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Quantification of Triphenyl-2H-tetrazoliumchloride Reduction Activity in Bacterial Cells

Roberto Defez, Anna Andreozzi and Carmen Bianco*

Institute of Biosciences and BioResources, Via P. Castellino 111, Naples, Italy
*For correspondence: carmen.bianco@ibbr.cnr.it

[Abstract] This protocol describes the use of the 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) salt to evaluate the cell redox potential of rhizobia cells. The production of brightly colored and insoluble 1,3,5-Triphenyltetrazolium formazan arising from TTC reduction is irreversible and can be easily quantified using a spectrophotometer. This protocol allows the production of reproducible results in a relatively short time for *Sinorhizobium meliloti* 1021 cells grown both in exponential and stationary phases. The results here presented show that the *S. meliloti* cells deriving from exponential-phase cultures had increased cell redox potential as compared to the ones deriving from stationary-phase cultures. This means that under exponential growth conditions the *S. meliloti* cells generate higher amount of reducing equivalents needed for TTC reduction.

Keywords: *Sinorhizobium meliloti* 1021, Cell redox potential, 2,3,5-triphenyl-2H-tetrazolium chloride (TTC), 1,3,5-Triphenyltetrazolium formazan, Bacterial cells

[Background] The TTC salt is a water-soluble and colorless compound that can be reduced to formazan, a highly colored compound. The irreversible formation of formazan can be quantified using a spectrophotometer. Owing to its property and its low reduction potential, this tetrazolium salt is widely used in both eukaryotes and prokaryotes as an indicator of cell redox activity, viability, drug susceptibility and substrate utilization assays (Byth *et al.*, 2001; Hayashi *et al.*, 2003; Raut *et al.*, 2008; Lin *et al.*, 2008). The net positive charge on tetrazolium salts facilitates cellular uptake due to the membrane potential, allowing their intracellular reduction (Berridge *et al.*, 2005). In prokaryotes, the main studies of TTC reduction have concerned the Gram-negative respiring bacterium *Escherichia coli*, while only a few studies have been reported for members of the *Rhizobiacea* family. In this protocol, one of the best genetically characterized members of this family, the *S. meliloti* 1021 *rhizobium* strain, was used. The respiratory activity, expression of cytochrome terminal oxidases, of this strain was analysed using TTC as an indicator of cell redox potential.

To enable the development of a measurable color intensity and, at the same time, to avoid any possible inhibition of bacterial growth, the bacteria were incubated in the presence of TTC for an appropriate period of time compared to those described by other authors (Tengerdy *et al.*, 1967; Byth *et al.*, 2001; Tachon *et al.*, 2009).

Materials and Reagents

1. Sterile inoculation loop with incorporated needle (NUOVA APTACA, catalog number:

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6001/SG/CS)

2. 14-ml polypropylene round-bottom tubes (Corning, Falcon®, catalog number: 352059)
3. 50-ml conical centrifuge tubes (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 339652)
4. Safe-lock 2-ml tubes (Eppendorf, catalog number: 0030120094)
5. *Sinorhizobium meliloti* 1021
6. 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) (Sigma-Aldrich, catalog number: T8877)
7. 1,3,5-Triphenyltetrazolium formazan (Sigma-Aldrich, catalog number: 93145)
8. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, catalog number: D5879)
9. Tryptone (Sigma-Aldrich, catalog number: T9410)
10. Yeast extract (Sigma-Aldrich, catalog number: Y0375)
11. Calcium chloride ($CaCl_2$) (Sigma-Aldrich, catalog number: C3306)
12. Na_2HPO_4 (Acantor® Performance Materials, J.T. Baker, catalog number: 4062-01)
13. NaH_2PO_4 (Acantor® Performance Materials, J.T. Baker, catalog number: 3818-05)
14. TYR broth medium (5 g/L tryptone, 3 g/L yeast extract, 6 mM $CaCl_2$) (see Recipes)
15. Sodium phosphate buffer (pH 7.5) (see Recipes)

Equipment

1. Incubator room (at 30 °C)
2. Rotary shaker
3. Spectrophotometer (Beckman Coulter, catalog number: DU800)
4. Cuvettes for spectrophotometry application in the visible spectrum (Kartell, catalog number: 1938)
5. Micro-centrifuge (SCILOGEX D3024 High Speed Micro-Centrifuge) (Scilogex, catalog number: 912015139999)
6. Centrifuge (Thermo Fisher Scientific, Thermo Scientific™, model: Heraeus™ Megafuge™ 16R)
7. Oven (at 65 °C)
8. Balance (Mettler Toledo, catalog number: B204-S)

Procedure

A. Growth of bacterial cells

1. Pure cultures of *S. meliloti* 1021 stored at -80 °C were taken by sterile loops, inoculated in 15-ml round-botton tubes containing 2.0 ml of TYR medium and incubated for 24 h at 30 °C on a rotary shaker (200 rpm).
2. Aliquots of the cultures (0.15 ml) were transferred into 50-ml conical centrifuge tubes containing 15 ml of fresh TYR medium (OD_{600} = 0.2) and the optical density measured at 600 nm using a spectrophotometer and cuvettes for spectrophotometry application in the visible spectrum.

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3. When the cultures reach the exponential growth phase (OD_{600} = 0.7), which requires about 4 h of incubation, stop the growth and split the cultures in 10 ml for biomass evaluation and 1.5 ml for TTC reduction measurement.

B. TTC assay

1. Centrifuge the 10 ml bacterial cultures in 15-ml polypropylene round-bottom at 5,000 *x g* for 20 min (at room temperature) and discard the supernatant.
2. Incubate the cells at 65 °C in an oven for 4 h until dryness and weigh the cells with a balance.
3. Centrifuge the 1.5 ml bacterial cultures in 2-ml Eppendorf tubes at 8,000 *x g* for 5 min (at room temperature) and discard the supernatant.
4. Wash the bacteria with 1 ml of 50 mM sodium phosphate buffer (pH 7.5), centrifuge at 8,000 *x g* for 5 min (at room temperature) and discard the supernatant.
5. Resuspend the cells in 1 ml of 50 mM sodium phosphate buffer (pH 7.5) containing 24 mM 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) and incubate at 30 °C for 1 h on a rotary shaker at 200 rpm (Figure 1).

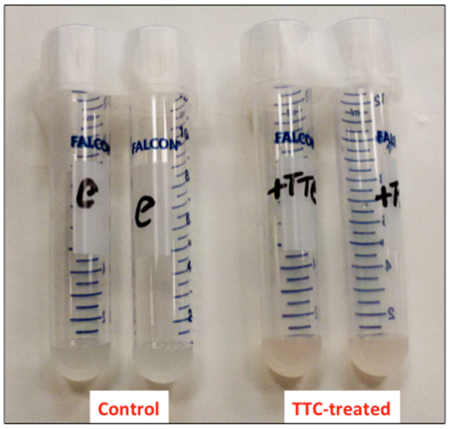

Figure 1. Picture of the cell suspensions after 1 h of incubation at 30 °C

6. Collect the cells by centrifugation at 8,000 *x g* for 5 min (at room temperature) and discard the supernatant (Figure 2).

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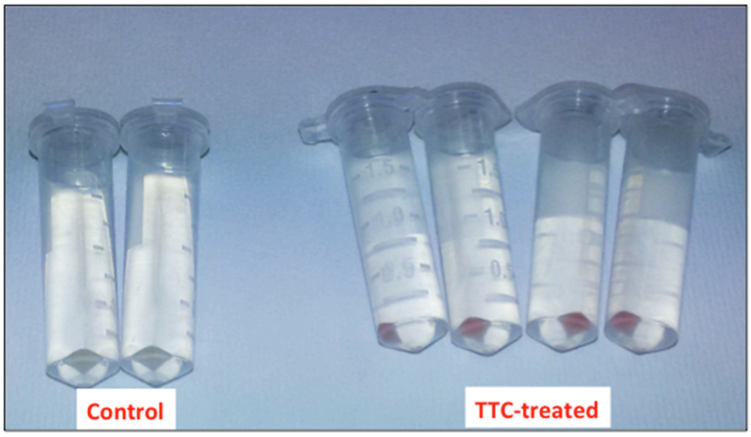

Figure 2. Picture of the bacterial cells after the centrifugation step

7. Resuspend the cells thoroughly in 1 ml 99.5 % DMSO (at room temperature) to dissolve the produced formazan.
8. Spin down the bacterial cells by one-minute centrifugation at 13,000 *x g* (at room temperature) and collect the supernatants.
9. Prepare formazan standard solutions at different known concentrations dissolving the powder in 99.5 % DMSO.
10. Read the absorbance of the samples (supernatants) and formazan standards at 510 nm using a spectrophotometer. Use the 99.5 % DMSO as blank control.
11. Plot the calibration curve of the formazan standards (mg/ml) against the absorbance of the solutions at 510 nm.
12. Determinate the amount of formazan produced from reactions with the bacterial cells directly from the formazan calibration curve.
13. Normalize the data to cell biomass (g) corresponding to 1.5 ml bacterial cells and determine the amount of formazan produced per g cells.

Data analysis

1. Perform at least five biological replicates, each conducted at different times.
2. An example of the excel Formazan standard curve is described in Table 1.
3. The readings of some experimental samples and the amount of formazan they produced are reported in Table 2 and Table 3.

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Table 1. Formazan standards and their relative absorbance at 510 nm

Formazan (mg/ml)	OD 510 nm
0.0075	0.04
0.015	0.066
0.03	0.120
0.06	0.234
0.09	0.382
0.12	0.452
0.15	0.612
0.24	0.964
0.3	1.173

Optical densities were determined using a spectrophotometer and data were plotted as shown in Figure 3.

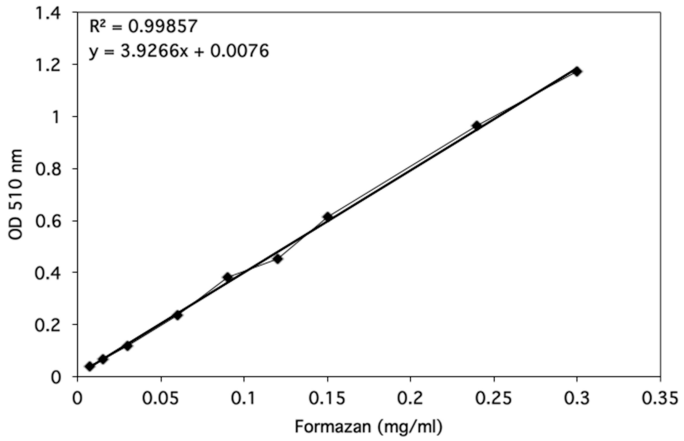

Figure 3. Formazan standard curve. The optical density at 510 nm was determined for a range of Formazan standards from 0.0075-0.3 mg/ml. The linear regression line was added by using the Excel 2011 for Mac (14.1.0 version). The equation for the regression line and the correlation coefficient are shown on the graph.

Table 2. Amount of formazan produced by *S. meliloti* 1021 cells grown up to exponential phase

OD 510 nm	Formazan (mg/ml)	mg Formazan/mg cells
0.19	0.046	0.057
0.22	0.054	0.056
0.22	0.061	0.067

The amount of formazan reported in this table was calculated by interpolating the standard solution points reported in Table 1 and elaborated in Figure 1.

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Table 3. Amount of formazan produced by *S. meliloti* 1021 cells grown up to stationary phase

OD 510 nm	Formazan (mg/ml)	mg Formazan/mg cells
0.14	0.034	0.029
0.14	0.033	0.029
0.13	0.032	0.026

The amount of formazan reported in this Table was calculated by interpolating the standard solution points reported in Table 1 and elaborated in Figure1.

Recipes

1. TYR broth medium (1 L)
   5 g tryptone
   3 g yeast extract
   Add ddH_2O to 1 L. Sterilize by autoclaving
   After the solution has cooled add 12 ml sterile 0.5 M $CaCl_2$
2. Sodium phosphate buffer (pH 7.5)
   a. Prepare 1 M NaH_2PO_4 solution by dissolving 1.2 g in a final volume of 10 ml double distilled water
   b. Prepare 1 M Na_2HPO_4 solution by dissolving 1.42 g in a final volume of 10 ml double distilled water
   c. Mix 1.6 ml 1 M NaH_2PO_4 solution and 8.4 ml 1 M Na_2HPO_4 solution and dilute to 200 ml with double distilled water to prepare 50 mM sodium phosphate buffer, pH 7.5

Acknowledgments

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