HPLC

1. HPLC setup

Goal: Run multiple HPLCs, some with our positive control laccase *T. versicolor* incubated with diclofenac and some with the *E. Coli/Chlamy* produced and purified enzymes

Methods: HPLC recorded at 276nm using RP18 column running at a flow rate of 1ml/min. Our methods were modeled off Hahn *et al.* 2018.

Gradient: Methanol (eluent A) and 0.1 % phosphoric acid (eluent B), starting from an initial ratio of 10 % A and 90 % B and reaching 100 % methanol within 14 min. Elution with methanol was continued for a further 6 min.

Column used:

- Endcapped LiChroCART 125-4 RP18-Column
- Particle size: 5 µm
- Pore Size (Å): 100
- Column length: 125 mm
- Inside diameter: 4 mm

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Preparing the Gradient:

Needed:

- 1 L of 0.1 % phosphoric acid (Eluent B)
- 1 L of 100 % Methanol (Eluent A)
- 1 L of MilliQ – Water

Have:

- 85 % phosphoric acid
- 100 % Methanol
- MilliQ - Water

$$c_1 * v_1 = c_2 * v_2$$

$$v_1 = \frac{c_1}{c_2} * v_2$$

$$v_1 = \frac{0.1\ \%}{85\ \%} * 1000\ ml$$

$$v_1 = 1.176\ ml \text{ phosphoric acid}$$

- Add 1.176ml of 85% phosphoric acid under a ventilation hood. Fill the phosphoric acid up to 1 L with MilliQ – Water.
- If every solution is prepared in 1 L, then you can start with filtrating the solutions (If the Methanol is an analysis solution you don´t need to filtrate it!).
- Using a sterile filter unit for each solution and be sure that every filter is clean.
- Screw tight each filter on top of a 1 L glass bottle, connect the filter with a vacuum pump to increase the filter speed and pour the respective solution onto the filter.

- After filtering the solutions, the solutions needed to be degassed. Put the bottles with the solutions into an ultrasonic bath with ice for 15 minutes and place the lid loosely on the bottle neck so that the gases can escape.
- Then tighten the lid, take the bottles out of the bath, dry them outside and avoid bumps and shakes.
- The solutions are now prepared for the HPLC.
- Run machine with solutions to prepare the column overnight before beginning the HPLC.

~~1.~~2. Preparation of stock solutions

a) Preparing diclofenac stock:

Our original ABTS assays had a concentration of 0.25 mM and Mehmood (2015) recommended this concentration as optimal concentration to visualize the spectra, so we aimed for this concentration of diclofenac in each reaction to compare effectiveness of the assay.

Diclofenac MW 318.13 g/mol

79.5325 µg/ml = 0.25mM

Initial stock should be 4mg/ml due to solubility

- 20 mg Diclofenac powder were added to 5 ml water making 4 mg/ml concentrated stock solution.
- Because the solutions will be added in a ratio of 1:1 for the Enzyme:Substrate, its important to create a working concentration double that of the final sample concentration needed. As the final sample will be 80 µg/ml, our working concentration will be 160 µg/ml (see Table 1 for comparisons of diclofenac and *T. versicolor*).

b) Preparing the Enzyme stock solution

0.5 U/mg enzyme written on the bottle
1 u = 1 µmol/min

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

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

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

3. Setup of an enzyme test:

- Prepare the working concentration from stock solutions
- Mix working concentrations to the final desired concentration

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This dilution will be added 1:1 to create a reaction, total volume of 400 µl (200 µl working concentration Diclofenac + 200 µl working concentration Laccase).

Table 1: Concentrations for stock, working, and final.

	Final desired concentration	Stock (solubility is 0.004 g/ml)	Working concentration (1:1 in final step)
Diclofenac	80 µg/ml	4 mg/ml	160 µg/ml
Laccase (*T. versicolor*)	100 µg/ml	20 mg/ml	200 µg/ml

a) Laccase dilution:

$\frac{20mg/ml}{100} = \frac{0.2mg}{ml} \rightarrow 200\ \mu g/ml$ (first dilution we want)
To do a dilution row, calculate for the concentrations you want the amount of enzyme from the stock solution you need:

We decided to measure an enzyme concentration of 100 µg/ml (1790 µM) due to it reaching optimal saturation within an hour in our ABTS tests.

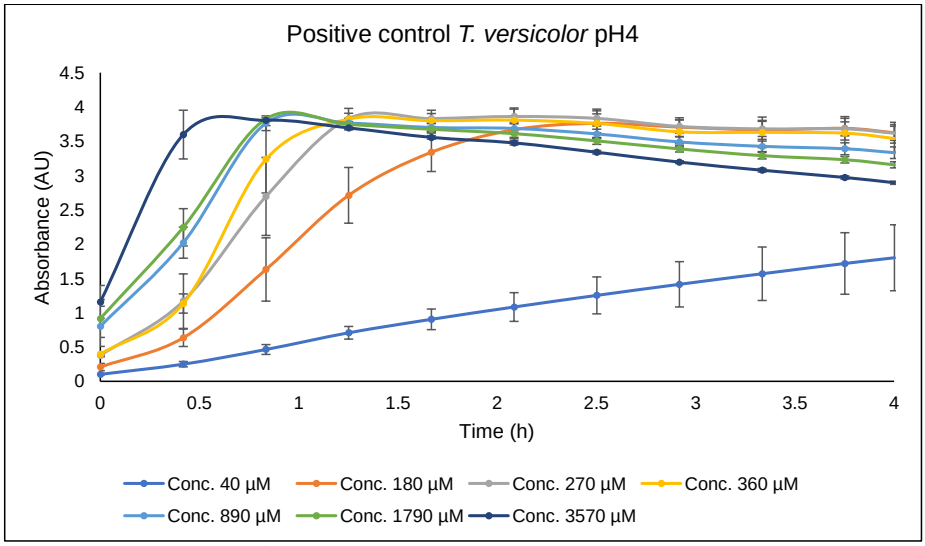

Fig. 1: ABTS assay data for positive control *T. versicolor* at pH 4.
Formula: $Dilution factor * Dilution\ concentration\ to\ use = Volume\ to\ use$

Calculation:
Concentration 100 µg/ml: $0.5 * 200 =\ \ 100\ \mu l\ from\ 200$

Laccase stock is diluted in pH 7 buffer prepared in ABTS assay, then in corresponding pH 4 buffer as needed during dilutions.

b) Diclofenac dilution:

To produce the dilution level needed to achieve 80 µg/ml of diclofenac, a working concentration of 160 µg/ml was needed initially from the 4 mg/ml diclofenac stock solution created previous.

To get this concentration it is again the Want over Have formula:

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

Calculation: $\frac{160\ \mu g/ml}{4000\ \mu g/ml} * 1\ ml = 40\ \mu g/ml$

Take 40 µl of the diclofenac 4mg/ml stock solution and add it to 960 µl pH 4 buffer to obtain the correct working dilution of 160 µg/ml diclofenac.

4. HPLC runs:

Diclofenac was tested at 3 different levels (10, 40 and 80 µg/ml) first to identify the correct level of concentration, then the optimal level will be added to 100 µg/ml of bought Laccase.

Items needed for 1 HPLC assay:

- Dilution of diclofenac (80 µg/ml determined by our experiments)
- Dilution of laccase (either positive control *T. versicolor* or produced enzyme)
- Running buffer 0.1 % phosphoric acid

Steps to prepare sample for injection:

- Take Laccase sample (either *T. vesicolor* or laccase purified from *E. coli/ C. Reinhardtii*) and dilute it to correct amount to be tested (see *T. versicolor* positive control dilution steps above).
- Then we will add 200 µl of the prepared 200 µg/ml T. versicolor laccase working concentration (or corresponding made laccase) and 200 µl of 160 µg/ml diclofenac working concentration in a 1:1 ratio at the optimal pH (*T. versicolor* at pH 5, BaLac pH 4, marLac pH 7). Final concentration 100 µg/ml T. versicolor - 80 µg/ml diclofenac. If performing a diclofenac control, substitute the laccase for pH 4 buffer.
- This will be left to sit and incubate at room temperature for the duration of its designated incubation period.
- At stopping point, heat sample to 95°C in preset hotplate to denature proteins and inactivate reaction. Centrifuge for 5 min at 13000 rpm. Remove supernatant and filter with 0.2 µm filter and syringe.
- Add 50 µl of filtered sample to 250 µl running buffer 0.1 % phosphoric acid and mix thoroughly.
- Clear HPLC line with at least 600 µl pure running buffer, then load 300 µl mixed sample and running buffer into the machine.
- Sample will be run in HPLC for 20 minutes or until complete and measured at 276nm.

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References

Hahn, V. *et al.* (2018) 'Enhanced laccase-mediated transformation of diclofenac and flufenamic acid in the presence of bisphenol A and testing of an enzymatic membrane reactor', *AMB Express*, 8(1), p. 28. doi: 10.1186/s13568-018-0546-y.

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