Uppsala iGEM 2019

Proof of concept Protocols

Enzyme assays

1. FOX-Assay

Purpose:
The oxidase that was used in the lab, was able to produce hydrogen peroxide. In order to measure the amount of the enzyme, an assay was performed to detect the amount of hydrogen peroxide that the enzyme produced. The assay is modified from Thermo Fisher. The presence of H_2O_2 allowed for detection of whether the desired oxidase was present. In this assay, hydrogen peroxide oxidizes ammonium ferrous (Fe^{2+}) ions into ammonium ferric (Fe^{3+}) ions. The ammonium ferrous ions were in a H_2SO_4 solution. This would be tested using a solution of sorbitol and xylenol orange.
Method:
Working reagent (WR) was made of:
Reagent A (25mN ammonium ferrous(II) sulfate and 2.5 M H_2SO_4)
Reagent B (100mM sorbitol, 125 µM xylenol orange in water)
The WR was made of 1 volume Reagent A and of 100 volume Reagent B (1:100). 1 mL of WR was prepared for each sample. The samples were prepared in cuvettes by adding 10 volumes of WR to 1 volume of sample, for example the ratio 1 mL of WR was added to 100 µL of sample. The sample that was used was undiluted supernatant from *P. Pastoris*. The cuvettes with the samples and WR was incubated in room temperature for 20 min and then measured in a spectrophotometry at 560 nm.

2. ABTS-assay

Purpose:
The peroxidase horseradish peroxidase is able to break down parts of the lignin, so a substrate was needed that had phenolic rings and could resemble lignin. The substrate used was 2,2'-Azino-bis(3-Ethylbenzthiazoline-6-Sulfonic Acid) (ABTS). The method is a continuous spectrophotometric assay. The assay is modified from Sigma.
Method:
Reagent 1: Prepare a 100 mM Potassium Phosphate buffer, pH 5.0, by making a 13.6 mg/mL solution in purified water using Potassium Phosphate, Monobasic. Adjust the pH of this solution to pH 5.0 using 1M KOH.
Reagent 2: (substrate) Prepare 9.1 mM ABTS by making a 5.0 mg/mL solution in Reagent 1 using ABTS. Check the pH of this solution and adjust to pH 5.0 as necessary. Prepare Fresh.

Reagent 3: Prepare a 0.3% (w/w) Hydrogen Peroxide Solution (H_2O_2) from a stock solution of hydrogen peroxide. Prepare the solution in purified water using Hydrogen Peroxide 30% (w/w) Solution. Prepare Fresh.
Reagent 4: (diluent) Prepare a 40 mM Potassium Phosphate Buffer with 0.25% (w/v) Bovine Serum Albumin and 0.5% (v/v) Triton X-100, pH 6.8.
The sample was undiluted for the *P. Pastoris* produced enzymes and diluted 1:200 for the bought HRP.

Tabel 1: Reagent and setup for the ABTS assay

Reagent	Test	Blank
Reagent 2 (substrate)	2.9 mL	2.9 mL
Reagent 3 (H_2O_2)	0.1 mL	0.1 mL
Reagent 4 (diluent)	-	0.05 mL
Enzyme sample	0.05 mL	-

Put the reagent 2 into the cuvette and let the substrate incubate in room temperature for 5 min. Then add reagent 3 and reagent 4 or the enzyme, depending on what sample is measured. Make the measurement of the sample in the cuvette, in a spectrophotometry for 3 minutes at 405 nm.

Calculate the units/mg solid with:

$$\frac{\Delta A_{405\,nm}/min(test) - \Delta A_{405nm}/min(blank) * 3.05 * DF}{36.8 * 0.05}$$

DF=Dilution factor of enzyme
3.05= Final volume(in milliliters) of reaction
36.8=millimolar extinction coefficient of oxidized ABTS at A405 nm
0.05= volume (in milliliters) of enzyme used

3. Manganese Peroxidase

Purpose:
The manganese peroxidase was needed to be tested in order to see whether it was active.
Method:
A reaction mixture of 25mM lactate, 0.1 mM $MnSO_4$, 1 mg BSA, 1 mg phenol red and 0.2 mL of culture filtrate from the yeast in 20 mM sodium acetate buffer(pH 4.5) into a total volume of 2 mL. The reaction was started in the cuvette with addition of 0.1 M H_2O_2 and stopped after one minute. The assay was measured at 610 nm, a blank was also performed at the same wavelength but without enzyme mixture.

4. Enzymatic assay for Manganese Peroxidase

Purpose:
The assay is designed to measure the oxidation of ABTS in order o get the laccase and MnP activity.
Method:
The assay is performed with a spectrophotometry at 420 nm. A 0.5 mM ABTS solution in 50 mM sodium acetate (pH 5.0). The MnP was mixed with 0.5 mM ABTS, 0.05 mM H_2O_2, 0.16 mM $MnCl_2$, in 40 mM sodium citrate buffer (pH 4.5), the enzyme sample is 100 µL in a 1 mL final volume. The cuvette was filled with the samples and run in a spectrophotometry at 420 nm.

Lignin assays

5. Fractionation of Lignin

Purpose:
In order to use organic solvents to degrade or “melt” the lignin and because of the lignins complex structure, a fractionation is needed. An organic media is used to make the lignin groups that are similar in properties, stick together and therefore become a new fraction. Kraft Lignin is the shape of the lignin as it is when it reaches the end of line at a paper-mill, for example. Fractionation was in addition carried out in preparation for detection of degradation using size exclusion chromatography, which was not used in the end.

Method:
Methanol sample: 2 grams of Kraft Lignin was mixed with 20 mL methanol. The solution was stirred for 2 hours, at 500 rpm, and then centrifuged for 5 minutes in 4000 rpm. The supernatant was saved for the next step.
The exact same setup was prepared but was run through a level 1 filter-paper. The supernatant was saved for the next step.

Acetone fraction: The pellet from the earlier centrifuge step was mixed with 20 mL of acetone and then stirred for 2 hour, at 500 rpm, and then centrifuged for 10 min at 4000 rpm. Supernatant and pellet was saved separately.

The pellet from the filtered fraction was mixed with 20 mL of acetone and then stirred for 2 hours, at 500 rpm. The mix was then filtered through a level 3 filter-paper. The pellet and supernatant was saved separately.

6. Native-PAGE

Purpose:
For the detection whether the lignin had been degraded or not several different methods were used. The Native-PAGE was a first step in figuring out how to detect the lignin. Because the lignin doesn't resemble proteins, a gel without SDS was considered a good start.
Method:
Sample buffer(2X)
49µL 1.5 M tris HCl, pH 8.8
50µL 25% glycerol
1µL 1% bromophenol Blue
All was mixed into eppendorf tube.
When the buffer was used, it was diluted 1:1 with ddH_2O

Running buffer(10X):
275 mL ddH_2O
125 mL Tris HCl pH8.8
100 mL glycerol (1.92M)
All was mixed a diluted to 1X when need to run the gel.
When the buffer was used, it was diluted 1:10 with ddH_2O

The Native-PAGE
Stacking gel:
4.275 mL 0.375 M Tris-HCl pH 8.8
0.67 mL Acrylamide/Bis-acrylamide(30%)
0.05 mL 10% ammonium persulfate
5 µL TEMED

Separating gel 10%
3.4 mL Acrylamide/Bis-acrylamide(30%)
6.49 mL 0.375 Tris-HCl ph8.8
100 µL 10% ammonium persulfate
10 µL TEMED

First, the separating gel was added to the glass plates held by the casting frame. 200 µL Isopropanol was on top of the gel in order to make the line straight. When the gel had polymerized the stacking gel was added on top and the comb was added to the gel. The glass was added into the chamber for the gel to run in and the samples was added. 1X running buffer was added to the chamber before it was closed. A voltage of 80V was the

initial voltage for the stacking gel and when the samples had reached the separating gel the volt was increased to 120 V. The samples ran until they reached the end of the glass.

7. SDS-PAGE

Purpose:
For the detection whether the lignin had been degraded or not several different methods were used. The SDS-PAGE was the second step in the gel usage to find out whether the lignin had been degraded or not. Several SDS-PAGE:s were done.
Method:
Separation gel:
Table 2: Separation gel with specifik percent

	20 %	18 %	15 %	10 %
ddH_2O	0.59 mL	1.334 mL	2.29 mL	4.1 mL
acrylamide/bis (30%)	6.7 mL	6.066 mL	5 mL	3.3 mL
Tris-HCl (1.5 M, pH 8.8)	2.5 mL	2.5 mL	2.5 mL	2.5 mL
SDS (10%)	100 µL	100 µL	100 µL	100 µL
TEMED	10 µL	10 µL	10 µL	10 µL
APS	100 µL	100 µL	100 µL	100 µL

The stacking gel that was used for all of the gels were a 4 % gel.

Stacking gel for 4%:
6.1 mL ddH_2O
1.3 mL Acrylamide/bis (30%)
2.5 mL Tris-HCl (0.5M pH6.8)
100 µL SDS (10%)
100 µL APS (10%)
10 µL TEMED

First the separating gel was added to the glass plates held by the casting frame. 200 µL Isopropanol was on top of the gel in order to make the line straight. When the gel had polymerized the stacking gel was added on top and the comb was added to the gel. The glass was added into the chamber for the gel to run in and the samples was added. 1X running buffer was added to the chamber before it was closed. A voltage of 80V was the initial voltage for the stacking gel and when the samples had reached the separating gel the volt was increased to 120 V. The samples ran till they reached the end of the glass.

8. Fractionating lignin in high pH

Purpose:
According to some articles, lignin is able to be degraded when exposed to a high pH.

Method:
A 0.5 M NaOH was prepared with 5.99 grams of solid NaOH in 300 mL ddH_2O.
2 grams of Kraft Lignin was mixed with 0.5M NaOH and stirred overnight.
Some of the pellet from the acetone fraction was also mixed with 0.5M NaOH.

9. Degrading lignin with HRP extracted from horse-radish

Purpose:
In order to see whether we could degrade lignin with our enzyme, a number of experiments were setup with different compounds. This experiment had a phosphate buffer to match the buffer that the HRP extracted from the horse-radish from the local supermarket (ICA). The samples were tested with a nanodrop and Platereader Tecan.
Method:
2 grams of sulfonated lignin was mixed with 20 mL of ddH_2O.
The following setup was made.

Tabell 3: Sample setup for the degradation of lignin with HRP in phosphate buffer.

Sample	Lignin	H_2O	Phosphate buffer	H_2O_2	Heat-inactivated enzyme	ICA HRP
Sample 1	100 µL	9.9 mL	-	-	-	-
Sample 2	100 µL	-	9.9 mL	-	-	-
Sample 3	100 µL	-	9.74 mL	-	0.16 mL	-
Sample 4	100 µL	-	9.74 mL	-	-	0.16 mL
Sample 5	100 µL	-	9.41 mL	0.33 mL	-	0.16 mL
Sample 6	100 µL	-	9.41 mL	0.33 mL	0.16 mL	-
Sample 7	100 µL	-	9.57 mL	0.33 mL	-	-

10. Sonicating lignin

Purpose:

We wanted to see if a change in the lignin could be achieved by using sonication as a method to change the lignin structure. Both the Kraft Lignin and the Sulfonated lignin was sonicated.

Method:

1 mL of lignin solution was mixed with 4 mL the correct buffer depending on the original solution. The fractionation with acetone had acetone and the fractionation with methanol had methanol and the solutions with water was mixed with water. When the lignin was being sonicated the amplitude was set to 50% and the pulse had a 5 second run and a 5 second pause that it alternated between. The lignin was sonicated for 30 min.

11. Degrading lignin with enzyme and Tris-buffer

Purpose:

In order to see whether we could degrade lignin with our enzyme, a number of experiments were setup with different compounds. This experiment had a Tris-HCl buffer to match the buffer that the HRP extracted from the horse-radish from the local supermarket (ICA). The samples was tested with nanodrop and Platereader Tecan.

Method:

2 grams of sulfonated lignin was added into 20 mL of ddH_2O. Then the following setup was made:

Tabel 4: Sample setup for degradation of lignin with HRP in Tris-HCl buffer

Sample	Tris-HCl	ICA HRP	Sigma HRP	Heat-inactivated ICA	Heat-inactivated Sigma	H_2O_2	Lignin
Sample 1	9.74 mL	-	160 µL	-	-	-	100 µL
Sample 2	9.74 mL	160 µL	-	-	-	-	100 µL
Sample 3	9.41 mL	-	160 µL	-	-	330 µL	100 µL
Sample 4	9.41 mL	160 µL	-	-	-	330 µL	100 µL
Sample 5	9.41 mL	-	-	-	160 µL	330 µL	100 µL
Sample 6	9.41 mL	-	-	160 µL	-	330 µL	100 µL
Sample 7	9.9 mL	-	-	-	-	-	100 µL

12. Degrading lignin with HRP with continuous addition of hydrogen peroxide

Purpose:
According to an article, continuous addition of hydrogen peroxide to the solution with enzyme could help the degradation of lignin. So this was done during a certain period of time. The reaction were detected in a full spectrum nanodrop analysis.
Method:
1 gram of sulfonated lignin was added to 10 mL of ddH_2O and stirred. HRP was added to a concentration of 0.03 mg/mL and hydrogen peroxide with a concentration of 90 µM was added every 120 minutes. The samples were stirred for 4 hours. To try the samples in the nanodrop, 1.5 µL of the sample from different timepoints was added to the nanodrop and measured in a full spectrum analysis.

13. Degrading lignin with HRP

Purpose:
To try different samples and to see whether the data could be affected by specific components in the sample.
Method:
The following setup was done in order to test the different components in the sample. Sample 2 would be the one with all the components in it.

Tabell 5: Sample setup of degradation of lignin with HRP with continuous addition of H_2O_2

Sample	Kraft Lignin	HRP	H_2O_2	ddH_2O
Sample 1	0.02 g	-	33 µL	9.67 mL
Sample 2	0.02 g	250 µL	33 µL	9.717 mL
Sample 3	0.02 g	-	-	10 mL
Sample 4	0.02 g	250 µL	-	9.75 mL

14. Lysation of yeast pellet with enzymes

Purpose:

One of the enzymes expressed by *Pichia Pastoris* was supposed to be secreted. When we didn't see any enzyme activity in the supernatant we suspected that the enzymes where still in the yeast cells, therefore we wanted to lyse the cells.

Method:

The pellet from the Pichia Pastoris samples were sonicated with an amplitude of 50% and a pulse of 5 seconds and paus of 5 seconds. The cells were sonicated for 30 minutes. The sonicated cells were vortexed with glass-beads and then centrifuged down for 10 minutes in 4000 rpm. The supernatant was then tested in the FOX-assay(see protocol for FOX-assay).