# The use of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide–based colorimetric assay in the viability analysis of the filamentous cyanobacterium Arthrospira platensis

AUTHOR(S):

Shiraishi, Hideaki; Toyoda, Akiko

This is a pre-copyedited, author-produced version of an article accepted for publication in *Bioscience, Biotechnology, and Biochemistry* following peer review. The version of record [Shiraishi, H. and Toyoda, A. The use of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide-based colorimetric assay in the viability analysis of the filamentous cyanobacterium *Arthrospira platensis*. *Biosci. Biotechnol. Biochem.* **85**, 739-742 (2021)] is available online at: https://doi.org/10.1093/bbb/zbaa050

NOTE

# The use of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide-based colorimetric assay in the viability analysis of the filamentous cyanobacterium *Arthrospira platensis*

**Hideaki Shiraishi* and Akiko Toyoda**
Graduate School of Biostudies, Kyoto University, Kyoto 606-8501, Japan

*To whom correspondence should be addressed.
*Email: siraisi@kuchem.kyoto-u.ac.jp*

Running head: MTT assay with *Arthrospira platensis*

**Abstract**

The applicability of MTT assay to an industrially valuable filamentous cyanobacterium *Arthrospira platensis* was examined. When it was applied to *A. platensis* NIES-39, as few as 10 viable trichomes were quantitatively detected. However, depending on the experimental conditions, it also generated artifactual viability signals. The results should help clarify the scope and limits of the MTT assay in viability analysis.

Key words: colorimetric viability assay; cyanobacteria; heat sensitivity; spirulina

Edible filamentous cyanobacteria belonging to the genera *Arthrospira* have been commercially propagated since the 1970s and are consumed worldwide as food and food additives under the name spirulina [1]. In the biotechnological study of microorganisms including *Arthrospira*, it is often necessary to examine cell viability after various experimental treatments. For many unicellular species, cell viability can be assessed by simply determining the number of colony-forming units in a unit volume. However, it is difficult or impossible to determine it for filamentous, multicellular cyanobacteria because the survival rate of individual filaments is not necessarily proportional to that of individual cells. Also, many filamentous cyanobacteria including *Arthrospira* spp. exhibit gliding motility on solid media and do not form distinct colonies [2], thus, further complicating quantitation of viability. As an alternative procedure, the cell viability of *Arthrospira* spp. was assessed by determining growth curves in a previous study [3]. However, the procedure was time-consuming and required more than 3 days to determine cell viability.

For viability measurement, dye-based assays are also used to differentiate between dead and viable cells [4]. For example, the fluorescent dye propidium iodide (PI) is often used as a dead cell indicator in eukaryotic systems as it permeates into non-viable cells but not in viable cells. However, it has been reported that in filamentous cyanobacteria, PI binds to both viable and non-viable cells, interfering with cell viability analysis [5]. A non-fluorescent dye, trypan blue, is also used to specifically stain non-viable cells [6]. When this dye is used, microscopic observation is needed to detect dead, stained cells. However, in our observation with *A. platensis*, microscopic inspection of individual cells was severely interfered with by filament aggregation, particularly after treatment with cytotoxic agents. Therefore, it was impossible to quantitatively analyze cell viability using this dye.

Metabolic staining is another strategy to differentiate between viable and dead cells. One of the most commonly used methods is based on the water-soluble yellow dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT); the dye is reduced to a water-insoluble purple formazan product in metabolically active cells [7]. After incubating cells with MTT, the formazan product can be solubilized in organic solvents and then quantitated using a spectrophotometer. In this assay, a reduction in viability results in a decrease in the purple formazan product. This assay is widely used in eukaryotic systems [8,9] and has also been applied to bacterial species [10]. As for cyanobacteria, it was successfully applied to a unicellular species *Microcystis aeruginosa* [11]. However, there has been no report that applied it to *Arthrospira* spp. Also, since it has been reported that MTT assay yields false-positive viability signals in

some biological systems [12–15], careful evaluation is necessary before applying it to a new system. Therefore, in this study, we evaluated the applicability of the MTT assay to *A. platensis*.

The former study with *M. aeruginosa* used dimethyl sulfoxide (DMSO) to dissolve the formazan product [11]. However, in our preliminary experiments with *A. platensis*, quantitation of the formazan product was not very reproducible when it was dissolved in DMSO, particularly when many samples were subjected to the assay. Photo-bleaching of the formazan product in DMSO was one of the possible causes of the relatively low reproducibility. Although photo-bleaching is suppressed by shielding the light [11], it is cumbersome and often tricky always to shield the light when a high number of samples are subjected to the assay. Since acid-isopropanol, which was used in the original report of MTT assay [7], did not cause photo-bleaching and was found to yield reproducible results with *A. platensis*, we used it throughout this study to dissolve the formazan product.

We first examined the time-course of the accumulation of the formazan product in *A. platensis* cells to determine the optimal incubation period with MTT. *A. platensis* NIES-39 obtained from the Microbial Culture Collection at the National Institute for Environmental Studies, Tsukuba (MCC-NIES) was cultured in SOT medium as described previously [16,17]. *A. platensis* filaments in 50 µL of SOT medium were mixed with 5 µL each of MTT solution (5 mg/mL in $H_2O$) and cultured for various periods. Then, filaments were collected by centrifugation, and the purple formazan product in the samples was dissolved in 100 µL of acid-isopropanol, which was prepared by mixing 2-propanol with 1/25 volume of 1N HCl. The amount of the formazan product was quantitated by determining absorbance at 570 nm. As a control for background absorbance contributed by cellular substances, heat-killed cells were prepared by incubating cells at 80°C for 30 min and treated in the same way.

As shown in Figure 1, the formazan product accumulated in the cells over a period of 120 min when viable cells were used (Figure 1, open circles). In contrast, the product was scarcely detectable when heat-killed cells were used (Figure 1, closed circles). The background absorbance generated with the heat-killed cells was considerably low, approximately 0.42% of the absorbance generated with viable cells when compared at 120 min (Figure 1). This level of background would be negligible in most applications of MTT assay. Furthermore, when precise quantitation is required, background absorbance determined with heat-killed cells can be subtracted from the assay data.

Since the accumulation of formazan product reached a plateau in 2 h, the incubation period with MTT was fixed to 2 h in the following experiments. A step-by-step protocol of MTT assay applied to *A. platensis* in this study was as follows.

*A. platensis* filaments in the late logarithmic phase ($OD_{730}$ = 0.1 – 0.3) were suspended in 50 µL of SOT medium and mixed with 5 µL of MTT solution (5 mg/mL in $H_2O$) in a 1.5-mL microfuge tube (flat-bottom microfuge tube ST-0150F; Ina-Optika, Osaka). Then, they were cultured at 30°C for 2 h. After 2 h, filaments were collected by centrifugation in a microcentrifuge equipped with an angle rotor at 14,000 rpm for 2 min at 4°C. Then, as much supernatant as possible was removed using a micropipette; this step was facilitated by the use of the flat-bottom microfuge tubes for centrifugation as the pellet binds tightly to one edge of the bottom after centrifugation. Then, 100 µL of acid-isopropanol was added and mixed with the pellet. The mixture was maintained at room temperature with intermittent mixing until the purple pellet was completely dissolved, which usually required 30 – 90 min. Then, 100 µL of water was added to the sample and mixed. The addition of water at this step reduced the evaporation of the solvent during the transfer of a high number of samples into the wells of a microtiter plate in the next step, thus, improving the reproducibility of quantitation. Samples were centrifuged in a microcentrifuge at 14,000 rpm for 2 min at 4°C, and 150 µL of the supernatant was transferred to a flat-bottom 96-well plate (AGC Technoglass, Shizuoka). Absorbance at 570 nm was measured using a microplate reader (Synergy H4 Hybrid Multi-Mode Microplate Reader; BioTek Instruments, Winooski, VT). Samples without cells were also prepared in the same way as mentioned above, and the data from them were used to subtract background absorbance.

Following the protocol described above, we next determined the dynamic range and sensitivity of the MTT assay, varying the number of *A. platensis* filaments used in the assay. As shown in Figure 2(a), absorbance at 570 nm was almost linearly correlated with the number of *A. platensis* filaments included in the assay ($R^2$ = 0.995). Even the smallest number of filaments used in this experiment (65 filaments) yielded considerable absorbance. The sensitivity of the assay was further examined using smaller numbers of filaments. As shown in Figure 2(b), when 3 to 70 filaments were subjected to the assay, the absorbance of the reaction product was still almost linearly correlated with the number of cyanobacterial filaments ($R^2$ = 0.997). The results in Figure 2 indicated that MTT assay was very sensitive and had a broad dynamic range of more than 2-orders of magnitude when the protocol employed in this study was used.

To examine whether the current MTT assay yields results similar to those of previously employed methods, *A. platensis* cells were heated at various temperatures,

and their viability was analyzed with the growth curve-based conventional method [3, 18] and the current MTT assay. To this end, *A. platensis* cells were incubated for 30 min at various temperatures (30–80°C), and then ratios of viable cells were first determined by analyzing growth curves as described previously [3]. As shown in Figure 3(a), when cells were treated at up to 40°C, there were no detectable changes in the viability. However, a slight loss of viability was observed when samples were treated at 45°C, and no viability was detected when they were treated at temperatures of ≥50°C. This result was consistent with a former report that showed that the growth rate of various strains of *A. platensis* was reduced at 45ºC and impaired at ≥50ºC [19].

Next, the viability of similarly treated cells was analyzed with the current MTT assay. As shown in Figure 3(b), the results of the MTT assay at 45ºC and 50ºC were considerably different from those obtained with the growth curve-based method. When MTT assay was used, samples treated at 45°C yielded higher values than those treated at 30–40°C (Figure 3(b)), whereas reduction in viability was observed with the growth curve-based method at this temperature (Figure 3(a)). Furthermore, in samples treated at 50°C, faint but reproducible absorbance was detected in the MTT assay (Figure 3(b)), whereas cell survival was never observed with the growth curve-based method at this temperature (Figure 3(a)). Although the viability signal detected in the 50ºC samples in Figure 3(b) was low, it was reproducible and significantly higher than the values obtained with the growth curve-based method (Student's *t*-test; $p < 0.005$).

A conceivable cause of the difference at these temperatures was that perturbation of cellular metabolism induced by the heat-treatment may have been retained after the heat-treatment irrespective of the viability status of cells. For example, if the enzymatic activity responsible for the formation of the formazan product was induced to a high level by incubation at 45°C, the induced activity may be retained for a while after the heat-treatment and would increase the amount of the formazan product as shown in Figure 3(b). Also, at 50°C, trace enzymatic activity may remain for a while, although cellular propagation activity was lost at this temperature, causing a slight positive viability signal at 50°C, as seen in Figure 3(b). If the observed inconsistency was caused by these transiently remaining enzyme activities, their effect would be eliminated by waiting for a sufficiently long period before performing MTT assay rather than performing it soon after the heat treatment. To test this hypothesis, in the next experiment, samples were maintained for 16 h under standard culture conditions after the heat-treatment, and then MTT assay was performed. As shown in Figure 3(c), the result was similar to that obtained with the conventional method; the viability signal decreased at 45°C, and it was undetectable at 50°C.

This result with the heat-treated cells suggested that cytotoxic treatment may sometimes induce transient perturbation of cellular metabolism, causing inconsistency in the result in MTT assay. Recently, inconsistencies have also been reported in eukaryotic systems [12–15]. In one case, inconsistency was observed when a cytotoxic treatment increased the intracellular concentration of NADH, thus, enhancing the nonspecific reduction of MTT [13]. Many factors would affect MTT assay also in *A. platensis* and other cyanobacteria, causing inconsistency in cell viability analysis. Notably, in photosynthetic organisms, strong oxidants and strong reductants are simultaneously present during oxygenic photosynthesis [20]. Although redox homeostasis is maintained by the cellular regulatory network under normal growth conditions, it may be broken when an impact of stress is rapid and intense, resulting in an excessive accumulation of photosynthetic electron supply, which would make the cells in a hyper-reduced state. It is possible that such an event took place at 45˚C in the sample in Figure 3(b), causing excessive reduction of MTT.

Thus far, we have used the MTT assay in several different experiments with *A. platensis*. MTT assay was especially useful in monitoring the growth of *A. platensis* when it was cultured under the conditions where it tended to form aggregate; under such conditions, the growth of *A. platensis* could not be monitored by optical density measurement. On the other hand, in survival rate determination, its reliability depended on the treatment's nature. For example, in cryopreservation experiments, survival rates could be determined correctly by the MTT assay by culturing cells for 16 h after thawing. In such experiments, most cells looked intact when samples were thawed, but cells that had been injured by the freezing and thawing process lysed during the 16 h incubation, thus yielding correct viability data. In contrast, when *A. platensis* cells were treated with rifampicin or streptomycin, MTT assay yielded higher viability signals than the actual ratio of viable cells even after cells were cultured for three days. It appeared that the cellular activity to yield the formazan product remained for a long period even after most cells lost viability. In these cases, the growth curve-based method was a better choice for survival rate determination.

As a final note, it is worth mentioning that MTT assay is often performed in 96-well plates using a multichannel pipette since it reduces the processing time. However, in this study, MTT assay was carried out in microfuge tubes. In the initial attempt, we also tried to perform it in 96-well plates. However, since the pellet of *A. platensis* was so loose after centrifugation in a 96-well plate that it was impossible to remove the supernatant completely after the centrifugation. To make matters worse, when acid-isopropanol was added to the mixture of the culture medium (SOT medium) and MTT,

purple substances were generated in the mixture independently of cells, severely reducing the reproducibility of the experimental results. Therefore, to obtain reproducible results, it was essential to remove the mixture of culture medium and MTT as much as possible before adding acid-isopropanol. It could be accomplished only when microfuge tubes were used for centrifugation.

## Author contributions

HS conceived and designed the experiments. HS and AT carried out experiments and analyzed data. HS drafted the manuscript.

## Funding

This work was supported by JSPS KAKENHI Grant Number 18K19338 from the Japan Society for the Promotion of Science.

## References

[1] Belay A. Biology and industrial production of *Arthrospira* (*Spirulina*). In: Richmond A, Hu Q, editors. Handbook of Microalgal Culture. West Sussex: John Wiley & Sons; 2013. p. 339–358.

[2] Hoiczyk E. Gliding motility in cyanobacteria: observations and possible explanations. Arch Microbiol. 2000;174:11–17.

[3] Shiraishi H. Cryopreservation of the edible alkalophilic cyanobacterium *Arthrospira platensis*. Biosci Biotechnol Biochem. 2016;80:2051–2057.

[4] Johnson S, Nguyen V, and Coder D. Assessment of cell viability. Curr Protoc Cytom. 2013;64:9.2.1–9.2.26.

[5] Johnson TJ, Hildreth MB, Gu L, et al. Testing a dual-fluorescence assay to monitor the viability of filamentous cyanobacteria. J Microbiol Methods. 2015;113:57–64.

[6] Strober W. Trypan blue exclusion test of cell viability. Curr Protoc Immunol. 2015;111: A3.B.1–A3.B.3. doi: 10.1002/0471142735.ima03bs111

[7] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65: 55–63

[8] Mshana RN, Tadesse G, Abate, G, et al. Use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide for rapid detection of rifampin-resistant *Mycobacterium tuberculosis*. J Clin Microbiol. 1998;36:1214–1219.

[9] Kumar P, Nagarajan A, Uchil PD. Analysis of cell viability by the MTT assay. Cold Spring Harb Protoc. 2018. DOI: 10.1101/pdb.prot095505

[10] Grela E, Kozłowska J, Grabowiecka A. Current methodology of MTT assay in bacteria – a review. Acta Histochem. 2018;120:303–311.

[11] Li J, Song L. Applicability of the MTT assay for measuring viability of cyanobacteria and algae, specifically for *Microcystis aeruginosa* (Chroococcales, Cyanobacteria). Phycologia 2007;46:593–599

[12] Jakštys B, Ruzgys P, Tamošiūnas M, et al. Different cell viability assays reveal inconsistent results after bleomycin electrotransfer *in vitro*. J Memb Biol. 2015;248:857–863.

[13] Jo HY, Kim Y, Park HW, et al. The unreliability of MTT assay in the cytotoxic test of primary cultured glioblastoma cells. Exp Neurobiol. 2015;24:235–245.

[14] Stepanenko AA, Dmitrenko VV. Pitfalls of the MTT assay: Direct and off-target effects of inhibitors can result in over/underestimation of cell viability. Gene. 2015;574:193–203.

[15] Karakaş D, Ari F, Ulukauya E. The MTT viability assay yields strikingly false-positive viabilities although the cells are killed by some plant extracts. Turk J Biol. 2017;41:919–925.

[16] Shiraishi H, Tabuse Y. The *Apl*I restriction-modification system in an edible cyanobacterium, *Arthrospira* (*Spirulina*) *platensis* NIES-39, recognizes the nucleotide sequence 5'-CTGCAG-3'. Biosci Biotechnol Biochem. 2013;77:782–788.

[17] Tadama S, Shiraishi H. Growth of the edible microalga *Arthrospira platensis* in relation to boron supply. Int J GEOMATE. 2017;12:90–95.

[18] Watanabe MM, Sawaguchi T. Cryopreservation of a water-bloom forming cyanobacterium, *Microcystis aeruginosa* f. *aeruginosa*. Phycol Res. 1995;43:111–116.

[19] Vonshak A. *Spirulina*: Growth, physiology and biochemistry. In: Vonshak A, editor. *Spirulina platensis* (*Arthrospira*): Physiology, Cell-biology and Biotechnology. London: Taylor & Francis; 1997. p. 43–65.

[20] Scheibe R, Backhausen JE, Emmerlich V, Holtgrefe S. Strategies to maintain redox homeostasis during photosynthesis under changing conditions. J Exp Bot. 2005;56:1481–1489.

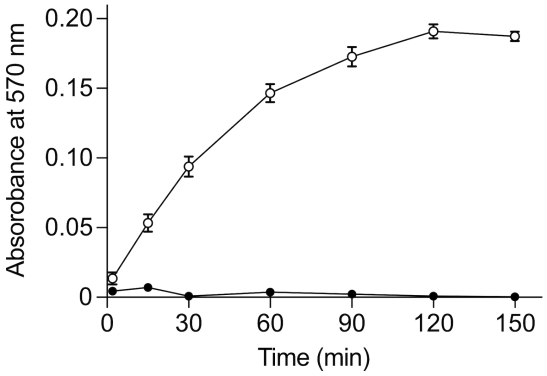


**Figure 1.** Time-course of the accumulation of the reaction product.
*A. platensis* NIES-39 filaments (291 ± 39 filaments in 50 µL of SOT medium) were mixed with 5 µL of MTT solution (5 mg/mL in $H_2O$) and cultured. After the indicated periods, filaments were recovered by centrifugation, and formazan product in the cells was dissolved in acid-isopropanol. Relative amounts of formazan product were determined by measuring absorbance at 570 nm. Viable (open circles) and heat-killed cells (closed circles) were used for the reactions. The means and standard errors of the means are shown ($n$ = 5).

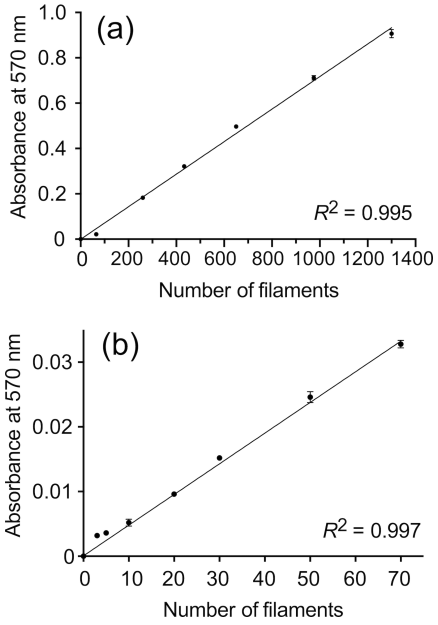


**Figure 2.** Dynamic range and sensitivity of MTT assay.
Varied numbers of *A. platensis* filaments were incubated with MTT in microfuge tubes, and accumulation of the reaction product was determined. (a) A dilution series of *A. platensis* filaments were subjected to the assay. (b) Filaments were individually picked using a micropipette, and the indicated numbers of filaments that were suspended in 50 µL of SOT medium were subjected to the assay. The means and standard errors of the means are shown ($n$ = 5). Linear regression analysis was performed using GraphPad Prism 8 (GraphPad Software, San Diego).

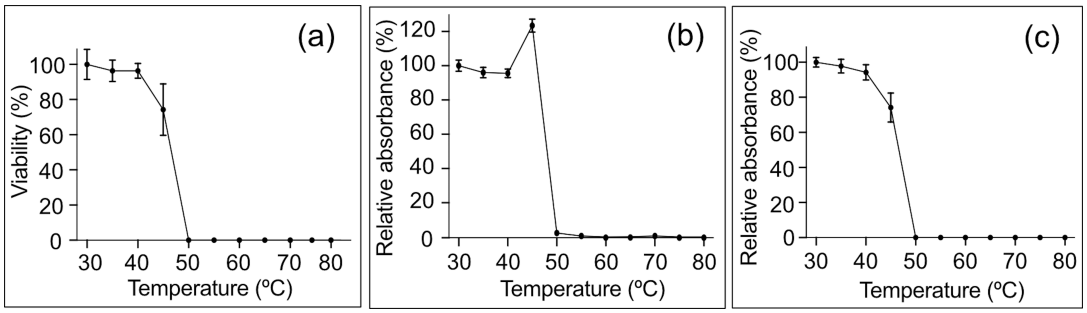


**Figure 3.** Viability analysis of *A. platensis* after heat-treatment.
(a) *A. platensis* filaments (approximately 400 filaments) were heated for 30 min at various temperatures in 50 µL of SOT medium and then cultured in 1 mL of the same medium. Viability was determined by comparing the growth curves of heat-treated and control samples. (b) MTT assay was performed soon after the heat-treatment in 50 µL of SOT medium. Relative $OD_{570}$ values relative to control samples (30°C) were calculated and plotted by setting the mean value of the control samples (0.207 at 30°C) as 100. (c) Samples were maintained for 16 h under standard culture conditions after heat-treatment. Then, MTT assay was performed. $OD_{570}$ values relative to the mean $OD_{570}$ value of the control samples (0.269 at 30ºC) were calculated and plotted as in (b). For each data point, the mean and the standard error of the mean is shown ($n$ = 5).