(1-naphthyl)tetrazolium chloride; WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt; WST-5, 2,2′-dibenzothiazolyl-5,5′-bis[4-di(2-sulfoethyl)carbamoylphenyl]-3,3′-(3,3′-dimethoxy-4,4′-biphenylene)ditetrazolium, disodium salt; WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt; XTT, 2,3-bis(2-methyloxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide inner salt

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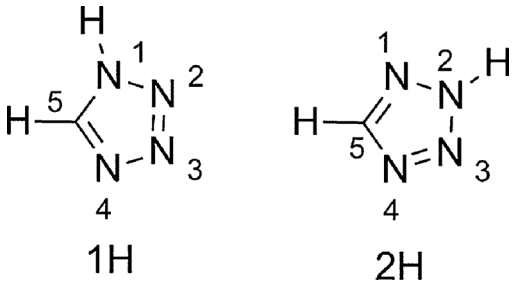


Fig. 1. Structure of the tetrazole rings characteristic for tetrazolium salts.

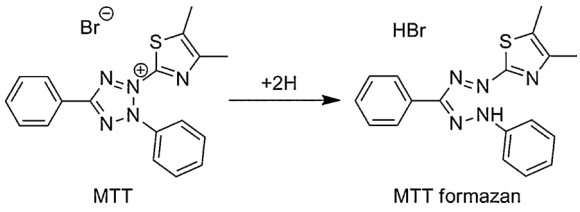


Fig. 2. Reduction of MTT bromide to its formazan.

reduction resulting in its cleavage. The product of the reaction is a corresponding formazan of intense color (Berridge et al., 2005).

Tetrazolium salts were first described in 1894. 2,3,5-triphenyl-2*H*-tetrazolium chloride (TTC) (Table 1) was the initial, prototypic compound that formed a water-insoluble formazan upon reduction. The subsequently developed tetrazolium salts, including MTT, were based on its structure (Altman, 1976; Tsukatani et al., 2008).

Chemical modifications of basic tetrazolium salts resulted in diversity of chemical properties and possible applicability of the newly generated compounds (Fedotcheva et al., 2017). Nowadays, generally used tetrazolium salts are MTT, inner salt MTS, NBT, XTT and a variety of WSTs (Water Soluble Tetrazolium salts) – WST-1, WST-5, WST-8 (Table 1). Tetrazolium salts with water-soluble formazans were developed in order to enable more reliable, continuous measurements of the progress of their reduction. They are, however, not obviously superior to salts that yield water-insoluble products - e.g. XTT and MTS require incorporation of an additional intermediate electron acceptor to advance production of their formazans (Goodwin et al. 1995). Also, cell lines and microbial strains may differ in their capability to reduce tetrazolium salts (Tachon et al., 2009). In microbial viability estimations MTT seems to be a more reliable choice, as it produces less background absorbance comparing to WST-5, WST-8 and XTT (Wang et al., 2010).

The first report on the possible application of MTT in estimating the viability of living cells was written by Mosmann (1983), who described the possible evaluation of mammalian cell lines response to cytotoxic compounds. This methodology was optimized for a mouse lymphoma cell line and was afterwards applied by researchers in countless experiments involving eukaryotic as well as prokaryotic cells. The application of an optimized MTT protocol allowed to measure culture growth and – in general - enzymatic activity. For many years, the MTT assay was one of the most frequently conducted tests of cells susceptibility (Liu et al., 1997).

Prior to Mosmann work (Slater et al., 1963) it had been long accepted, despite the lack of biochemical understanding of tetrazole enzymatic reduction, that catalysis of the reaction might occur only in living cells, as MTT salt could not be reduced by dead cells or erythrocytes. Early works of Slater et al. (1963), Altman (1976) and Burdon et al. (1993) indicated that the main enzymes capable of MTT reduction were mitochondrial dehydrogenases, their nonmitochondrial isoenzymes and even flavin oxidases. Recently, major role of eukaryotic NADH-dependent oxidoreductases in the reduction process was evidenced (Li and Song, 2007; Stockert et al., 2012). It was supposed that in eukaryotic cells MTT was reduced only in active mitochondria (Mosmann, 1983). Recently, this approach was questioned as mitochondria are generally not characterized with reducing properties and MTT salt has higher affinity to other organelle, especially the endoplasmic reticulum (Stockert et al., 2012).

The specification of the cellular site engaged in the MTT reduction was a crucial step towards the unraveling of the possible mechanism. In mixtures with eukaryotic cells, MTT is transported through the membrane by endocytosis (Lü et al., 2012). The net positive charge of MTT facilitates its cellular uptake (Berridge et al., 2005). Once in the cytoplasm, the MTT bromide is transported and accumulated in the preferable anionic reduction site - endoplasmic reticulum (Stockert et al., 2012). Here, accumulation of MTT salt has no short-term cytotoxic effect (Lü et al., 2012) and the reduction rate is dependent on glycolytic NADH production. Accumulation of the produced formazan is unfavorable, as microcrystals precipitation in the cytoplasm may increase cell permeability, induce the apoptosis or even mechanically disrupt the cell envelope (Lü et al., 2012). Detailed mechanism of formazan excretion remains uncertain (Stockert et al., 2012). Crystals depletion also may lead to mechanical cell injuries (Lü et al., 2012). Exocytosed formazan can form extracellular deposits and crystals may continue to build up due to extracellular MTT reduction (Berridge et al., 2005). Formation of formazan deposits on the outer cell surface may as well be facilitated by membrane-bound NADPH oxidases, which are known to produce metabolites that reduce other tetrazolium salts (Honoré et al., 2003).

Scientific literature provides the information mostly on the mechanism of MTT reduction in eukaryotic cell, briefly summarized above. As far as prokaryotic reduction of MTT is concerned the available research is scarce, however, interesting reports on other water-soluble tetrazolium salts can be found. In the work on tetrazole reduction in lactic acid bacteria it was proven using mutagenesis approach in *Lactococcus lactis*, that in growing cells menaquinones were necessary for electron transfer from intracellular NADH to tetrazolium violet (TV) substrate, whereas in resting cells TV was reduced directly by the membrane NADH dehydrogenases (Tachon et al., 2009). This determined the final location of formazan deposits either outside the cell or in the inner part of the plasma membrane, respectively.

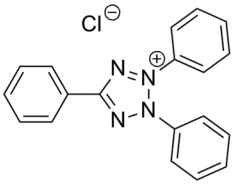
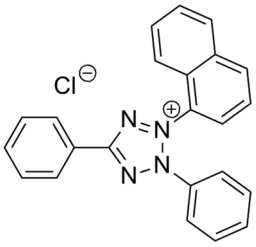
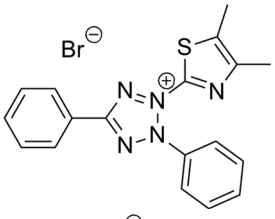
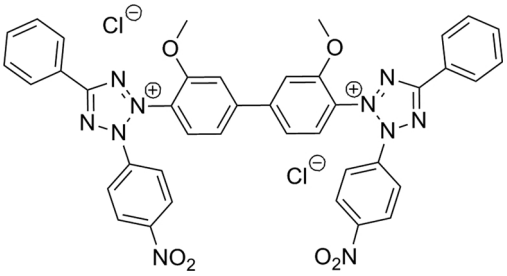
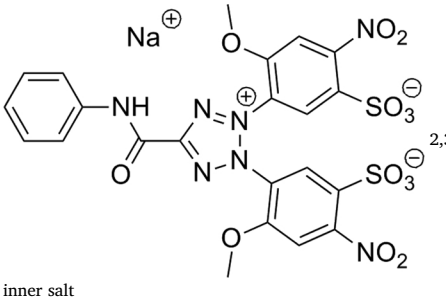
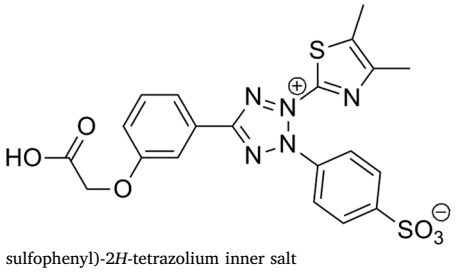
The reduction efficiency depends on the kind of tetrazolium salt, even in a single bacterial strain – e.g. in the same work by Tachon et al. (2009) *Lactococcus lactis* strain inactivated for NADH dehydrogenase was unable to reduce tetrazolium violet (TV) but it was still active regarding MTT reduction. It is probably the effect of engaging separate electron transport chains (often simultaneously) for biotransformations of structurally different tetrazolium salts.

Thus, biochemical characteristics of tetrazole reduction in procaryota is influenced by structure- diffusibility characteristics of the tetrazolium salt, culture conditions, microbial species and the phase of strain growth. All these factors need to be taken into consideration as they may contribute to misestimating of assay results.

3. MTT in microbiological assays

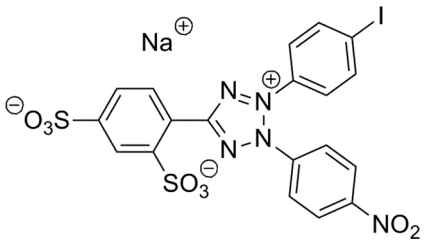
Although studies of enzymatic tetrazolium salts reduction were first applied in eukaryotic cell research, this type of assays are now widely used for viability estimations of microbial cells. The mechanism of MTT reduction by bacteria is, however, still poorly understood (Tachon et al., 2009) and that creates a danger of its misinterpretation. MTT assay results indeed often do not agree with data obtained by other cell-growth or viability estimation methods. Nevertheless, numerous reports on bacterial strains tested for viability and respiratory activity with MTT reduction-based techniques were released throughout the years. Predictably, basic microplate susceptibility estimations are the most common practice (Table 2). In addition, MTT is applied in other protocols e.g. for multidrug-resistant bacteria determination (Foongladda et al., 2002; Montoro et al., 2005; Mshana et al., 1998), biofilm formation evaluation (Brambilla et al., 2014) or even indirect

Table 1
Tetrazolium salts most commonly used in bioassays.

Tetrazolium salt	Full name and structure	Formazan characteristics
TTC	 2,3,5-Triphenyl-2*H*-tetrazolium chloride	• red • water-insoluble
TV	 2,5-Diphenyl-3-(1-naphthyl)tetrazolium chloride	• violet • water-insoluble
MTT	 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide	• purple • water-insoluble
NBT	 2,2′-bis(4-Nitrophenyl)-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-diphenylene)ditetrazolium chloride	• deep blue • water-insoluble
XTT	 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl) -2*H*-tetrazolium-5-carboxanilide inner salt	• orange • water-soluble
MTS	 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium inner salt	• blue • water-soluble

(*continued on next page*)

Table 1 (*continued*)

Tetrazolium salt	Full name and structure	Formazan characteristics
WST-1	 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate, monosodium salt	• dark red • water-soluble

quantification of antibacterial compounds (Wang et al., 2007).

Application of MTT reduction test allows the increase of sensitivity of other broth-dilution methods of antibacterial activity evaluation, e.g. the MTT assay can serve as an alternative/additional method of Minimal Inhibitory Concentration evaluation (Shi et al., 2007). In brief, instead of standard turbidity measurements after incubation, MTT solution may be added directly into the undiluted culture samples, followed by organic solvent addition and absorbance measurements. As the desired result of the MIC assay is usually the determination of the lowest concentration of compound that completely restrains the bacterial growth, spectroscopic quantification of solubilized formazan is believed a more sensitive method than optical density measurements, allowing detecting of lower cell density. Moreover, with this method dead bacterial cells do not interfere with result.

Besides the application in liquid bacterial cultures, MTT salt was described as a valuable tool in microorganisms' growth evaluation on solidified media. After spraying the water-based MTT solution over the plate of agar-diffusion susceptibility assay, inhibition zones can be measured with high accuracy due to intensity of formazan saturation of the grown culture (Corrado and Rodriques, 2004; Rahalison et al., 1991).

The MTT assay was also proposed as a possible method of determination of multidrug-resistant (MDR) *Mycobacterium tuberculosis*. The assay presents a variety of advantages above standard growth

Table 2
Examples of bacterial strains tested using the MTT assay.

Bacterial strain	Comment
Aeromonas hydrophilia	Test optimization for flatbed scanner (Gabrielson et al., 2002)
Alcaligenes faecalis	Test optimization for flatbed scanner (Gabrielson et al., 2002)
Bacillus licheniformis	Bacteria susceptibility testing (Dalai et al., 2012)
	Optimization for continuous assay (Stowe et al., 1995)
Bacillus subtilis	TLC agar-overlay method (Rahalison et al., 1991)
	MIC evaluation (Shi et al., 2007)
	Tetrazolium salt selection (Thom et al., 1993)
Brucella sp.	Test optimization for 71 *Brucella* strains (Broughton and Jahans, 1997)
Brucella abortus	Neutrophil bactericidal activity (Stevens and Olsen, 1993)
Comamonas denitrificans	Test optimization for flatbed scanner (Gabrielson et al., 2002)
Enterococcus faecalis	Test optimization for flatbed scanner (Gabrielson et al., 2002)
	Tetrazolium salt selection, *E. faecalis* does not produce detectable formazan after 20 min of incubation (Thom et al., 1993)
Enterococcus sulfureus	Test optimization for flatbed scanner (Gabrielson et al., 2002)
Escherichia coli	Bacteria used for test optimization (Gabrielson et al., 2002; Stowe et al., 1995; Wang et al., 2012)
	MIC evaluation (Shi et al., 2007)
	Neutrophil bactericidal activity (Stevens and Olsen, 1993)
	Tetrazolium salt selection (Thom et al., 1993)
Listeria monocytogenes	Neutrophil bactericidal activity (Stevens and Olsen, 1993)
Micrococcus luteus	Nisin concentration quantification by estimation of number of viable bacteria (Wang et al., 2007)
Mycobacterium smegmatis	Tetrazolium salt selection (Thom et al., 1993)
Mycobacterium tuberculosis	Faster alternative to growth methods (Foongladda et al., 2002; Montoro et al., 2005)
	Detection of rifampin-resistant strains (Mshana et al., 1998)
Proteus mirabilis	Bacteria susceptibility testing (Grela et al., 2016)
Pseudomonas aeruginosa	Tetrazolium salt selection (Thom et al., 1993)
	Biofilm quantification (Wu et al., 2010; Deb and Vimala, 2017)
Pseudomonas cepacia	Optimization for continuous assay (Stowe et al., 1995)
Pseudomonas fluorescens	MIC evaluation (Shi et al., 2007)
Salmonella enteritidis	Tetrazolium salt selection (Thom et al., 1993)
Serratia marcescens	Biofilm quantification (Wu et al., 2010)
Staphylococcus aureus	MIC evaluation (Shi et al., 2007)
	Neutrophil bactericidal activity (Stevens et al., 1991; Stevens and Olsen, 1993)
	Macrophages bactericidal activity (Thanawongnuwech et al., 1997)
	Tetrazolium salt selection (Thom et al., 1993)
	Biofilm quantification (Walecka et al., 2007; Shillaci et al., 2008; Wu et al., 2010)
Staphylococcus epidermis	Biofilm susceptibility assay (Nuryastuti et al., 2009)
	Biofilm quantification (Walecka et al., 2007; Shillaci et al., 2008)
Streptococcus mutans	Biofilm formation evaluation (Brambilla et al., 2014)
Vibrio alginolyticus	Test optimization for flatbed scanner (Gabrielson et al., 2002)
Vibrio fluvalis	Test optimization for flatbed scanner (Gabrielson et al., 2002)

Table 3
Bacteria density applied in MTT assay mixtures.

Bacteria density	Comment
10^5 CFU mL^{-1}	*M. luteus*, minimal bacteria density that allows formazan quantification (Wang et al., 2007)
10^7 CFU mL^{-1}	*E. coli* (Stowe et al., 1995)
10^7–10^8 CFU mL^{-1}	*P. mirabilis* (Grela et al., 2016)
OD_{600} = 0.1–0.18 1.4*10^9 CFU mL^{-1})	*E. coli*; final absorbance readings proportional to amount of used bacterial cells (Wang et al., 2010)
4*10^8–2*10^{10} CFU mL^{-1}	*M. tuberculosis*; 10^9 CFU per well on the microplate (Mshana et al., 1998)
Fully grown 24 h culture	MIC evaluation (Shi et al., 2007)
24 h biofilm	*S. aureus*, *S. marcescens* and *P. aeruginosa* biofilm on contact lens storage cases (Walecka et al., 2007; Wu et al., 2010)
	S. epidermis and *S. aureus* biofilm quantification (Shillaci et al., 2008)
24 h and 72 h biofilm	*S. mutans* biofilm formation evaluation (Brambilla et al., 2014)

methods or staining with radioactive compounds. Usually, it is faster, cheaper and less toxic, as well as sufficiently accurate and allowing test condition optimization. In brief, *M. tuberculosis* isolates cultured in the presence of antimicrobials can be tested for viability with the MTT assay within 5–7 days of growth, in comparison to 3–4 weeks that usually takes to perform the standard colony counts procedure with this slowly growing microorganism (Foongladda et al., 2002; Montoro et al., 2005; Mshana et al., 1998). The microbial population must contain at least 1% of drug-resistant *M. tuberculosis* to successfully apply detection using MTT method (Abate et al., 1998). So far, the MTT assay offers no possibility of replacing standard colony counts or MIC/MBC evaluation but it definitely represents a valuable supporting diagnostic method in tuberculosis. Conflicting information can be found concerning adequacy of MTT reduction method for isoniazid-resistant *M. tuberculosis* research. According to some studies, high formazan production rates, inconsistent with live-cell density were observed in this type of mycobacteria (Abate et al., 1998; Mshana et al., 1998). Contrarily, 100% specificity of the MTT assay for isoniazid susceptibility testing of *M. tuberculosis* was recently reported (Hundie et al., 2016). Also, it was noted that drug-resistant strains expressed lower overall enzymatic activity (Foongladda et al., 2002).

In recent years, the MTT assay has been adapted for cyanobacteria viability evaluation. For example, the method was precisely optimized for *Microcystis aeruginosa* (Li and Song, 2007). Poor light stability of formazan, however, forms an important disadvantage. In the work of Li and Song (2007), half-life of only 8 min was described for reduced MTT. Such characteristics may generate major inaccuracies, as cyanobacteria require intense light exposition for optimal activity. It was suggested that MTT formazan may be reoxidized to the tetrazolium salt, as similar photooxidation was observed for 2,3,5-triphenyl-2*H*-tetrazolium bromide (Graham et al., 1977). Alternatively, azo groups of formazan can be further reduced to amines, resulting in the cleavage of the molecule and its discoloration (Stoward, 1967). Lastly, there is a possibility of stereoisomerization of formazan molecule – with the shift in absorbance spectra (Al-Araji et al., 2015). In contrary, precipitating formazan crystals – especially forming larger deposits - are thought to be considerably light resistant (Stoward, 1967).

The MTT assay was also proposed as an alternative method for microbial biofilm quantification. Comparing to a standard crystal violet staining and extraction, MTT allows not only to localize and quantify this structure, but mainly to determine the presence of live bacteria. As the result, the MTT assay may be applied in biofilm susceptibility testing, even with MIC establishment as the final effect (Nuryastuti et al., 2009).

Similarly to the standard MTT assay in planktonic cultures, the biofilm quantification protocol requires initial bacterial growth, subsequent incubation with MTT and final solubilization of formed formazan. Bacterial biofilm may be washed off before incubation with MTT (Nuryastuti et al., 2009) or alternatively - after this step, when formazan is already formed in the mixture (Deb and Vimala, 2017). It must be remembered that the second approach may lead to removal of part of formazan formed by biofilm-bound cells. Most experiments investigating artificial biofilm formation require a specific surface material. The microplate MTT assay may be easily adjusted to biofilm formation test if investigated material can be cut into small enough pieces. As the example, indirect but reliable measurements were prepared for bacterial biofilm on the enamel specimens. Results remained with good correspondence to plate counts (Brambilla et al., 2014). Also, as biofilm forming in catheters represents an important medical problem, its prevention was also investigated with MTT (Deb and Vimala, 2017).

4. Methodological aspects

The most popular media for MTT reduction assay are phosphate buffers of neutral pH and different growth media (Montoro et al., 2005; Tachon et al., 2009). Its appropriate choice seems to be crucial in most experiments. If rich growth medium is used it allows longer incubations, but the change of cell density/MTT concentration ratio during assay needs to be taken into consideration (Tables 3–5) t was reported that certain types of buffered reaction media can negatively influence the accuracy of the MTT assay. Sodium acetate, KH_2PO_4-NaOH, glycine-NaOH buffer interfere with final measurements of DMSO-dissolved formazan. The strongest interferences was observed in case of Tris-HCl, in which A_{550} measurement was decreased even at pH = 9.0 (Wang et al., 2012).

In some protocols it is advised to remove growth medium prior to

Table 4
Working concentrations of MTT salt in chosen protocols.

Final MTT concentration	Comment
1 mg mL^{-1}	*M. tuberculosis* viability evaluation (Mshana et al., 1998)
	Biofilm quantification (Shillaci et al., 2008)
0.5 mg mL^{-1}	*P. mirabilis* viability estimation (Grela et al., 2016)
	Biofilm formation evaluation (Brambilla et al., 2014)
	Biofilm susceptibility assay (Nuryastuti et al., 2009)
0.45 mg mL^{-1}	Original protocol for eukaryotic cells (Mosmann, 1983)
	(Wang et al., 2010)
	Biofilm quantification (Wu et al., 2010)
0.4 mg mL^{-1}	MIC evaluation (Shi et al., 2007)
	Bovine neutrophil bactericidal activity (Stevens et al., 1991; Stevens and Olsen, 1993)
0.25 mg mL^{-1}	OD is proportional to MTT concentration 0.03–0.5 mg mL^{-1} (Broughton and Jahans, 1997)
	Production of formazan was proportional to bacteria density and time of incubation (Wang et al., 2007)
0.14 mg mL^{-1}	Protocol for cyanobacteria viability estimation (Li and Song, 2007)
0.09 mg mL^{-1} (217 μM)	Continuous MTT assay (Stowe et al., 1995)
0.05 mg mL^{-1}	MDR *M. tuberculosis* detection (Foongladda et al., 2002; Montoro et al., 2005)
0.01% (w/v)	Tetrazolium salts comparison (Gabrielson et al., 2002)
0.5 μg mL^{-1}	MDR *M. tuberculosis* detection (Abate et al., 1998)

Table 5
Duration of MTT reduction and formazan dissolution steps in chosen protocols.

Incubation time (with MTT)	Incubation time (with solvent)	Comment
overnight	3 h	Detection of MDR *M. tuberculosis* (Montoro et al., 2005)
4–5 h	12 h	MIC evaluation (Shi et al., 2007)
4 h	overnight	Detection of MDR *M. tuberculosis* (Mshana et al., 1998)
4 h	1 h	Original protocol for eukaryotic cells (Mosmann, 1983)
		Detection of MDR *M. tuberculosis* (Abate et al., 1998; Foongladda et al., 2002)
4 h	–	TLC agar-overlay assay (Rahalison et al., 1991)
3 h	1 h	Biofilm formation evaluation (Brambilla et al., 2014)
2 h	15 min	Biofilm quantification (Walecka et al., 2008)
2 h	10 min	Nisin concentration quantification by estimation of number of viable *M. luteus* cells (Wang et al., 2007)
2 h	2 min	Cyanobacteria viability estimation (Li and Song, 2007)
2 h	not mentioned	Biofilm quantification (Wu et al., 2010)
2 h	–	TLC agar-overlay assay (Corrado and Rodriques, 2004)
1 h	2-4 h	*Brucella* sp. (Broughton and Jahans, 1997)
1 h	1 h	*P. mirabilis* (Grela et al., 2016)
30–40 min	–	Continuous assay with Triton X-100 (Stowe et al., 1995)
30 min	overnight	Neutrophil bactericidal activity (Stevens and Olsen, 1993)
20 min	10 min	Method standardized for *E. coli* (Wang et al., 2010, 2012)
10 min	10 min	Method applied for *S. aureus* (Stevens et al., 1991)

the addition of organic solvent and absorbance reading as certain medium components may precipitate or disturb formazan dissolution (Denizot and Lang, 1986; Li and Song, 2007; Shi et al., 2007; Wang et al., 2010, Young et al., 2005). On the other hand, well aspiration itself is a step with a possibility of creating errors, and some researchers suggest to avoid this step if possible (Niks and Otto, 1990).

MTT concentration should be individually calibrated taking into account cell density used in assay to ensure saturation (Table 4). To some extent, application of higher concentration of MTT salt results in higher final results (Wang et al., 2010). On the other hand, however, possible negative effect of tetrazolium salt upon the microorganism viability should be considered and checked out. Toxic effect of high MTT concentration disturbs the assay as it must result in lower readings (Stowe et al., 1995).

It was observed that MTT reduction in whole bacterial cells is not characterized with standard enzymatic kinetics (stable enzyme amount and changing substrate concentration). During the assay bacteria can become entrapped in precipitating formazan and the reaction rate is limited (Wang et al., 2010). Also, prolonged incubation can cause discoloration of the probe, as bacteria may transform formazan to its colorless derivative (Stowe et al., 1995).

During the MTT assay, yellow, water-soluble salt is reduced to purple, water-insoluble formazan. The assay usually requires a final formazan extraction step – with an organic solvent of choice - and is regarded an endpoint assay (Berridge et al., 2005; Stockert et al., 2012).

Acid isopropanol was first proposed by Mosmann and still is considered a good, but not ideal solvent for the MTT assay. Many other organic solvents has been tested (Table 6) but a lot of them cannot provide satisfactory stability of final measurements or even causes major inaccuracies. For example, ethanol that easily dissolves formazan crystals also causes significant protein precipitation (Mosmann, 1983). The search for an ideal solvent of MTT formazan continued throughout the years, so far one of the best of proposed mixtures is alkaline DMSO supplemented with ammonia (Wang et al., 2012). Its most important advantages are elimination of pH-derived inaccuracies and improvement of solution time-stability. Such characteristics – to a lesser extent - can be obtained with pure DMSO that provides a lower light sensitivity of formazan solution than isopropanol. Also, isopropanol tends to leave a little amount of undissolved formazan residue, while DMSO allows a full dissolution even within 2 min. DMSO-formazan solution kept in darkness provides stable absorbance readings for up to an hour (Li and Song, 2007). In some protocols alternative SDS-based mixtures for formazan dissolution are proposed as they decrease the possible protein precipitation (Young et al., 2005).

Aside from MTT concentration and bacteria enzymatic activity, the extent of precipitation of formazan crystals during the reduction step highly depends on the type of medium. It was observed by Wang et al. (2010) that in buffered LB medium with 1% (w/v) glycerol no needle-like crystals are formed, while Grela et al. (2015) pictured them in assays conducted in standard Mueller-Hinton Broth. In both cases, formazan tended to form complexes with bacterial cells. Earlier work of Thom et al. (1993) indicated, that formazan crystals continued to enlarge even after probes fixation.

Attempts were made to create modified protocols to allow continuous MTT assay, in which formazan solubilization was achieved without the use of organic solvents (Grela et al., 2015; Stowe et al., 1995). With the use of low concentration of detergent in cell-MTT mixture, a homogenous microemulsion is formed during the reduction reaction and no additional dissolution steps are necessary. As detergents possess antibacterial properties their concentration must be chosen carefully, high enough to provide microemulsion formation without affecting the condition of incubated microorganisms. The main advantage of this kind of protocol is the possibility of continuous measurement of one sample but a set of control assays need to be properly conducted. If this is reassured, absorbance measurements are proportional to bacteria density. Compared to typical end-point reaction including dissolution with organic solvent, results obtained in continuous version of the MTT assay may likely be higher - the reaction is not limited with membrane formazan transport (Stowe et al., 1995). The accuracy of assay is improved due to avoiding organic solvents which may interact with reaction medium and cell suspension causing cloudiness and precipitation.

MTT salt water solution is characterized with the peak of absorbance at 375 nm (Stockert et al., 2012). Upon reduction spectrum of absorbance can drastically vary under the presence of solvents and metal ions (Carmichael et al., 1987; Stowe et al., 1995), with shoulders shifting from 512 to 587 nm and a maximum of 562 nm in sunflower oil (Stockert et al., 2012), 570 nm in acid isopropanol (Grela et al., 2015) (Fig. 3), 570 nm in mineral oil and 540 nm in DMSO (Carmichael et al., 1987) (Table 7). It is suggested that the measurement wavelength should be defined for each new set of assays taking into account cell line/microbial strain or solvent used.

Except spectrophotometric measurements, some protocols describe the evaluation of MTT assay results with a flatbed scanner (Gabrielson et al., 2002) or even by naked-eye observing (Abate et al., 1998).

5. Main sources of inaccuracies

The mechanism of MTT reduction in bacterial cell is not entirely studied yet. This lack of detailed knowledge of the process has caused

Table 6
Solvents proposed for formazan solubilization in the MTT assay.

Solvent	Comment
DMSO	Provides more stable readings than isopropanol (Li and Song, 2007; Wang et al., 2007) pH is critical for the accuracy of obtained results (Wang et al., 2012) Biofilm quantification (Walecka et al., 2008; Wu et al., 2010)
Alkaline DMSO with 8–800 mM ammonia	Eliminates pH-related interferences and provides relatively constant final reading (Wang et al., 2012)
DMSO with 8–40 mM HCl	Significant drop of A_{550} after an hour after dissolution (Wang et al., 2012)
Ethanol	Causes protein precipitation (Mosmann, 1983)
40% formaldehyde	Water solution (Broughton and Jahans, 1997)
Isopropanol	Also, 50 µL of PBS was added to the probe after formazan dissolution (Stevens et al., 1991; Stevens and Olsen, 1993)
Isopropanol with 4 N HCl	Originally applied for eukaryotic cells, HCl incorporated to reduce pH-dependent inaccuracies (Mosmann, 1983)
Isopropanol with 0.1 N HCl	MDR *M. tuberculosis* detection (Foongladda et al., 2002)
Isopropanol with 5% of 1 mol L^{-1} HCl	MIC evaluation (Shi et al., 2007; Grela et al., 2016)
10% SDS and 10 mM HCl in PBS	Tetrazolium salts comparison (Gabrielson et al., 2002)
10% SDS and 50% DMF in distilled water	Biofilm formation evaluation (Brambilla et al., 2014)
1:1 (v/v) 20% SDS in 50% DMF	pH = 4.7 (Montoro et al., 2005; Mshana et al., 1998)
17% SDS in 40% DMF	MDR *M. tuberculosis* detection (Abate et al., 1998)
Triton X-100	Added at the beginning of the assay, allows the microemulsion formation and prevents formazan from discoloration (Stowe et al., 1995)

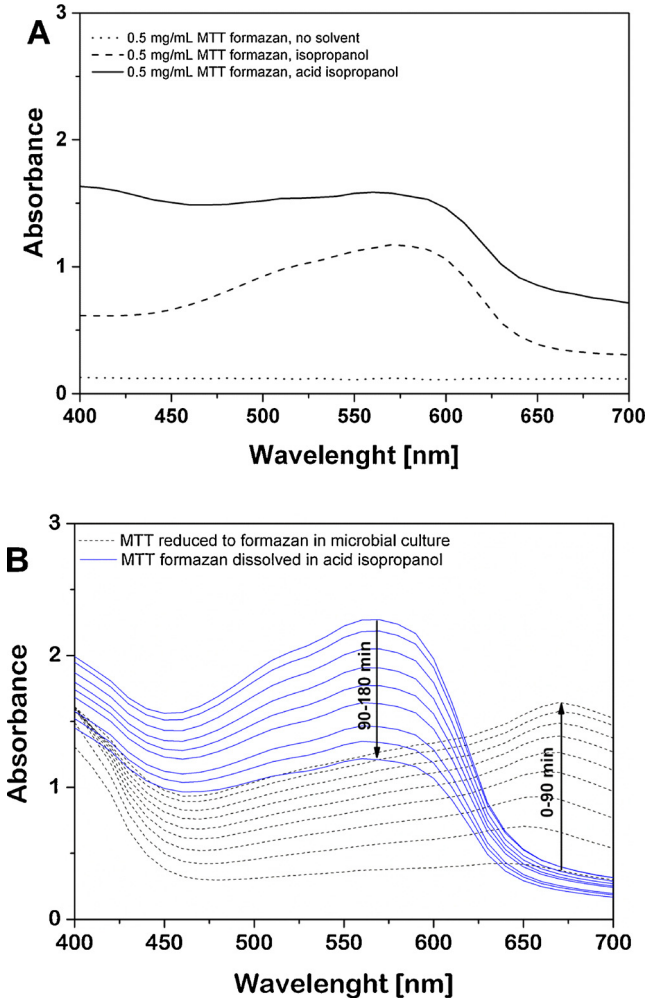


Fig. 3. A) Absorbance spectrum (400–700 nm) of undissolved MTT formazan suspended in water in comparison with MTT formazan dissolved in isopropanol and acid isopropanol **B)** Time – dependent alternations in the absorbance spectrum during MTT reduction by *Proteus mirabilis* (0-90 min in Mueller-Hinton Broth, 0.5 mg mL^{-1} MTT) and subsequent dissolution in acid isopropanol (90–180 min in Mueller-Hinton Broth-acid isopropanol mixture). Both MTT assay steps were run with intense shaking in darkness (Grela et al., 2015).

method variability that is noticeable to anyone who attempts to employ MTT test in microbiology. As the MTT assay has been exploit since 1983 in numerous alternations, different factors that affected the final results were spotted (Table 8).

Several chemicals cause abiotic MTT reduction in cell-free medium. Here, compounds of antioxidant and reducing activity can be listed e.g. flavonoids, with the examples of luteolin, quercetin and plant extracts in general (Peng et al., 2005), indicating that any search for new antimicrobials utilizing the MTT assay must very carefully optimized. Next, a variety of antimutagenic, chemopreventive and anti-inflammatory agents (Jaszczyszyn and Gąsiorowski, 2008) etc. may alter the result of the MTT test as the effect of free radical scavenging activity. Their antibacterial characteristics with the MTT assay must be taken under special consideration.

The optimum wavelength of final formazan solution absorbance readings can vary importantly depending on the solvent chosen to terminate the assay (Carmichael et al., 1987). As the effect, throughout the years a considerably wide range of conditions have been applied (Table 7). Recently, salts and complexes containing copper (II) were described to alter the MTT formazan absorbance spectrum (Perez et al., 2017). Since the copper (II) complexes of good stability – such as Cu(II) His_2 – do not create similar inaccuracies, complexation of formazan is proposed as a mechanism of the phenomenon.

Aside from compounds directly reducing MTT salt or changing formazan spectroscopic characteristics, enzyme inhibitors represent a vast group of compounds that affect the MTT assay (Weyermann et al., 2005). The overall effect upon microorganism's metabolic efficiency should be considered even if the tested culture remains viable in the presence of the biologically active compound tested.

Nevertheless, the MTT assay will continue to be widely used in microbiology and with proper optimization steps it is a valuable source of information. It is important that in most cases ruling out the interaction of tested chemicals with MTT must not be avoided before main part of the assay can be performed (Jaszczyszyn and Gąsiorowski, 2008).

6. Perspectives

Certainly, future applicability of the MTT assay in microbial studies requires a better understanding of the underlying biochemical process and establishment of uniform, widely accepted protocols. Researchers have already proposed a variety of MTT-based methods for bacteria characteristics. At present, only data obtained within one coherent set of measurements can be analyzed with sufficient precision. Making comparisons or concluding from other results may be misleading, because results reproducibility depends strongly not only on chosen test

Table 7
Optimum wavelength values used for formazan solution measurements.

Wavelength [nm]	Comments
630	*Brucella* sp. (Broughton and Jahans, 1997)
590	Standard method applied for formazan quantification (Stowe et al., 1995)
582	Applied after formazan dissolution with DMSO (Stowe et al., 1995)
576	Continuous method with Triton X-100 (Stowe et al., 1995)
570	Originally applied for eukaryotic cells (Mosmann, 1983); Optimized assay for multi-resistant *M. tuberculosis* (Abate et al., 1998; Foongladda et al., 2002; Mshana et al., 1998) Biofilm quantification (Wu et al., 2010)
568	Applied after formazan dissolution with isopropanol (Stowe et al., 1995)
560	Applied after formazan dissolution with 150 µL isopropanol and 50 µL PBS (Stevens et al., 1991; Stevens and Olsen, 1993)
556	Protocol for cyanobacteria viability estimation (Li and Song, 2007)
550	Applied after formazan dissolution with DMSO (Wang et al., 2012) Applied after formazan dissolution with isopropanol/HCl (Shi et al., 2007) Bactericidal activity of macrophages (Thanawongnuwech et al., 1997) Biofilm formation evaluation (Walecka et al., 2007; Brambilla et al., 2014)
540	Biofilm quantification (Deb and Vimala, 2017) Tetrazolium salts comparison (Gabrielson et al., 2002)
510	Applied after formazan dissolution with DMSO (Wang et al., 2007)

Table 8
Factors affecting the final result of MTT assay.

Factor	Interferences
Prolonged incubation	Possible transformation of formazan to colorless derivative (Stowe et al., 1995)
Presence of drug-resistant strains	Possibly lower observed metabolic activity (Foongladda et al., 2002)
pH after dissolution	Shift in the spectrum of absorbance, lower readings in acidic pH after dissolution in DMSO (Plumb et al., 1989)
pH of reaction buffer	Low pH decreases the reading result (e.g. in sodium acetate, KH_2PO_4-NaOH, glycine-NaOH and especially Tris-HCl buffer) (Wang et al., 2012)
Type of reaction medium	KH_2PO_4–NaOH and glycine–NaOH may cause increased turbidity after formazan dissolution, resulting in unstable readings (Wang et al., 2012)
Microorganism characteristics	E.g. strain type, growth phase, level of metabolic activity and growth conditions (Berridge et al., 2005)

Chemicals	Interferences
Albumins	False positive (higher absorbance) results (Funk et al., 2007)
Antimutagenic, chemopreventive and anti-inflammatory agents	Drugs of free-radical scavenging activity (Jaszczyszyn and Gąsiorowski, 2008)
Cu(II) compounds	Cu(II)Ser_2, Cu(II)$Urea_2$ and $CuCl_2$ interfere with formazan absorbance readings (Perez et al., 2017)
Glucose	Accelerates the rate of MTT reduction (Liu et al.,1997; Stockert et al., 2012)
Exogenous electron carriers	Accelerate the rate of MTT reduction (Bernas and Dobrucki, 2000)
Flavonoids	Especially flavonoids hydroxylated in 3 position (Bruggisser et al., 2002; Peng et al., 2005)
Free radicals	And compounds that generate them intracellularly (Jaszczyszyn and Gąsiorowski, 2008)
Inhibitors of glucose transport	Decreasing the rate of MTT reduction (Stockert et al., 2012)
Lactic acid	Possibility of tetrazolium salt reduction inhibition (Tachon et al., 2009)
Lipid and 'lipid-like' molecules	Accelerates the rate of MTT reduction (Massa and Farias, 1982)
Phenol red	Interferes with the final absorbance reading at pH > 6 (Denizot and Lang, 1986)
Plant extracts	Reduction of MTT compound reactivity (Karakas et al., 2017)
Pyridine nucleotides	Accelerate the rate of MTT reduction (Liu et al., 1997)
Reducing agents	Such as ascorbic acid, dihydrolipoic acid, α-tocopherol and sulfhydryl-containing compounds accelerate the rate of MTT reduction (Bruggisser et al., 2002; Chakrabarti et al., 2000; Stockert et al., 2012)
Retinol	Vitamin A acts as a reductase, catalyzing MTT reduction Chakrabarti et al. (2000)
Triton X-100	Accelerates the rate of MTT reduction (Massa and Farias, 1982)
Tris-HCl buffer	Drastic reduction of dissolved formazan absorbance (Wang et al., 2012)

principles but also the microbial culture homogeneity and metabolic activity (Lü et al., 2012; Young et al., 2005).

Nevertheless, the MTT assay have gained much attention as the effect of its numerous advantages. Properly executed, it allows to obtain the results with accuracy similar to the one of traditional plate counts (Brambilla et al., 2014; Dalai et al., 2012) with supreme rapidity. In comparison to most protocols for bacteria vitality estimations the MTT assay classifies as one of the cheapest and simple tests - considering reagents and equipment required. Another noticeable advantage is the applicability as the microplate assay, useful both in advanced experiments and screening assays.

It can safely be assumed that the MTT assay still will be widely and successfully used in the future years. Existing gaps in our knowledge considering application of MTT in bacterial protocols represents additional necessity for individual test optimizations, but also create further possibilities of evaluating novel, better implemented methodologies. So far, existing information on bacterial reduction of MTT does not allow to design a universal, microbiological MTT assay's protocol.

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