1. Hydrolase enzyme activity

Chemicals used:

Substrates (stored in -20 °C freezer)

- β-glucosidase (4-methylumbellyferyl-β-D-glucopyranoside - **MUF**G)
- cellobiohydrolase (4-methylumbellyferyl-N-cellobiopyranoside – **MUF**C)
- phosphatase (4-methylumbellyferyl-phosphate – **MUF**P)
- leucin aminopeptidase (L-leucine-7-amido-4-methylcoumarin – **AMC**L)
- chitinase (4-methylumbellyferyl-N-acetglucosaminide – **MUF**N)

Solutions (stored at room temperature)

- Dimethyl sulfoxidase (**DMSO**)
- Ultrapure water (**MilliQ- H_2O**)
- Distilled water

Standard/substrate preparation:

- Prepare the stock solutions of substrates (in DMSO; see the table below). Allow substrates to dissolve at room temperature (you will need to shake the tubes for some time or use shaker for few minutes).
- Make dilutions of the stock solutions, using ultrapure water (see the table below).
- Store stock solution (stable 3 months) and dilution series in the dark (covered in the aluminium foil), in the cold room or refrigerator.
- Stock solutions solidify in cold - put them into lukewarm water to melt them

Stock solutions:				Dilutions:		
standards (calibration)	concentration	substrate wt	solution added	**5 uM/L (1)**	**25 uM/L (5)**	**50 uM/L(10)**
MUF	(5 mM)	0.00880 g	+ 10 ml DMSO	0.1 ml, add H2O to 100 ml	0.5 ml, add H2O to 100 ml	0.5 ml, add H2O to 50 ml
AMC	(5 mM)	0.00875 g	+ 10 ml DMSO	0.1 ml, add H2O to 100 ml	0.5 ml, add H2O to 100 ml	0.5 ml, add H2O to 50 ml
substrates	concentration	substrate wt	solution added	**250 uM/L (50)**	**500 uM/L (100)**	**1500 uM/L (300)**
MUFG	(2.5 mM)	0.01692 g	+ 20 ml DMSO	2 ml + 18 ml H2O	4 ml + 16 ml H2O	12 ml + 8 ml H2O
MUFC	(2.5 mM)	0.02503 g	+ 20 ml DMSO	2 ml + 18 ml H2O	4 ml + 16 ml H2O	12 ml + 8 ml H2O
MUFP	(2.5 mM)	0.01281 g	+ 20 ml DMSO	2 ml + 18 ml H2O	4 ml + 16 ml H2O	12 ml + 8 ml H2O
MUFN	(2.5 mM)	0.01897 g	+ 20 ml DMSO	2 ml + 18 ml H2O	4 ml + 16 ml H2O	12 ml + 8 ml H2O
AMCL	(2.5 mM)	0.01624 g	+ 20 ml DMSO	2 ml + 18 ml H2O	4 ml + 16 ml H2O	12 ml + 8 ml H2O

Sample preparation:

- Samples are stored in the -20°C freezer. Take the samples that you will use today and let them melt for few minutes.
- Weigh 0,5 g of sample into 100 ml glass beakers.
- Add 50 ml distilled H_2O (if there are bigger pieces break them with the spoon and mix it)
- Put 4 samples into the sonication bath and sonicate them for 4 min – this will release the enzymes into the solution.
- Filter the samples into plastic dish using the small kitchen sifter. Insert magnet (these samples can be stored for max. 8 hours in cold room)

Test of the optimal concentration:

We test the optimal concentration of substrates at the beginning of the measurement sample series. We need to know the concentration in which enzymatic activities are the highest. We test first sample of each treatment as a representative sample (type of soil, plant dominants, interference, season ...).

- 1 representative sample = one black 96-well microplate.
- Pipette 50µl of each substrate/standard solutions and blanks on a **black microplate**, follow the outline below (black microplates can be used 4 times). Use multichannel pipette.
- Store the plate in dark at room temperature until ready to add the samples.
- Mix the sample using the magnet.
- Pipette 200 µl of sample into separate wells on a black microplate (follow the outline below). When pipetting, mix the samples properly by sucking the samples in and out few times.
- Cover the plate (aluminium foil), let it incubate in dark and measure the fluorescence in the given intervals.

	1	2	3	4	5	6	7	8	9	10	11	12
MUFG	50uM	50uM	50uM	50uM	100uM	100uM	100uM	100uM	300uM	300uM	300uM	300uM
MUFC	50uM	50uM	50uM	50uM	100uM	100uM	100uM	100uM	300uM	300uM	300uM	300uM
MUFP	50uM	50uM	50uM	50uM	100uM	100uM	100uM	100uM	300uM	300uM	300uM	300uM
AMCL	50uM	50uM	50uM	50uM	100uM	100uM	100uM	100uM	300uM	300uM	300uM	300uM
MUFN	50uM	50uM	50uM	50uM	100uM	100uM	100uM	100uM	300uM	300uM	300uM	300uM
MUF	H2O	H2O	H2O	1uM	1uM	1uM	5uM	5uM	5uM	10uM	10uM	10uM
AMC	H2O	H2O	H2O	1uM	1uM	1uM	5uM	5uM	5uM	10uM	10uM	10uM
Control + H2O	MUFG 50 uM	MUFG 300uM	MUFC 50 uM	MUFC 300 uM	MUFP 50 uM	MUFP 300 uM	AMCL 50 uM	AMCL 300 uM	MUFN 50 uM	MUFN 300 uM	H2O	H2O

Pipetting:

- 50µl of each of the substrate/standard solutions (rows)
- 200 µl of sample (columns), row H – distilled water - mix it
- Change tips for every concentration, substrate and column

Application on microplates:

- Measure 4 samples on 1 plate, use 4 plates (4 samples x 4 plates = 16 samples).
- At the beginning prepare 16 samples, optimal concentration of substrates and standards.
- Pipette 50µl of each substrate/standard solutions and 200 µl of sample and mix it (follow the outline below).
- Change tips for every concentration, substrate and each sample (=every 4th column).
- Cover the plate, let it incubate in dark and measure the fluorescence in the given intervals.

	Sample 1			Sample 2			Sample 3			Sample 4		
A	MUFG	MUFG	MUFG	MUFG	MUFG	MUFG	MUFG	MUFG	MUFG	MUFG	MUFG	MUFG
B	MUFC	MUFC	MUFC	MUFC	MUFC	MUFC	MUFC	MUFC	MUFC	MUFC	MUFC	MUFC
C	MUFP	MUFP	MUFP	MUFP	MUFP	MUFP	MUFP	MUFP	MUFP	MUFP	MUFP	MUFP
D	AMCL	AMCL	AMCL	AMCL	AMCL	AMCL	AMCL	AMCL	AMCL	AMCL	AMCL	AMCL
E	MUFN	MUFN	MUFN	MUFN	MUFN	MUFN	MUFN	MUFN	MUFN	MUFN	MUFN	MUFN
F	H2O	H2O	H2O	H2O	H2O	H2O	H2O	H2O	H2O	H2O	H2O	H2O
G	MUF5	MUF25	MUF50	MUF5	MUF25	MUF50	MUF5	MUF25	MUF50	MUF5	MUF25	MUF50
H	AMC5	AMC25	AMC50	AMC5	AMC25	AMC50	AMC5	AMC25	AMC50	AMC5	AMC25	AMC50

Fluorescence measurement:

- Measure fluorescence in the given time intervals:
    1. T0 = 30 min after pipetting
    2. T1 = 60 min after T0 (1,5 h after pipetting)
    3. T2 = 60 min after T1 (2,5 h after pipetting, 120 min after T0)

Spectrophotometer settings:

- switch on the computer and the spectrophotometer (spektrofotometr TECAN Infinite F200)
- programme „ENZYMY“.
- workbook „MUF bez cyklu.mdfx“, with the following parameters:

Plate: [NUN96fb_LumiNunc FluoroNunc] Nunclon 96 Flat Black
Plate with cover *no*
Fluorrescence Intensity:
    Excitation: 360(35) nm
    Emission: 465(35) nm
Mode: Top
Integration
    Lag time: 0 us
    Integration time: 20 us
Multiple Reads per Well: *no*
Read
    Number of flashes: 10
    Settle time: 0 ms
Gain:
    Manual: 32

Calculation and results:

- The excel workbook converts the fluorescence values into units of enzyme activity.
- Enzyme activity is expressed in nmol/h/g DW.
- For calculation of testing samples use excel workbook MUSTR_koncentrace and for measuring samples with known optimal concentration use MUSTR_MUF.

2. Phenol oxidase and peroxidase (lignolytic enzymes) activity

Chemicals used:

Substrate (stored in room temperature)

- L-DOPA (L-3,4-dihydroxyphenylalanine)

Solutions

- acetate buffer – pH 5
- H_2O_2 (stored in refrigerator)
- Ultrapure water (**MilliQ- H_2O**)

Preparation:

- Buffer: 1,5 ml of 100% acetic acid (bottle in digestor), add milli-q water to 500 ml, adjust pH with concentrated NaOH solution
    - Stability: 3 months
- L-DOPA: Prepare a 25 mM solution of L-DOPA by adding 0,246 g of L-DOPA compound to 50 ml of buffer. Use sonication bath covered with aluminium foil to fully dissolve all compound (more than 30 minutes). Store in the cold room or refrigerator.
    - Stability: max 5 days (this amount is sufficient for 2 measurements)
- H_2O_2: 0,25 ml of 30% H_2O_2 (bottle in refrigerator), add milli-q water to 25 ml.
    - Stability: max 5 days
- These 3 solutions store in the cold room or refrigerator.
- Samples – use the filtrates left from the Hydrolase enzyme activity (stored in the cold room).

Application on microplates:

- We can measure 6 samples on 1 plate (we have prepared 16 filtrates).
- Use 4 deep plates – need to be balanced on centrifuge

Pipetting (DEEP plate):

- 20µl of H_2O_2
- 100 µl of buffer
- 100 µl of L-DOPA
- 400 µl (2 x 200) of sample

	1	2	3	4	5	6	7	8	9	10	11	12
A	DOPA	DOPA+H2O2	DOPA	DOPA+H2O2	DOPA	DOPA+H2O2	DOPA	DOPA+H2O2	DOPA	DOPA+H2O2	DOPA	DOPA+H2O2
B	DOPA	DOPA+H2O2	DOPA	DOPA+H2O2	DOPA	DOPA+H2O2	DOPA	DOPA+H2O2	DOPA	DOPA+H2O2	DOPA	DOPA+H2O2
C	DOPA	DOPA+H2O2	DOPA	DOPA+H2O2	DOPA	DOPA+H2O2	DOPA	DOPA+H2O2	DOPA	DOPA+H2O2	DOPA	DOPA+H2O2
D	DOPA	DOPA+H2O2	DOPA	DOPA+H2O2	DOPA	DOPA+H2O2	DOPA	DOPA+H2O2	DOPA	DOPA+H2O2	DOPA	DOPA+H2O2
E	buffer	buffer +H2O2	buffer	buffer +H2O2	buffer	buffer +H2O2	buffer	buffer +H2O2	buffer	buffer +H2O2	buffer	buffer +H2O2
F	buffer	buffer +H2O2	buffer	buffer +H2O2	buffer	buffer +H2O2	buffer	buffer +H2O2	buffer	buffer +H2O2	buffer	buffer +H2O2
G	buffer	buffer +H2O2	buffer	buffer +H2O2	buffer	buffer +H2O2	buffer	buffer +H2O2	buffer	buffer +H2O2	buffer	buffer +H2O2
H	buffer	buffer +H2O2	buffer	buffer +H2O2	buffer	buffer +H2O2	buffer	buffer +H2O2	buffer	buffer +H2O2	buffer	buffer +H2O2

20µl of H_2O_2

	1	2	3	4	5	6	7	8	9	10	11	12
A		H2O2		H2O2		H2O2		H2O2		H2O2		H2O2
B		H2O2		H2O2		H2O2		H2O2		H2O2		H2O2
C		H2O2		H2O2		H2O2		H2O2		H2O2		H2O2
D		H2O2		H2O2		H2O2		H2O2		H2O2		H2O2
E		H2O2		H2O2		H2O2		H2O2		H2O2		H2O2
F		H2O2		H2O2		H2O2		H2O2		H2O2		H2O2
G		H2O2		H2O2		H2O2		H2O2		H2O2		H2O2
H		H2O2		H2O2		H2O2		H2O2		H2O2		H2O2

100 µl of buffer – do not touch the liquid (=you can use the same tips)

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E	buffer	buffer	buffer	buffer	buffer	buffer	buffer	buffer	buffer	buffer	buffer	buffer
F	buffer	buffer	buffer	buffer	buffer	buffer	buffer	buffer	buffer	buffer	buffer	buffer
G	buffer	buffer	buffer	buffer	buffer	buffer	buffer	buffer	buffer	buffer	buffer	buffer
H	buffer	buffer	buffer	buffer	buffer	buffer	buffer	buffer	buffer	buffer	buffer	buffer

100 µl of L-DOPA – do not touch the liquid (= you can use the same tips)

	1	2	3	4	5	6	7	8	9	10	11	12
A	DOPA	DOPA	DOPA	DOPA	DOPA	DOPA	DOPA	DOPA	DOPA	DOPA	DOPA	DOPA
B	DOPA	DOPA	DOPA	DOPA	DOPA	DOPA	DOPA	DOPA	DOPA	DOPA	DOPA	DOPA
C	DOPA	DOPA	DOPA	DOPA	DOPA	DOPA	DOPA	DOPA	DOPA	DOPA	DOPA	DOPA
D	DOPA	DOPA	DOPA	DOPA	DOPA	DOPA	DOPA	DOPA	DOPA	DOPA	DOPA	DOPA
E												
F												
G												
H												

400 µl (2 x 200) of sample - mix it (when adding the second 200µl sample), change tips for each column

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6
B	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6
C	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6
D	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6
E	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6
F	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6
G	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6
H	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6

- Cover the finished plate with parafilm, check that the weights of the plates are close to equal (< 1 g difference).
- Centrifuge for few (8s) seconds (drops on the walls will blend with all the liquids)
- Mark the time into lab diary.
- Store the plate in dark at room temperature to incubate for 18h.

After 18 hours:

- Centrifuge the plates, 8 min, 2500 rpm

Pipetting (SHALLOW transparent microplate):

- Transfer 250µl (2x125) of solution from DEEP plate – into SHALLOW transparent microplate, be careful not to take any of the solid particles. Check before that the shallow plates are clean and without bad scratches.

Fluorescence measurement:

- Measure the fluorescence - workbook „DOPA.mdfx“, with the following parameters:

Plate: [NUN96fw_LumiNunc FluoroNunc] – Nunclon 96 Flat White
Plate: Part of Plate: celá
Shaking:
    Parameter
        Duration: 10 sec
        Mode: Linear
        Amplitude: 1 mm
        Frequency: neaktivní
Wait (Timer)
    Timer

Wait time: 00:00:10 (hh:mm:ss)
Options: neaktivní

Absorbance

Wavelength

Measurement: 450(10) nm
Reference: ne

Multiple Reads per Well: ne

Read

Number of flashes: 5
Settle time: 0 ms

Calculation:

- The excel workbook converts the absorbance values to units of enzyme activity.
- Enzyme activity is expressed in nmol/h/gDOM.
- For calculation of measuring samples use excel workbook MUSTR_DOPA.