Laboratory Notebook

Figure 1. A picture of our laboratory.

July

5.7. Preparation of LBA plates
Present: Malin, Julia, Waltteri

8 LBA plates with 50 ug/ml kanamycin were made in 200 ml LB media (LB broth, BactoAgar). The plates were stored at +4°C.

6.7. *E. coli* transformation with empty pET36b and preparation of 1xBG11 media for autoclaving
Present: Malin, Julia, Waltteri

100 µl competent *E. coli* DH5α cells (from the bioenergy -80 °C freezer) were transformed with the empty pET36b(+) expression vector (Novagen). This was done to obtain a higher quantity of the plasmid for construct assembly.

1000 ml of 1xBG11 media was prepared for autoclaving based on the SBC protocol.
The DNA volume was 1 µl and the cell culture was placed in a water bath.
2 x about 80 ml MQ H_2O bottles were also prepared for the autoclavation, as well as tips in different sizes and microcentrifuge tubes.

7.7. Preparation of *E. coli* cultures for pET36b plasmid isolation

Present: Malin, Julia

A picture was taken of the transformation plates from 6.7.2021. Both plates contained hundreds of colonies (Figure 2).

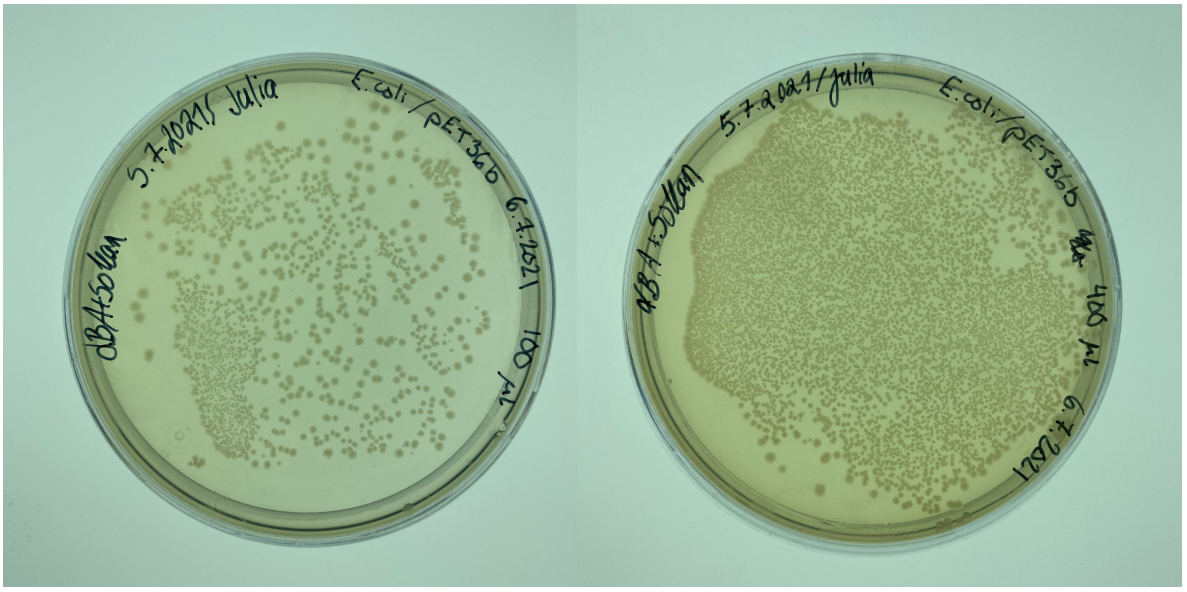

Figure 2. Plates containing *E. coli* DH5α cells that are transformed with the empty pET36b(+) expression vector.

Two single colonies were selected and marked on the plate on which 100 µl of cells were plated. 6 ml of LB media was added to two 12 ml culture tubes. Cells from the selected single colonies were inoculated into each tube, and the tubes placed at +37°C on 220 rpm shaker overnight. The next step is to isolate the pET36b plasmid from these two cultures.

8.7. Isolation of pET36b plasmids

Present: Malin, Julia

The empty pET36b plasmids were isolated from the cultures made on 7.7.2021 (see Preparation of *E. coli* cultures for pET36b plasmid isolation) using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. The elution was done with 40 µl water in the first elution round, and with 30 µl in the second round.
The DNA concentrations were measured using BioDrop as follows:

- after the first elution round:

sample 1: 84.08 ng/µl
sample 2: 47.52 ng/µl

- after the second elution round (only done for sample 1):
  sample 1: 17.79 ng/µl

The samples from the first elution round were combined. The DNA concentration of this combined sample was measured to be 62.75 ng/µl and the volume 72 µl. Hence, the obtained quantity of the pET36b plasmid is 4518 ng ~ 4.5 µg. This should be enough for construct assembly where the plan is to do 3 x 1 µg digestion reactions of the plasmid.

The combined sample and the small sample from the second elution round are saved and stored at RT in the lab.

9.7. Filtration of wastewater samples from Turun seudun puhdistamo
Present: Julia, Malin

The wastewater samples were taken on 8.7.2021. The sample was very separated so first we filtered the clear part of it through a filtering paper. The part from the wastewater containing most of the biomass was at the bottom of the sample. It was put in tubes, centrifuged and filtered through the filtering paper.

After filtering water through a filtering paper, syringe filtering was performed. The water was filtered by pouring it into a 60 ml filtering pipe (i.e. syringe) with a 0.2 µm filter. When 300 ml of water was filtered, the 0.2 µm filter detached from the filter pipe and approximately 15 ml of the unfiltered water contaminated the filtered water. A small 50 ml batch of the end product (i.e. filtered water) was saved and it was frozen. The filtered water was combined with the contaminated water and stored at +4°C for three days.

12.7. Filtration of wastewater samples from Turun seudun puhdistamo
Present: Jenni, Julia, Malin, Wade

The combined water (from 9.7.2021) was filtered through a 60 ml filtering pipe (i.e. syringe) with a 0.2 µm filter. A 500 ml sample was produced and it was frozen in an 1 l borosilicate bottle to be analyzed at a company we work with.

Figure 3. Wastewater samples. On the right side, there is an original sample with separated layers. In the middle, there is water filtered through a filtering paper. On the left side, there is the final product i.e. water which has been filtered through a filtering paper and syringe filtered.

13.7. Preparation of LBA plates with and without kanamycin and preparation of $MgCl_2$ and $CaCl_2$ solutions
Present: Julia, Laura, Malin

LBA plates were made without antibiotics. In addition, LBA plates with 285 µl kanamycin were made in 285 ml LBA. They were incubated at RT for an hour. After that, *E. coli* DH5α cells were added to plates which didn't contain antibiotics.

0.1M $MgCl_2$ and 0.1M $CaCl_2$ solutions were also made. For $MgCl_2$, 0.5050 g of it was added in a tube and then 25 ml MQ water was filtered through a 0.45 µl filter. For $CaCl_2$, 0.3683 g of it was added in a tube and then filtered 25 ml MQ water through a 0.45 µl filter.

15.7. *E. coli* transformation with laccase genes
Present: Julia, Malin

20 µl of MQ water was added to the plasmids pUCIDT-CotA, pUCIDT-CueO and pUCIDT-Yak (received from IDT). They were then vortexed and incubated at RT for 30 min. After that, the plasmids were transformed into *E. coli* DH5α cells by following the *E. coli* heat-shock transformation protocol.

19.7. Preparation of *E. coli* cultures for pUCIDT plasmid isolation
Present: Julia

Two single colonies were selected and marked on the plates with 100 µl of cells. 6 ml of LB broth (received from our advisor Hariharan Dandapani) and 6 µl of kanamycin were added into two 12 ml culture tubes. Cells from the selected single colonies were inoculated into each tube, and the tubes placed at +37°C on 220 rpm shaker overnight. The next step is to isolate the pUCIDT plasmid from these two cultures.

20.7. Isolation of pUCIDT plasmids and production of glycerol prep
Present: Julia

A glycerol prep that contained 200 µl of 50% glycerol and 800 µl culture including laccase plasmids was made. The glycerol preps were stored at -80°C.

The pUCIDT plasmids containing the laccase DNA were isolated from the cultures made on 19.7.2021 (see *E. coli* cultures for pUCIDT plasmid isolation) using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. The elution was done with 40 µl water.

The DNA concentrations were measured using BioDrop as follows:

pUCIDT-CotA-NdeI-XhoI: 764.5 ng/µl
pUCIDT-CotA-NsiI-XhoI: 579 ng/µl
pUCIDT-CueO-NdeI-XhoI: 323.9 ng/µl
pUCIDT-CueO-NsiI-XhoI: 375 ng/µl

pUCIDT-Yak-NdeI-XhoI: 495.8 ng/µl
pUCIDT-Yak-NsiI-XhoI: 436.8 ng/µl

Samples were saved and stored at RT in the lab.

27.7. Preparation of digestion samples
Present: Julia, Malin

Altogether 5 samples were made and two of them included a pET36b plasmid. The total volume of a mixup was 52 µl and it was made as follows:

- 2 µg plasmid / 1 µg pET36b plasmid
- 2 µl NdeI
- 2 µl XhoI
- 5 µl CutSmart Buffer (NEB)

Consequently, the total volume of plasmids is: 52 µl - 2 µl - 2 µl - 5 µl = 43 µl

The sample volumes based on concentrations were calculated as follows:

PLASMID	CONCENTRATION	VOLUME OF DNA	VOLUME OF MQ
CotA-NdeI-XhoI	764.5 ng/µl	2 µg / 0.7645 µg/µl = 2.6 µl	43 µl - 2.6 µl = 40.4 µl
CueO-NdeI-XhoI	323.9 ng/µl	2 µg / 0.3239 µg/µl = 6.2 µl	43 µl - 6.2 µl = 36.8 µl
Yak-NdeI-XhoI	495.8 ng/µl	2 µg / 0.4958 µg/ml = 4 µl	43 µl - 4 µl = 39 µl
pET36b	62.75 ng/µl	1 µg / 0.06275 µg/µl = 15.9 µl	43 µl - 15.9 µl = 28.1 µl (NB! calculating error)

The samples were incubated at 37°C for 4h, at 80°C for 20 min and then stored at 4°C overnight.

28.7. Separation and analysis of digestion samples
Present: Julia, Malin

A buffer (500 ml 1xTAE, 50 ml 10xTAE and the rest MQ) and 1,2 % agarose gel (65 ml 1xTAE buffer, 0.78 g agarose, 6.5 µl of SyBR Safe DNA gel stain 10 000x) for agarose gel electrophoresis were made.

10 µl of NEB purple loading dye 6x was added to the samples (done on 27.7.21). The gel chart can be seen below:

1	2	3	4	5	6	7	8	9	10	11	12	13
Ladder	1	1	2	2	3	3	Ladder	P1	P1	P2	P2	Empty

1. CotA-NdeI-XhoI, 2. CueO-NdeI-XhoI, 3. Yak-NdeI-XhoI, P1 = pET36b, P2 = pET36b

The gel was run at 50V for 90 min. After that, the DNA containing (numbers 1-3) plasmids were cut out of the gel and put in the freezer until ligation. The pET36b plasmids were put back to electrophoresis at 50V for two more hours. Finally, they were cut out as well and put in the freezer.

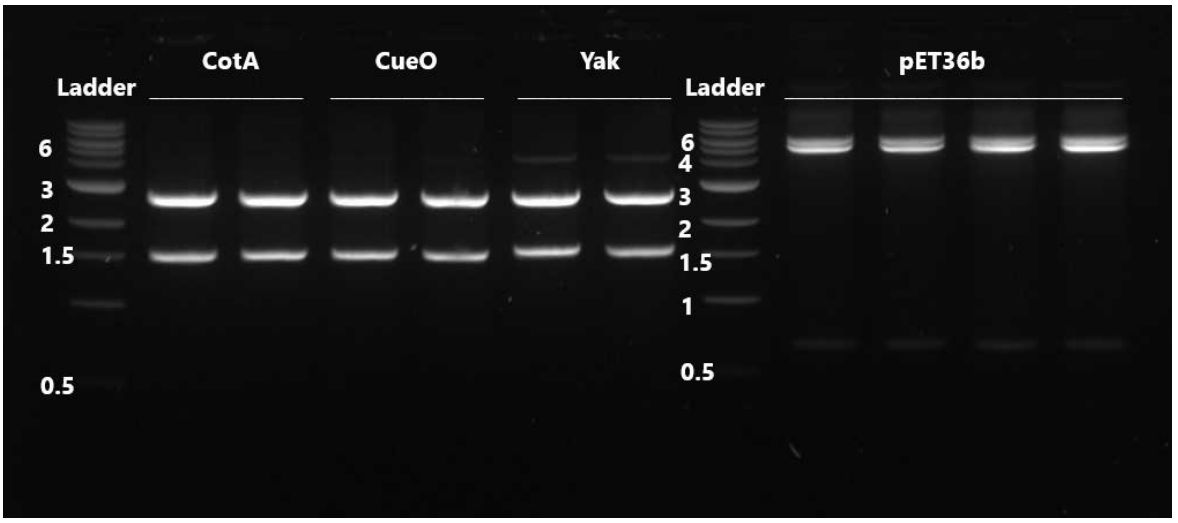

Figure 4. Agarose gel electrophoresis of the digestion products of the pUCIDT-Kan plasmids carrying cotA, cueO and yak, and pET36b(+) expression vector.

29.7. Gel extraction and ligation

Present: Julia, Malin

QIAquick® Gel Extraction Kit was used following the manufacturer's instructions to isolate DNA from a gel (made on 28.7.2021). The DNA concentrations of samples were measured using BioDrop. Two of the plasmid samples were combined because their concentrations were too low.

The concentrations measured using BioDrop:

- CotA:
  - m = 150 mg, c = 13.7 ng/µl
- CueO:
  - m = 180 mg, c = 10.56 ng/µl
- yak:
  - m = 190 mg, c = 15.77 ng/µl
- pET36b:
  - m1 = 160 mg, c1 = 7.946 ng/µl, m2 = 170 mg, c2 = 7.046 ng/µl, c = 8.695 ng/µl

Ligation samples were made in a 3:1 ratio. Volumes of DNA and a pET36b plasmid were calculated as follows:

- CotA:
    - m = (1542 / 5248) * 150 ng = 44.07393293 ng
    - V = m / 13.7 ng/µl = 44.07393293 ng / 13.7 ng/µl = 3.2170754 µl
- CueO:
    - m = (1551 / 5248) * 150 ng = 44.3311738 ng
    - V = m / 10.56 ng/µl = 44.3311738 ng / 10.56 ng/µl = 4.19802782 µl
- Yak:
    - m = (1602 / 5248) * 150 ng = 45.788872 ng
    - V = m / 15.77 ng/µl = 45.788872 ng / 15.77 ng/µl = 2.90354293 µl
- pET36b:
    - m = 50 ng
    - V = m / 8.695 ng/µl = 50 ng / 8.695 ng/µl = 5.75043128 µl

The samples were done as follows:

	CotA	C1	CueO	C2	yak	C3
Insert, µl	3.22	0	4.2	0	2.9	0
Vector, µl	5.75	5.75	5.75	5.75	5.75	5.75
T4 DNA Ligase Reaction Buffer, µl	2	2	2	2	2	2
T4 DNA Ligase, µl	1	1	1	1	1	1
mqH2O	8.03	11.25	7.05	11.25	8.35	11.25

C1, C2 and C3 = controls

The samples were incubated at 19°C for 16h, at 65°C for 10 min and then stored at 4°C.

2.8. Making of competent cells
Present: Julia, Malin

5 ml of LB media was put in growing tubes and 5 µl of ampicillin was added to pNiv(S3) tubes. *E. coli* BL21(DE3) cultures with antibiotics were also made. Tubes were incubated at 37°C on 220 rpm shaker overnight.

3.8. Making of competent *E. coli* BL21(DE3) cells, isolation of pNiv plasmids and digestion of *Synechocystis* plasmids
Present: Julia, Laura, Malin

pNiv(S3) cells (from 2.8.2021) were centrifuged and pellets were frozen. pNiv plasmids were isolated using QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions.

The pNiv concentrations were measured using BioDrop:
- After combining the two pNiv samples: 477.7 ng/µl

The DNA concentrations of CotA-NsiI and CueO-NsiI were measured using Biodrop on 20.7.21 (see Isolation of pUCIDT plasmids).

Three 50 µl digestion samples were made according to this:

- 2 µg DNA / 1 µg pET36b
- 2 µl NsiI
- 2 µl XhoI
- 2 µl BspHI
- 5 µl CutSmart Buffer (NEB)

Consequently, the total volume of plasmids is: 50 µl - 2 µl - 2 µl - 2 µl - 5 µl = 39 µl

The sample volumes based on concentrations were calculated as follows:

PLASMID	CONCENTRATION	VOLUME OF DNA	VOLUME OF MQ
CotA-NsiI	0.579 µg/µl	2 µg / 0.579 µg/µl = 3.5 µl	39 µl - 3.5 µl = 35.5 µl
CueO-NsiI	0.375 µg/µl	2 µg / 0.375 µg/µl = 5.3 µl	39 µl - 5.3 µl = 33.7 µl
pNiv	0.4777 µg/µl	1 µg / 0.4777 µg/µl = 2.1 µl	39 µl - 2.1 µl = 36.9 µl

Samples were run at 37°C for 4h, at 80°C for 20 min and stored at 4°C.

25 ml cultures with 23 ml LB media and 2 ml *E. coli* BL21(DE3) cells were made. They were grown in the incubator at 37°C on 220 rpm shaker.

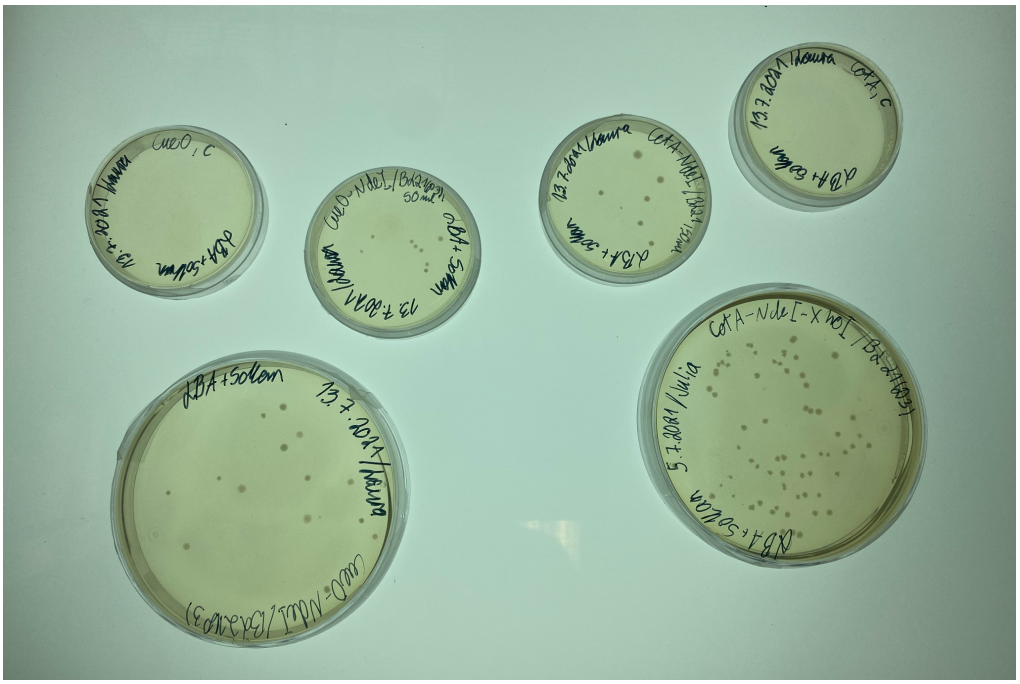

Figure 5. CueO-pET36b and CotA-pET36b plasmids in competent *E. coli* BL21(DE3) cells and control plates.

To determine the stage of growth, OD600 (i.e. the optical density of a sample measured at a wavelength of 600 nm) values of the samples were measured every 30 min until OD600 was between 0.4A - 0.5A. The value shouldn't rise above 0,5A.

The measured OD660 values:

- 0.160A
- 0.235A
- 0.453A

The samples were kept on ice for 30 min. Then, they were centrifuged for 2 min at 5000 rpm at +4°C. They were put on ice and the supernatant was discarded. Pellets were resuspended in 1 ml 0.1M $MgCl_2$ and transferred to a 1.5 ml microcentrifuge tube. They were centrifuged for 30s at 13000 rpm. The washing process was repeated twice. The pellets were resuspended in 100 µl 0.1M $CaCl_2$ and put on ice.

A transformation into the competent *E. coli* BL21(DE3) cells was started. This was done to CotA and CueO as well as their control reactions. 3 µl of the laccase-pET36b plasmid was added to the cells and they were incubated on ice for 30 min. After that, they were placed in +44°C for 30 s and then on ice for 2 min. 500 µl of LB media was added and the samples were incubated at 37°C on 220 rpm shaker for an hour.

50 µl of transformed cells were put on one LA plate and the rest of them on another LA plate. Then they were incubated at 37°C on 220 rpm shaker for 16 h.

4.8. Making and transformation of competent *E. coli* BL21(DE3) cells, digestion of Yak-NsiI plasmids and agarose gel electrophoresis of digestion samples
Present: Julia, Malin

One 50 µl digestion sample was done as follows:

- 2 µg Yak
- 2 µl NsiI
- 2 µl XhoI
- 2 µl PvuII
- 5 µl CutSmart Buffer (NEB)

Consequently, the total volume of Yak plasmid is: 50 µl - 2 µl - 2 µl - 2 µl - 5 µl = 39 µl

The sample volume of Yak-NsiI is calculated as follows:

- concentration = 0.579 µg
- DNA volume: 2 µg / 0.579 µg/µl = 4.6 µl
- volume of MQ: 39 µl - 4.6 µl = 34.4 µl

The samples were incubated at 37°C for 4h, 80°C for 20 min and left at 4°C.

1,5% agarose gel (65 ml 1xTAE, 0.98 g agarose, 6.5 µl SyBR Safe DNA gel stain 10 000x) was done for electrophoresis. 10 µl of NEB purple loading dye 6x was added to all digested samples.

A gel chart:

1	2	3	4	5	6	7	8	9	10	11	12	13
4	4	5	5	6	6	Ladder (100bp)	P11	P11	P21	P21	P31	P31

4. CotA-NsiI, 5. CueO-NsiI, 6. Yak-NsiI, P11, P21, P31 = pNiv

The samples were run at 50V for 100 min. All the samples were cut out and put in the freezer.

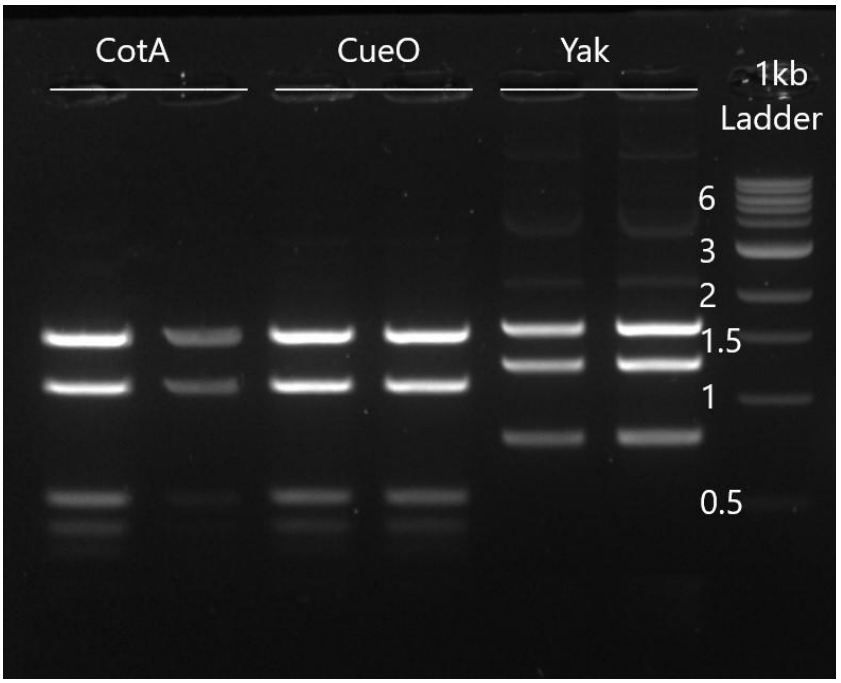

Figure 6. Agarose gel electrophoresis of the digestion products of the pUCIDT-Kan plasmids carrying *cotA*, *cueO* and *yak* (NsiI-XhoI).

New digestion samples were done for the addition of BspHI to the plasmids. The samples were done as follows:

- 1 µg (2.1 µl) pNiv
- 5 µl CutSmart Buffer (NEB)
- 1 µl NsiI
- 1 µl XhoI
- 40.9 µl MQ

Samples were incubated at 37°C for 4h, at 80°C for 20 min and stored at 4°C.

20 ml culture was made by mixing 18,5 ml LB media and 1,5 ml *E. coli* BL21(DE3) cells. The culture was incubated at 37°C on 220 rpm shaker and OD600 values were measured every 30 min until the values were between 0.4A-0.5A.

The measured OD600 values:

- 0.165A
- 0.224A

- 0.438A

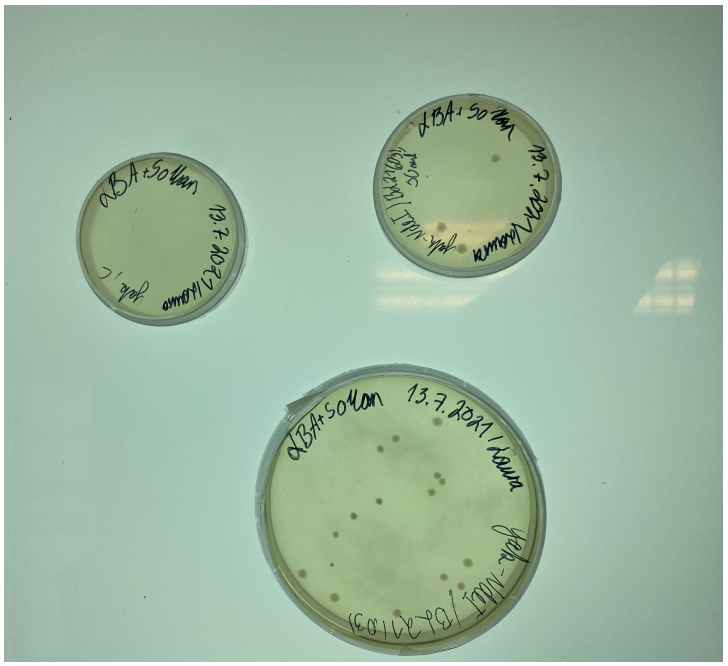

Figure 7. Yak-pET36b plasmids in competent *E. coli* BL21(DE3) cells and a control plate.

Samples were put on ice for 5 min. They were centrifuged for 2 min at 5000 rpm at +4°C and then put back on ice. The supernatant was discarded and the pellet was resuspended in 1 ml 0.1M $MgCl_2$ and transferred to a 1.5 ml microcentrifuge tube. Then the samples were centrifuged for 30 s at 13000 rpm. The washing process was repeated twice and the pellet was resuspended in 100 µl 0.1M $CaCl_2$, after which the tube was put on ice.

Transformation into the competent *E. coli* BL21(DE3) cells was started. This was done to Yak as well as to its control reaction. 3 µl of the yak-pET36b plasmid was added to the cells and they were incubated on ice for 30 min. After that, they were placed in +42°C for 45 s and then put on ice for 2 min. 500 µl of LB media was added and the samples were incubated at 37°C on 220 rpm shaker for an hour.

50 µl of transformed cells were put on one LA plate and the rest of them on another LA plate. Then, they were incubated at 37°C on 220 rpm shaker for 16h.

5.8. Gel electrophoresis of pNiv digested plasmids, extraction of NsiI and pNiv DNA from gel and ligation of pNiv plasmids and laccase DNA

Present: Julia, Malin

1,5% agarose gel (65 ml 1x TAE, 0,98 g agarose, 6,5 µl SyBR Safe DNA Gel Stain 10000x) was made according to a protocol. 10 µl of NEB purple loading dye was added to digested pNiv plasmids.

Gel chart:

1	2	3	4	5	6	7	8	9	10	11	12	13
			Ladder (100bp)	P11	P11	P21	P21	P31	P31			

(All are digested pNiv plasmids.)

The samples were run at 50V for an hour after which plasmids were cut at 2468 bp.

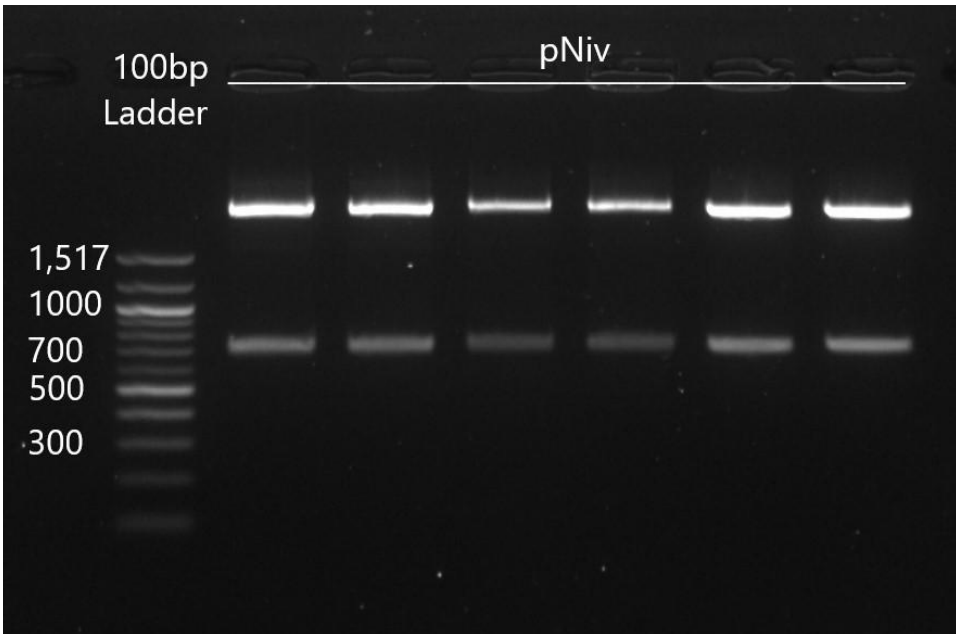

Figure 8. Agarose gel electrophoresis of the digestion products of the carrier plasmid pNiv(S3) (SpeI-SalI).

QIAquick® Gel extraction kit was used according to the manufacturer's instructions to extract DNA (from 3.8. and 4.8.) from a gel. The DNA concentrations were measured using Biodrop. Two of the pNiv plasmid samples were combined.

The concentrations measured using Biodrop:

- CotA:
  - m = 210 mg, c = 7.427 ng/µl
- CueO:
  - m = 220 mg, c = 15.08 ng/µl
- Yak:
  - m = 170 mg, c = 9.123 ng/µl
- pNiv:
  - m1 = 120 mg, m2 = 150 mg, m3 = 240 mg, c1 = 7.169 ng/µl, c2 = 11.93 ng/µl, c3 = 15.85 ng/µl, added p21 and p31 to each other c = 10.35ng/µl

Ligation samples were made in a 3:1 ratio. Volumes of DNA and a pNiv plasmid were calculated as follows

- CotA:
  - m = (1541 / 2468) * 150 ng = 93.6588331 ng
  - V = m / 7.427 ng/µl = 12.6105875 µl
- CueO:

    - m = (1550 / 2468) * 150 ng = 94.2058347 ng
    - V = m / 15.08 ng/µl = 6.24707127 µl
- Yak:
    - m = (1598 / 2468) * 150 ng = 97.1231767 ng
    - V = m / 9.123 ng/µl = 10.6459692 µl
- pNiv:
    - m = 50 ng
    - V = 50 ng / 10,35 ng/µl = 4.83091787 µl

The samples were done as follows:

	CotA	C4	Cue O	C5	yak	C6
Insert, µl	12.6	0	6.2	0	10.6	0
Vector, µl	4.8	4.8	4.8	4.8	4.8	4.8
T4 Ligase Reaction Buffer, µl	2	2	2	2	2	2
T4 DNA Ligase, µl	1	1	1	1	1	1
MQ, µl	0	12.2	6	12.2	1.6	12.2

C4, C5 and C6 = controls

Samples were incubated at 25°C for 1h, at 65°C for 10 min and stored at 4°C. After that, the samples were frozen.

9.8. Preparation of LBA plates with ampicillin and liquid cultures of both *E. coli* BL21(DE3) cells and *E. coli* DH5α cells

Present: Julia, Malin

11 LBA plates with 100 µg/ml ampicillin were made in 300 ml of LB media (LB broth, BactoAgar). The plates were stored in the fridge.

Liquid cultures of *E. coli* BL21(DE3) cells were made by adding 5 ml of LB media, 5 µl of ampicillin and three colonies (from plates made on 3.8. and 4.8.) to falcon tubes. Liquid cultures of *E. coli* DH5α cells were made similarly. The cultures were incubated at 37°C on 220 rpm shaker for 16 h.

10.8. Preparation of glycerol preps of *E. coli* BL21(DE3)-pET36b cells and isolation and measurement of the plasmids. Making of competent *E. coli* DH5α cells and their transformation with the ligation products.

Present: Julia, Malin

First glycerol preps of *E. coli* BL21(DE3)-pET36b cells were made. 800 µl of cells and 200 µl of glycerol were added to microcentrifuge tubes and put in -80°C freezer. The rest of the cells were centrifuged for 30s at 13000 rpm. Supernatant was discarded and the pellet was frozen.

Competent *E. coli* DH5α cells were made by adding 32 ml of LB media and 3 ml of *E. coli* DH5α cells to the erlenmeyer tube. To determine the stage of growth, OD600 values were measured every 30 min until the values were between 0.4A-0.5A but still under 0.5A.

The measured OD600 values:

0.237A
0.265A
0.443A

The $MgCl_2$ washing buffer was made by mixing 25 ml $MgCl_2$ ($m(MgCl_2)$=0.505g) with 25 ml MQ. After that, the buffer was filtered through a 0.45 µm filter. The rest was done following the Competent cell protocol and the *E. coli* heat-shock transformation protocol. The plates were incubated at 37°C for 16 h.

The plasmids were isolated by using the QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's instructions.

The DNA concentrations were measured by using BioDrop as follows:

- CotA-pET36b:
  - 237.3 ng/µl
  - 346.0 ng/µl
  - 530.1 ng/µl
- CueO-pET36b:
  - 299.9 ng/µl
  - 326.0 ng/µl
  - 204.3 ng/µl
- yak-pET36b:
  - 359.9 ng/µl
  - 587.9 ng/µl
  - 358.9 ng/µl

After that, the samples were put in the freezer.

11.8. Analysis of plates (from 10.8.), preparation of digestion samples of laccase-pET36b plasmids and preparation of liquid cultures of *E. coli* DH5α-laccase-pNiv cells.

Present: Julia, Malin

Plates done on 10.8. were analyzed. The Yak control plate was full of colonies which means that results are not reliable. The Yak 1 plate had also broken due to falling under the incubator so it was discarded. The CotA control plate also had a few colonies. (Figure 9.)

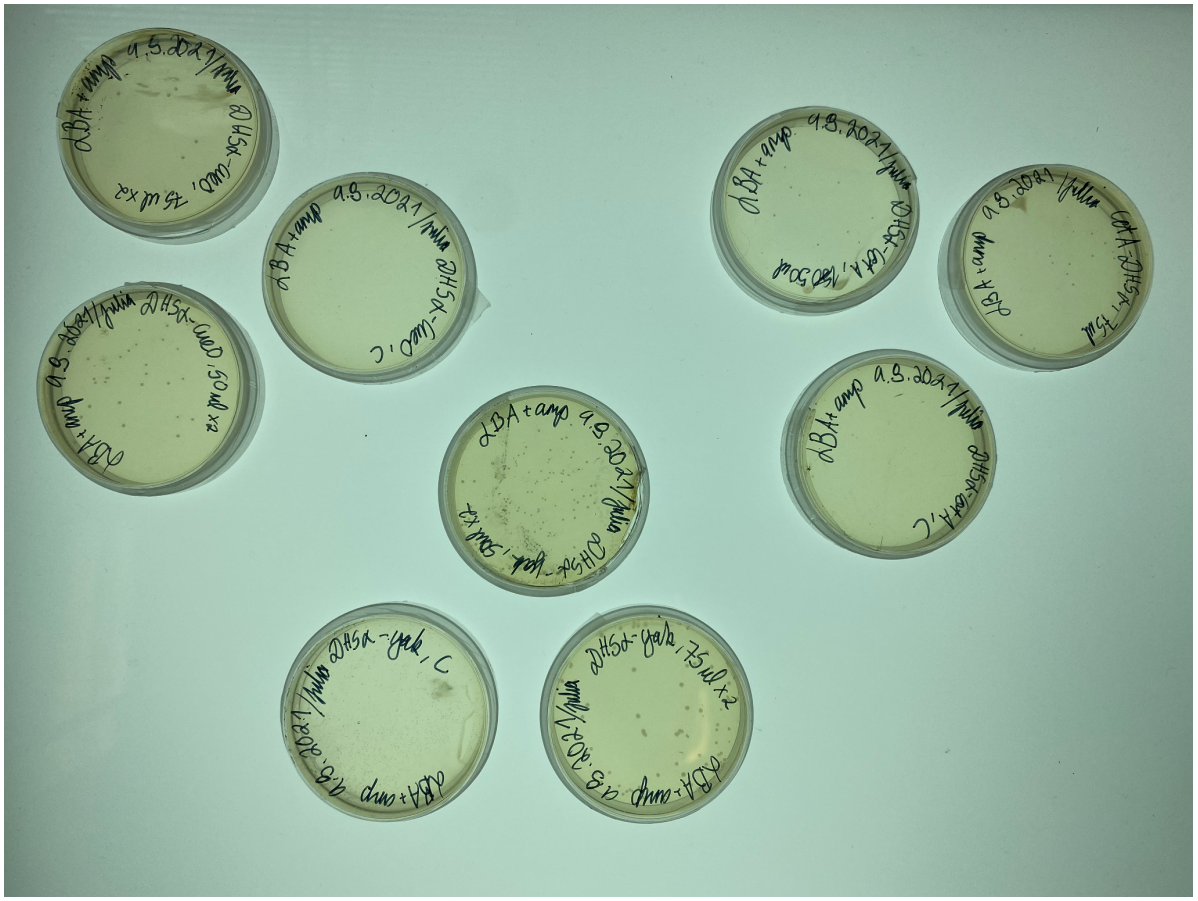

Figure 9. CotA-pNiv, CueO-pNiv and Yak-pNiv plasmids in competent *E. coli* DH5α cells and control plates.

50 µl digestion samples for CotA/CueO/Yak-pET36b plasmids were made as follows:

- 2 µg DNA
- 2 µl NdeI
- 2 µl XhoI
- 5 µl CutSmart Buffer (NEB)

Consequently, the total DNA value is: 50 µl - 2 µl - 2 µl - 5 µl = 41 µl

The DNA volumes of samples were calculated as follows:

PLASMID	CONCENTRATION	VOLUME OF DNA	VOLUME OF MQ
CotA-pET36b			
	237.3 ng/µl	2 µg / 237.3 ng/µl = 8.4 µl	41 µl - 8.4µl = 32.6 µl
	346.0 ng/µl	2 µg / 346.0 ng/µl = 5.8 µl	41 µl - 5.8 µl = 35.2 µl
	530.1 ng/µl	2 µg / 530.1 ng/µl = 3.8 µl	41 µl - 3.8 µl = 37.2 µl

CueO-pET36b			
	299.9 ng/µl	2 µg / 299.9 ng/µl = 6.7 µl	41 µl - 6.7 µl = 34.3 µl
	326.0 ng/µl	2 µg / 326.0 ng/µl = 6.1 µl	41 µl - 6.1 µl = 34.9 µl
	204.3 ng/µl	2 µg / 204.3 ng/µl = 9.8 µl	41 µl - 9.8 µl = 31.2 µl
Yak-pET36b			
	359.9 ng/µl	2 µg / 359.9 ng/µl = 5.6 µl	41 µl - 5.6 µl = 35.4 µl
	587.9 ng/µl	2 µg / 587.9 ng/µl = 3.4 µl	41 µl - 3.4 µl = 38.6 µl
	358.9 ng/µl	2 µg / 358.9 ng/µl = 5.6 µl	41 µl - 5.6 µl = 35.4 µl

Samples were incubated at 37°C for 4h, at 80°C for 20 min and left at 4°C.

Two colonies were chosen from 10.8. plates and they were incubated at 37°C on 220 rpm shaker for 16 h.

12.8. Check of pET36b-CotA/CueO/Yak plasmids, isolation of the plasmids from liquid cultures (made on 11.8.) and digestion of pDF-lac2 and pNiv-CotA/CueO/Yak plasmids.
Present: Julia, Malin

1.2% agarose gel (65 ml of 1xTAE, 0.78 g of agarose, SyBR Safe DNA Gel Stain 10000x) was made. 30 µl samples with NEB purple loading dye 6x were made.

Gel chart:

1	2	3	4	5	6	7	8	9	10	11	12	13
	Ladder	1.1	1.2	1.3	2.1	2.2	2.3	3.1	3.2	3.3	Ladder	

Wells 3-5 contained CotA, wells 6-8 contained CueO and wells 9-11 contained Yak.

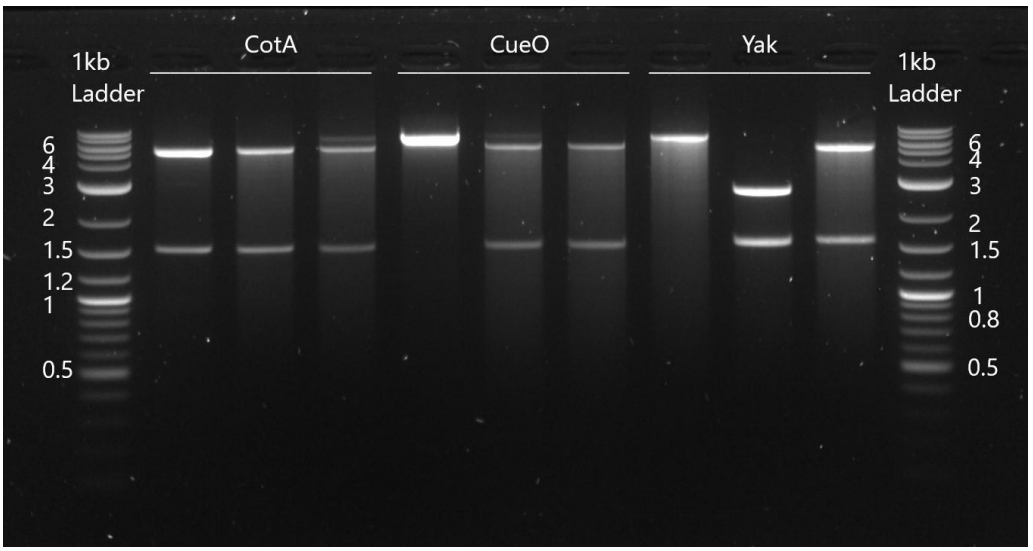

Figure 10. Agarose gel electrophoresis of the verification digestion of the pET36b-*cotA*, pET36b-*cueO* and pET36b-*yak* expression constructs. The expression constructs were digested with NdeI and XhoI restriction enzymes. The 1 kb DNA ladder (NEB) was used as a molecular weight standard, the DNA was colored with SyBR Safe DNA Gel Stain (ThermoFisher), and the gel was visualized under UV light. The agarose gel used was 1.2%.

NEB plasmid extraction kit was used to extract pNiv-CotA/CueO/Yak plasmids from a gel according to the manufacturer's instructions.

Concentrations of laccase constructs and pDF-lac2 were measured by using BioDrop as follows:

- CotA: 390.7 ng/µl
- CueO: 222.5 ng/µl
- Yak: 427.8 ng/µl
- pDF-lac2: 523.1 ng/µl

50 µl digestion samples were made as follows:

- 2 µg DNA/1 µg pDF-lac2
- 2/1 µl SalI
- 2/1 µl SepI
- 5 µl CutSmart Buffer (NEB)

Consequently, the total volume of DNA is: 50 µl - 2 µl - 2 µl - 5 µl = 41 µl

And for a pDF-lac2 plasmid: 50 µl - 1 µl - 1 µl - 5 µl = 43 µl

The DNA volumes of samples were calculated as follows:

PLASMID	CONCENTRATION	VOLUME OF DNA	VOLUME OF MQ
pNiv-CotA	390.7 ng/µl	2 µg / 390.7 ng/µl = 5.1 µl	41 µl - 5.1 µl = 35.9 µl
pNiv-CueO	222.5 ng/µl	2 µg / 222.5 ng/µl =	41 µl - 9 µl = 32 µl

		9 µl	
pNiv-Yak	427.8 ng/µl	2 µg / 427.8 ng/µl = 4.7 µl	41 µl - 4.7 µl = 36.3 µl
pDF-lac2	523.1 ng/µl	1 µg / 523.1 ng/µl = 1.9 µl	43 µl - 1.9 µl = 41.1 µl

Digestion was performed by incubating the samples at 37°C for 4h, at 80°C for 20 min and left at 4°C.

13.8. Gel electrophoresis of pDF-lac2 and laccase digestion products, extraction of samples from gel and preparation of ligation samples.
Present: Julia, Malin, Waltteri

1.5% agarose gel (65 ml 1xTAE, 0.98 g agarose, SyBR Safe DNA Gel Stain 10000x) was made. 60 µl samples were made and NEB purple loading dye 6x was added to them.

Gel chart:

1	2	3	4	5	6	7	8	9	10	11	12	13
	Ladder	1	1	2	2	3	3	pDF-lac2	pDF-lac2	pDF-lac2	pDF-lac2	Ladder

1. CotA, 2. CueO, 3. Yak

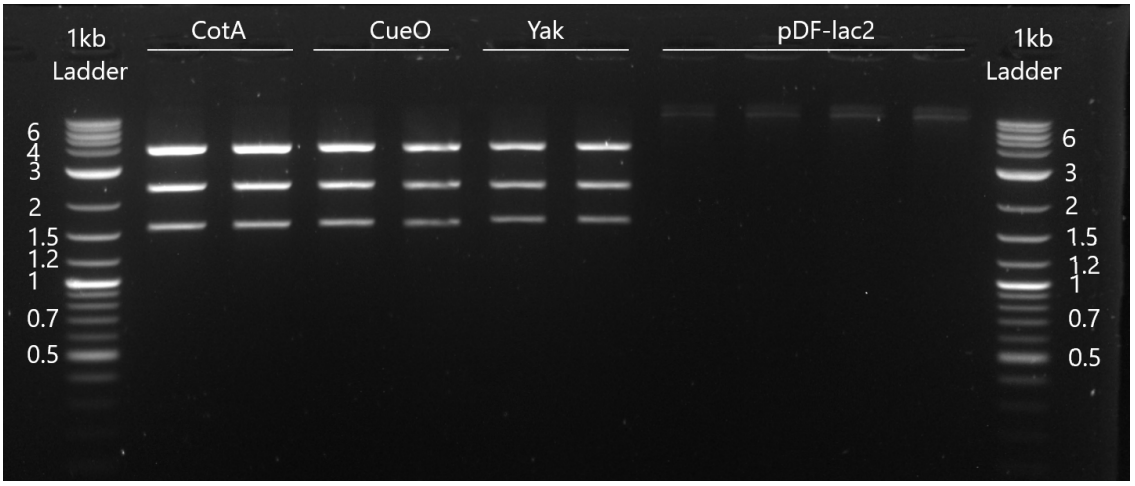

Figure 11. **Agarose gel electrophoresis of digestion products of the pNiv(S3)-cotA, pNiv(S3)-cueO, pNiv(S3)-yak and pDF-lac2 plasmids.** Wells 2 and 13 are NEB 1kb ladders. Wells 3 and 4 contain CotA, wells 5 and 6 contain CueO, wells 7 and 8 contain yak, and wells 9-12 contain pDF-lac2.

The samples (from 12.8.) were run at 50V for 80 min, after which the gel was pictured and gel pieces were cut and weighed. NEB gel extraction kit was used to extract DNA from a gel and the DNA concentrations of samples were measured by using BioDrop as follows:

- CotA: 26.55 ng/µl
- CueO: 24.96 ng/µl

- Yak: 15mg/µl
- pDF-lac2: 14.58 ng/µl

3:1 ligation samples were made as follows.The total sample volume was 10 µl.

	CotA	C1	Cue O	C2	Yak	C3
Insert, µl	1	0	1.1	0	not enough pDF-lac2	not enough pDF-lac2
Vector, µl	3.4	3.4	3.4	3.4		
T4 Ligase Reaction Buffer, µl	1	1		1		
T4, Ligase	1	1	1	1		
MQ, µl	3.6	4.6	3.5	4.6		

(C1, C2 and C3 are controls.)

The samples were incubated at 19°C for 16h, at 65°C for 10 min and left at 4°C.

16.8. Production of laccases
Present: Julia, Malin

pET36b-CotA/CueO/Yak plasmids containing glycerol preps were taken from -80°C freezer and plated. CotA 1, CueO 2 and Yak 3 were chosen and they were grown in the incubator at 37°C for 16h.

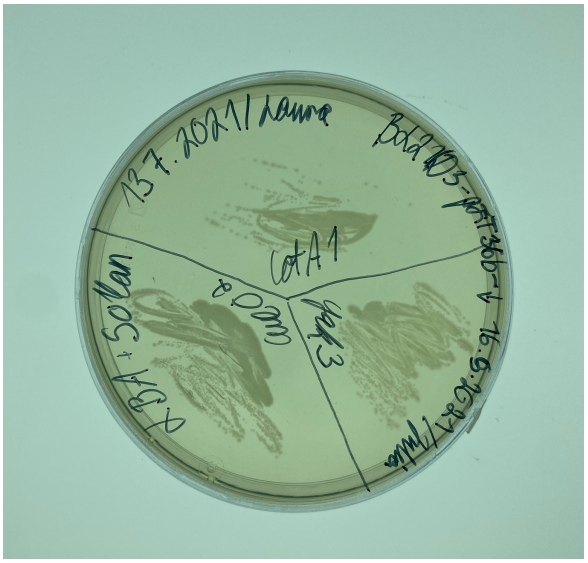

Figure 12. Grown *E. coli* BL21(DE3) cells with pET36b-CotA, pET36b-CueO and pET36b-Yak plasmids.

17.8. Production of laccases
Present: Julia, Malin

200ml of LB medium and 300ml of TRIS A buffer (20mM Tris-Cl, 150mM NaCl, imidazole 20mM, pH 7.5) were made.

Liquid culture from cells plated on 16.8.2021 was started. They were incubated at 37°C on 220 rpm shaker for 16 h.

18.8. Production of laccases
Present: Julia, Malin

400 µl kanamycin and 5 ml of cell culture were added to LB media (from 17.8.). The mixture was incubated at 37°C on 220 rpm shaker for an hour. The OD600 values were measured. After that, the cultures were put back to the incubator with the same settings and OD600 values were measured every 30 minutes until they were over 0.6A.

The measured OD600 values:

- CotA:
  - 0.080A
  - 0.169A
  - 0.458A
  - 0.604A
- CueO:
  - 0.371A
  - 0.154A
  - 0.350A
  - 0.554A
  - 0.783A
- Yak:
  - 0.298A
  - 0.203A
  - 0.411A
  - 0.671A

1M IPTG was made. There was some calculation error and 4 ml of IPTG was added instead of 400 µl. Because of that the cultures were discarded. The absorbance was not measured.

New liquid cell cultures were started from the cells plated on 16.8.2021. They were incubated at 37°C on 220 rpm shaker for 16h.

19.8. IPTG induction
Present: Julia, Malin

5 ml of liquid culture (made on 18.8.) was added to 300 ml of LB media and 300 µl of kanamycin. It was incubated at 37°C on 220 rpm shaker for an hour. The OD600 values were measured. After that the cultures were put back to the incubator with the same settings and OD600 values were measured every 30 minutes until they were over 0.6A.

The measured OD600 values:

- CotA:
    - 0.296A
    - 0.144A
    - 0.286A
    - 0.446A
    - 0.745A
- CueO:
    - 0.068A
    - 0.122A
    - 0.229A
    - 0.384A
    - 0.642A
- Yak:
    - 0.050A
    - 0.334A
    - 0.202A
    - 0.396A
    - 0.604A

300 µl of IPTG was added and the samples were put back to the incubator at 37°C on 220 rpm shaker for three hours. After that, the OD600 values were measured.

The measured OD600 values:

- CotA: 0.228A
- CueO: 0.204A
- Yak: 0.175A

The values indicate that the IPTG induced the growth of the cells. After measurements, the cell cultures were added to centrifuge bottles and centrifuged at 4000 x g for 10 minutes at +4°C. The supernatant was discarded and 15 ml of Tris A buffer was added to pellets. The samples were stored at -20°C.

20.8. Protein purification

Present: Julia, Malin

The cells were put in a sonicator for 4 x 30 s cycles to break cell membranes. The amplitude peak was between 8 and 12. After that, the samples were centrifuged at 20 000 rpm for 30 minutes at +4°C. Pellets were discarded and the supernatant volumes were measured.

The measured volumes:

- CotA: 31 ml
- CueO: 29 ml
- Yak: 29.5 ml

Protein purification was started by using affinity chromatography. A filter and 1 ml Ni2+ -affinity hartz (Ni Sepharose 6 Fast Flow, GE Healthcare) were added to the bottom of the column. Because the hartz is stored in ethanol, the ethanol was removed from the column by adding 3 CV (i.e. column volume) of MQ and balanced by adding 3 CV of Tris A buffer. After that, the samples were added to the column and the column was washed with 3 CV of Tris A buffer.

10 ml of B buffer (20 mM Tris-Cl pH 7.5, 150 mM NaCl, 300 mM imidazole) was made. 7 CV of B buffer was added to the column and the run through was collected to ten microcentrifuge tubes. After collection the hartz was washed with 10 ml MQ.

6.9. Measurement of laccase activity
Present: Malin, Julia, Waltteri

1/100, 1/50 and 1/33 dilutions were prepared of both purified protein and raw extract for Bradford assay, as well as ten BSA dilutions of known concentrations (10-100 µg/ml). The Bradford reagent was added and the absorbance was measured at 595 nm after 10 minutes.

Known concentrations' results were plotted to create a standard which was used to find out the correct dilution of proteins for the SDS-PAGE gel. Samples were prepared of raw extract and purified protein for the gel and the gel was run with previously mentioned samples and ladder at 180 V for about 30 minutes. The gel was then scanned. A prestained gel and the ladder (PageRuler Unstained Protein Ladder, ThermoFisher) were used for protein molecular weight confirmation.

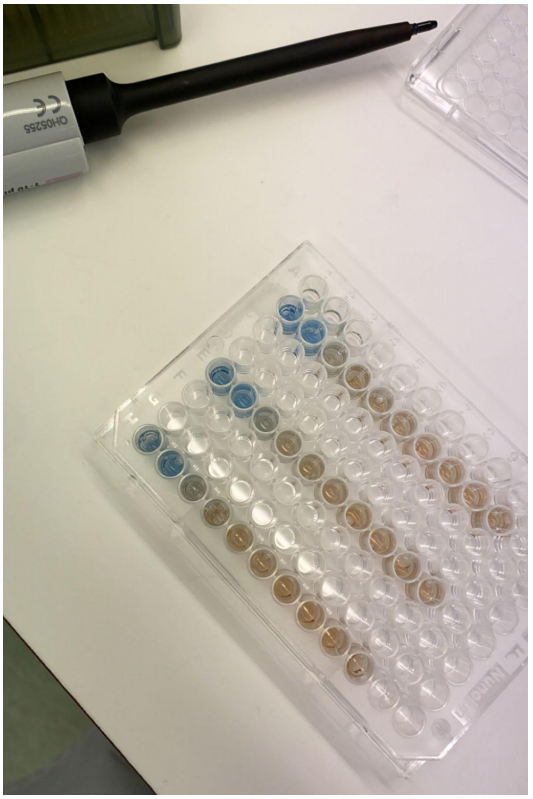

Figure 13. Bradford method results.

SDS-PAGE well chart:

1	2	3	4	5	6	7	8	9	10	11
CotA Raw extract	CueO Raw extract	Yak Raw extract	Ladder	Purified CotA	Purified CueO	Purified Yak	Ladder	Purified CotA	Purified CueO	Purified Yak

7.9. Measurement of laccase activity
Present: Malin, Julia, Waltteri

An ABTS assay was performed to measure laccase activities. First, ABTS was dissolved in water using manufacturer's recommendations. A paper test was performed to try to measure laccase activity. A filter paper was soaked to a mixture of ABTS (2 mM) and phosphate-citrate-buffer (100 mM, pH 4) and it was dried at 60°C. 10 µl of enzymes glycerol

preps were added and it was incubated at 30°C for 10 min. Measured absorbance at OD530.

A 96-well plate was done to test activity. The experiments were done in two different pHs of phosphate-citrate buffer (pH 5, orange, and pH 7, blue). The substrate (ABTS, blue) and enzyme (CotA and CueO, pink) concentrations/volumes were as follows:

		CotA	CotA	CotA	CotA	CotA	CotA	CueO	CueO	CueO	CueO	CueO	CueO	
pH		1	2	3	4	5	6	7	8	9	10	11	12	
5	A	0 mM	2 mM	4 mM	6 mM	8 mM	10 mM	0 mM	2 mM	4 mM	6 mM	8 mM	10 mM	No enz
5	B	0 mM	2 mM	4 mM	6 mM	8 mM	10 mM	0 mM	2 mM	4 mM	6 mM	8 mM	10 mM	1µl
5	C	0 mM	2 mM	4 mM	6 mM	8 mM	10 mM	0 mM	2 mM	4 mM	6 mM	8 mM	10 mM	5µl
5	D	0 mM	2 mM	4 mM	6 mM	8 mM	10 mM	0 mM	2 mM	4 mM	6 mM	8 mM	10 mM	10µl
7	E	0 mM	2 mM	4 mM	6 mM	8 mM	10 mM	0 mM	2 mM	4 mM	6 mM	8 mM	10 mM	No enz
7	F	0 mM	2 mM	4 mM	6 mM	8 mM	10 mM	0 mM	2 mM	4 mM	6 mM	8 mM	10 mM	1µl
7	G	0 mM	2 mM	4 mM	6 mM	8 mM	10 mM	0 mM	2 mM	4 mM	6 mM	8 mM	10 mM	5µl
7	H	0 mM	2 mM	4 mM	6 mM	8 mM	10 mM	0 mM	2 mM	4 mM	6 mM	8 mM	10 mM	10µ

It was noticed that the ABTS used was faulty and, consequently, all of the experiments with it failed. The ABTS should have been colourless at the beginning, and then changed to more greenish. However, our ABTS was yellow at the beginning so we concluded that it was already oxidized (Figure 14).

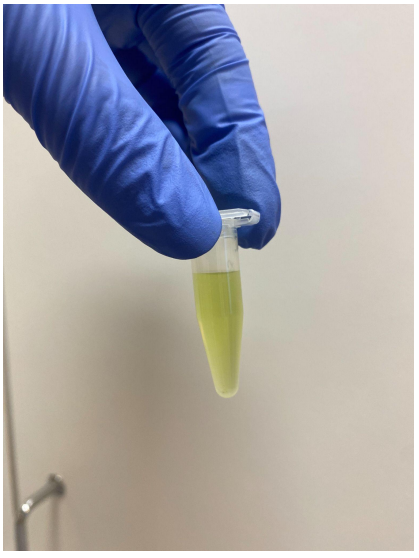

Figure 14. There was no relevant change in the colour of ABTS when compared to its colour at the beginning.

28.9. Measurement of laccase activity

Present: Julia and Malin

Due to a faulty ABTS, we decided to measure laccase activities with syringaldazine. The procedure is otherwise similar, but the absorbance is measured at 530 nm instead of 420 nm. First, 0.0778 mg of syringaldazine (0.216 mM) was dissolved in methanol. The potassium-phosphate buffer (0.1 mM) was made and pH was set at 6.5.

A similar 96-well plate was made as on 7.9. The substrate was synringadalzine and the control was a commercial SAE0050-laccase from *Aspergillus SP.*. The Potassium-phosphate

buffer was used. The substrate (syringaldazine, blue) and enzyme (CotA and CueO, pink) concentrations/volumes were as follows:

	Cont	Cont	Cont	Cont	CotA	CotA	CotA	CotA	CueO	CueO	CueO	CueO	
	1	2	3	4	5	6	7	8	9	10	11	12	
A	0 mM	5 mM	10 mM	20 mM	0 mM	5 mM	10 mM	20 mM	0 mM	5 mM	10 mM	20 mM	No enz
B	0 mM	5 mM	10 mM	20 mM	0 mM	5 mM	10 mM	20 mM	0 mM	5 mM	10 mM	20 mM	1µl
C	0 mM	5 mM	10 mM	20 mM	0 mM	5 mM	10 mM	20 mM	0 mM	5 mM	10 mM	20 mM	5µl
D	0 mM	5 mM	10 mM	20 mM	0 mM	5 mM	10 mM	20 mM	0 mM	5 mM	10 mM	20 mM	10µl

(Cont = control)