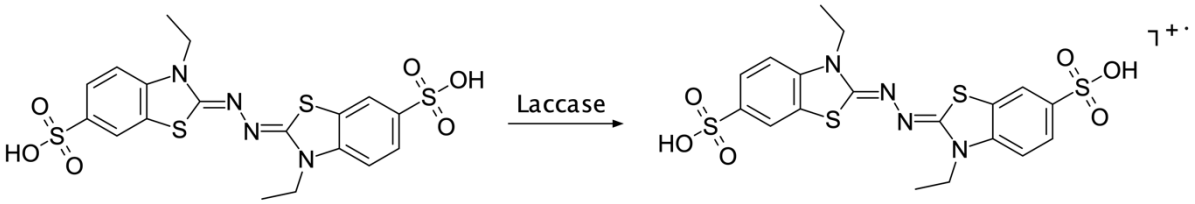
ABTS ASSAY

General:



Colorless

M=514.62 g/mol

Green

- 2,2'-Azino-di(3-ethylbenzthiazolin-6-sulfonacid)
- CAS: 28752-68-3; 30931-67-0 (Diammonium salt)

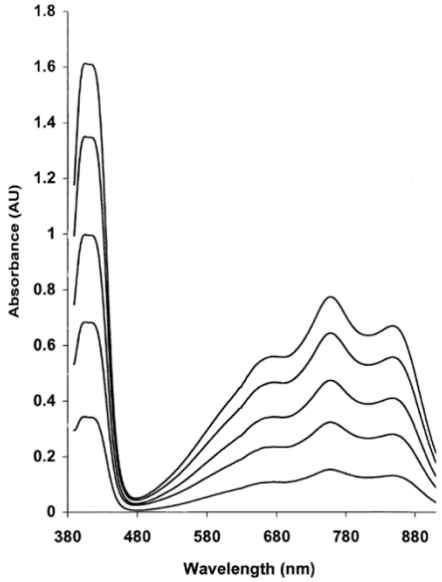


Fig. 1: Absorption spectrum of $ABTS^{+\cdot}$ [1]

Conditions of paper [2]:

- Paper on BaLac
- θ variable
- $c = 1\ \mathrm{mM}$ ABTS
- Buffer: $c = 100\ \mathrm{mM}$ Citrat-Phosphat; $\mathrm{pH} = 4.0$
- Air-saturated
- Spectroscopic parameter: $\lambda = 420\ \mathrm{nm}$; $\varepsilon_{420\ \mathrm{nm}} = 36000\ \mathrm{M^{-1}cm^{-1}}$

Conditions of paper [3]:

- Paper on marLac
- Spectroscopic parameter: See above
- ABTS concentration unknown
- Buffer: $40\ \mathrm{mM}$ Britton-Robinson buffer; $\mathrm{pH} = 7.0$
- $\theta = 60°\mathrm{C}$
- Rest: standard conditions

Preparing the buffers

0.2 M Na_2HPO_4 needed

0.1 M Citric acid needed

For the basic solution with a volume of 200 ml, use Na_2HPO_4 with a molecular weight of 177.99 g/mol

Formula: $m\ [g] = M\ \left[\frac{g}{mol}\right] * c\ [M] * V\ [l]$

Calculation: $177.99\ \frac{g}{mol} * 0.2\ M * 0.20\ l = 7.119\ g$ for a 0.2 M solution

Weigh the calculated amount of Na_2HPO_4 and fill the bottle to 200 ml with ultra filtrated (UF) H_2O.

For the acidic solution with a volume of 200 ml, use Citric acid **monohydrate** with a molecular weight of 210.14 g/mol.
Calculation: $210.14\ \frac{g}{mol} * 0.1\ M * 0.25\ l = 4.203\ g\ per\ 200\ ml$ for a 0.1 M solution

Weigh the calculated amount of Citric acid monohydrate and fill the bottle to 200 ml with UF H_2O.
Mix both solutions until they are clear.

V = 1000 mL (Multiplied all values by 50 from 20 ml values)

Tab. 1: Mixing ratios to achieve the desired pH values

pH	**0.2 M Na_2HPO_4 in ml**	**0.1 M Citric acid in ml**
4.0	385.5	614.5
5.0	515	485
7.0	823.5	176.5

Preparing ABTS

ABTS → $C_{18}H_{16}N_4O_6S_4$-$(NH_4)_2$-Mr548.7

548.7 g = 1 mol

54.87 g = 100 mmol

$$c = \frac{n}{V}$$

$$c = \frac{54.87}{1000}$$

$$c = 0.05487\ g/ml$$

Weigh 0.05487 g out on a fine scale. If you can´t weigh the exact amount, calculate the amount of water in which you want to dilute it with the following formula:

$$Amount\ of\ Water\ to\ add = \frac{actual\ weighted}{c}$$

Add the amount of water you calculated and mix it. ABTS Stock solution is ready and should be stored cooled and protected from light.

The final concentration per each well will be 0.25mM.

Dilution of enzyme

0.5 U/mg enzyme written on the bottle of *Trametes versicolor* from Sigma-Aldrich.

1 u = 1 µmol/min

We need 10 U/ml as concentration for the enzyme stock.

Formula: $Concentration\ of\ the\ enzyme = \frac{Want}{Have} * endvolume$

Calculation: $\frac{10\ U/ml}{0.5\ U/mg} * 1\ ml = 20\ mg/ml$

$\frac{20\ mg/ml}{100} = \frac{0.2\ mg}{ml} \rightarrow 200\ \mu g/ml$ (first dilution we want)

To do a dilution series, calculate for the concentrations you want the amount of enzyme from the stock solution you need.

We decided to measure the following enzyme concentrations:

Tab. 2: Concentrations of *T. versicolor* in µg/ml and µM

Concentration of *T. versicolor* [µg/ml]	Concentration of *T. versicolor* [µM]
200	3570
100	1790
50	890
20	360
15	270
10	180
2	40

Working dilution

Formula: $dilution\ factor * dilution\ concentration\ to\ use = Volume\ to\ use$

Calculation examples:

Concentration 2000 µg/ml: $0.1 * 200 = 20\ \mu l\ from\ stock$

Concentration 1000 µg/ml: $0.5 * 200 = \ \ 100\ \mu l\ from\ 200$
And so on.
The final dilution of 200 µg/ml was reached in the well where the amount was diluted by a factor of 10, so the final volume matches the table below. 5 µl of working dilution was added to each well to achieve the final concentration. The calculation is explained in more detail below the table.

Tab. 3: Calculations to achieve the final concentrations in the wells

Concentration [µg/ml] enzyme in the well	Working dilution
200	200 total volume = 20 µl stock enzyme + 180 µl buffer
100	200 total volume = 100 µl 200 µg/ml enzyme + 100 µl buffer
50	200 total volume = 100 µl 100 µg/ml enzyme + 100 µl buffer
20	200 total volume = 80 µl 50 µg/ml enzyme + 120 µl buffer
10	100 total volume = 50 µl 20 µg/ml enzyme + 50 µl buffer
2	100 total volume = 20 µl 20 µg/ml enzyme + 80 µl buffer

The volume of each well in a 96 well plate is 200 µl.
And because the enzyme converts 0.5 µmol/min (because 0.5 U/ml and 1 U = 1 µmol/min) there should be at least 0.5 µl enzyme in each well, but because pipetting 0.5 µl is not very accurate, you need to adjust the dilutions with buffer and increase to an amount to 5 µl enzyme for each well.

➔ So, in each well there are 5 µl of the enzyme of each concentration + 5 µl ABTS + 190 µl of buffer!

Tab. 4: Example how the wells of the 96 well plates were filled

Column	Well	3	4
	Enzyme Concentration	0 µg/ml	2 µg/ml
	pH		
a	4	5 µl ABTS + 195 µl buffer	5 µl enzyme 2µg/ml + 5 µl ABTS + 190 µl buffer
b	4	5 µl ABTS + 195 µl buffer	5 µl enzyme 2µg/ml + 5 µl ABTS + 190 µl buffer
c	ABTS Control	5 µl buffer + 195 µl buffer	5 µl enzyme 2µg/ml + 195 µl buffer
d	5	5 µl ABTS + 195 µl buffer	5 µl enzyme 2µg/ml + 5 µl ABTS + 190 µl buffer
e	5	5 µl ABTS + 195 µl buffer	5 µl enzyme 2µg/ml + 5 µl ABTS + 190 µl buffer
f	ABTS Control	5 µl buffer + 195 µl buffer	5 µl enzyme 2µg/ml + 195 µl buffer
g	7	5 µl ABTS + 195 µl buffer	5 µl enzyme 2µg/ml + 5 µl ABTS + 190 µl buffer
h	7	5 µl ABTS + 195 µl buffer	5 µl enzyme 2µg/ml + 5 µl ABTS + 190 µl buffer

Caution! Make sure to keep enzyme and ABTS separated until you are performing the assay!

Once ready, mix ABTS into each well according to scheme using a multichannel pipette, mix each well thoroughly, and place plate in plate reader at settings 420nm at 30˚C. Allow to run for 4 hours and photograph well plate after completion.

References:

(1) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant Activity Applying an Improved ABTS Radical Cation Decolorization Assay. *Free Radical Biology and Medicine* **1999**. https://doi.org/10.1016/S0891-5849(98)00315-3.

(2) Scheiblbrandner, S.; Breslmayr, E.; Csarman, F.; Paukner, R.; Führer, J.; Herzog, P. L.; Shleev, S. V.; Osipov, E. M.; Tikhonova, T. V.; Popov, V. O.; Haltrich, D.; Ludwig, R.; Kittl, R. Evolving Stability and PH-Dependent Activity of the High Redox Potential Botrytis Aclada Laccase for Enzymatic Fuel Cells. *Scientific Reports* **2017**. https://doi.org/10.1038/s41598-017-13734-0.

(3) Yang, Q.; Zhang, M.; Zhang, M.; Wang, C.; Liu, Y.; Fan, X.; Li, H. Characterization of a Novel, Cold-Adapted, and Thermostable Laccase-like Enzyme with High Tolerance for Organic Solvents and Salt and Potent Dye Decolorization Ability, Derived from a Marine Metagenomic Library. *Frontiers in Microbiology* **2018**. https://doi.org/10.3389/fmicb.2018.02998.