Advanced Synthesis & Catalysis

Supporting Information

Supporting online information

Experimental

Modeling of *Pa*HNL5 substrate complexes

Based on the X-ray crystal structure of *Pa*HNL1[1] (PDB accession number: 1ju2), the structure of *Pa*HNL5 was modeled using the program Modeller v6.2[2], as described previously.[3,4]

Molecular models of **1a**, **(*R*)-2** and **(*S*)-2** were built and optimized using the program Sybyl v6.8 (Tripos Inc.). The substrates were docked into the active site of *Pa*HNL5 using the program AutoDock v3.0.[5] During the simulations, the protein was kept rigid, whereas the position and orientation of the substrates as well as four torsion angles were allowed to vary. A hybrid genetic algorithm[5] with phenotypic local search (designated as a Lamarckian genetic algorithm) was applied in both cases. A cluster analysis with an rmsd-cutoff of 1.5 Å was performed.

The resulting *Pa*HNL5 substrate complexes were further optimized using programs from the Amber suite.[6]

Site-saturation mutagenesis

Site-saturation mutagenesis libraries (F72X, V113X, V317X, V329X, L331X, L343X, V360X) were created by overlap extension PCR (see Figure S1) using template plasmid pGAPZA *Pa*HNL5[7] and partly complementary mutagenic primers containing a degenerated codon (N N (G/T)) at the desired site:

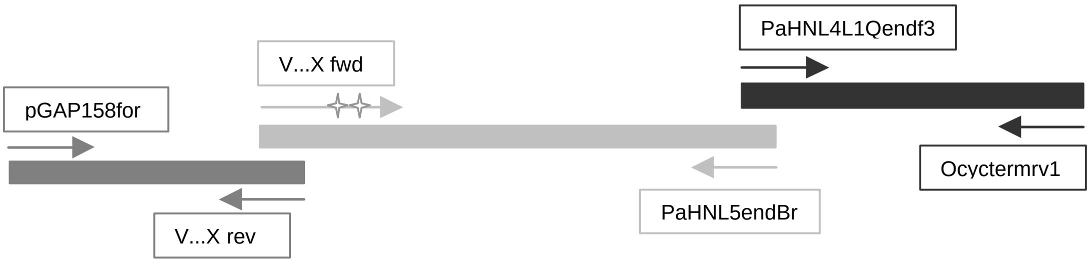

Figure S1: Strategy 2 - overlap extension PCR for site-saturation mutagenesis library construction.

Fragment amplification – PCRI, PCRII, PCRIII

The PCR was performed in 100 µL with 60 ng template plasmid pGAPZA *Pa*HNL5,[7] 0.2 mM dNTP, 40 pmol of each primer, 1.2 U Phusion High-Fidelity DNA Polymerase (Finnzymes) and 1x Phusion HF reaction buffer. The following program was used: 30 sec 98°C, 30 cycles (10 sec 98°C, 20 sec 60°C, 1 min 72°C) and a final extension at 72°C for 7 min. The template DNA was digested with *Dpn*I and the PCR product was purified applying the QIAquick PCR Purification Kit from Qiagen. The following primers were used:

PCRI:

pGAP158for: 5'-ccttctctctccttccacc-3'

F72Xrev: 5’-gattatatgcmnncccatctgcagtcaacgtgttcgg-3’

V113Xlongrev: 5'-gaatgaaatgttagctctggcgtamnngcctgcattgattatggtcgtgcc-3'

V317Xshortrev: 5'-aacagaggcttcaattggatttgg-3'

V329Xshortrev: 5'-ttgataataatcacttctaatgcctaaaacag-3'

L331Xrev: 5’-ggaaaatggcaagcttgamnnagaaacttgataataatcacttc-3’

L343Xrev: 5’-gtaagatgttgtaggaaamnnactaaagggtggagtgg-3’

V360Xshortrev: 5'-aatatgagcaaaagtcgaatttgg-3'

PCRII

F72Xfwd: 5’-ccgaacacgttgactgcagatgggnnkgcatataatc-3’

V113Xshortfwd: 5'-tacgccagagctaacatttcattc-3'

V317Xlongfwd: 5'-cccaaatccaattgaagcctctgttnnkactgttttaggcattagaagtgattattatcaag-3'

V329Xlongfwd: 5'-ctgttttaggcattagaagtgattattatcaannktctctgtcaagcttgccattttcc-3'

L331Xfwd: 5’-agtgattattatcaagtttctnnktcaagcttgccattttccac-3’

L343Xfwd: 5’-ccactccaccctttagtnnktttcctacaacatcttac-3’

V360Xlongfwd: 5'-ccaaattcgacttttgctcatattnnkagccaagttccaggaccattgt-3'

PaHNL5endBr: 5'-gactgagtgacctcgctatctgactcacatggactcttgaat-3'

PCRIII

PaHNL4L1Qendf3: 5'-gtcagatagcgaggtcactcagtccgaacaaaaactcatctcagaag-3'

Ocyctermrv1: 5'-tgctcacatgttggtctccagcttgc-3'

Overlap extension PCR for the construction of linear integration cassettes

Step 1: The PCR was performed in 50 µL with 45-60 ng of the PCRI product, 25-50 ng of the PCRII product, 25-30 ng of the PCRIII product, 0.2 mM dNTP, 1x Phusion HF reaction buffer and 0.6 U Phusion High-Fidelity DNA Polymerase (Finnzymes). The following program was used: 30 sec 98°C, 10 cycles (10 sec 98°C, 20 sec 60°C, 1 min 72°C) and a final extension at 72°C for 7 min.

Step 2: Reagents were added to the mixture of step 1 to get a final volume of 100 µL: 10 µl of the 5 × HF reaction buffer, 1 µL of a 10 mM dNTP-mix, 40 pmol of each primer (pGAP158for, Ocyctermrv1), 0.6 U Phusion High-Fidelity DNA Polymerase (Finnzymes). Then the following temperature program was used: 30 sec 98°C, 20 cycles (10 sec, 98°C, 20 sec 65°C, 1 min 72°C) and a final extension at 72°C for 7 min. The overlap extension PCR product was purified applying the QIAquick PCR Purification Kit or the QIAquick Gel Extraction Kit from Qiagen and then digested with *Xma*JI (*Avr*II) from Fermentas. The DNA was precipitated with ethanol, redissolved in nuclease-free water and used for *P. pastoris* X33 transformation (0.5-1µg/transformation). The cells were plated on YPDS plus zeocin plates according to the standard protocol from Invitrogen.

Cultivation of *Pichia pastoris* X33 transformants and screening for improved HPA (1a) conversion

*Pa*HNL5 variants were tested for their activity to convert **1a** into the corresponding **(*R*)-2**. Therefore, recombinant *Pichia pastoris* X33 strains secreting *Pa*HNL5 muteins were cultivated in 96-well deep well plates using 700 µL BMD7.5% medium per well (0.4 M potassium phosphate, pH 6.0; 13.4 g/L Yeast Nitrogen Base; 75 g/L D-glucose; 0.4 mg/L biotin; 25 µg/mL zeocin). The cultures were incubated at 28°C, 80% humidity and 320 rpm for 110-120 hours. Then, they were harvested through centrifugation (Centrifuge 5810 R, Eppendorf AG) using the following settings: 4000 rpm, 10 min, 4°C.

Conversion of HPA

The hydrocyanation reaction was then performed in 96-well deep well plates at pH 2.4 and room temperature: 400 µL of the culture supernatant were transferred to a clean deep well plate. 150 µL of a 3 M citrate phosphate buffer (pH 2.4) and 200 µL of a **1a**-stock solution were added. The **1a-**stock solution was prepared as followed: 1.2 mL of **1a** were added on 46.8 mL of a 3 M citrate phosphate buffer (pH 2.4). After that, 22 µL of a 12 M NaCN solution were added to start the reaction. To prevent HCN evaporation, deep well plates were finally covered with a SILVERseal^{TM} Sealer aluminium foil (Greiner Bio-One GmbH, Germany). To agitate the reaction solution, a magnetic stirrer for 96-well deep-well plates (Alligator Tumble Stirrer from V&P Scientific) was used together with one PTFE encapsulated stir stick (VP 734-2) per well. During the reaction, a frequency of 25 Hz was applied. After one hour, the reaction was stopped removing the silver seal foil and adding a 50% (v/v) solution of H_2SO_4 in H_2O. The addition of the acidic solution shifted the pH to pH < 1.0 removing the remaining HCN and preventing a continuing enzymatic reaction. The agitation frequency was set to 15 Hz and after the volatilization of HCN was finished, the reaction mixture was diluted 1:10 in 3 M citrate phosphate buffer (pH 2.4). All steps including the possible evaporation of HCN were carried out under a well ventilated hood.

Screening for conversion

The fluorimetric analysis was performed with the plate reader FLUOstar OPTIMA from BMG LABTECH GmbH applying the 485-P filter as excitation filter and the 520-P filter as emission filter. The fluorimetric reaction was carried out in 96-well PP-microplates (V-bottom, clear) from Greiner Bio-One GmbH. 10 µL of a 1:10 diluted reaction solution were mixed with 150 µL of a 3 M citrate phosphate buffer (pH 2.4). The conversion of NBDH to the corresponding hydrazone was started by adding 60µL of a NBDH/EtOH stock solution. (NBDH stock solution: 8 mg of 4-Hydrazino-7-nitrobenzofurazane (NBDH) were mixed with 50 mL of ethanol (99.8%) through sonication (15 min). The remaining solid NBDH was removed through centrifugation.) The increase in the strongly fluorescent hydrazone was tracked and the slope of this chemical reaction was evaluated. It could be directly correlated to the amount of aldehyde **1a** remaining in the reaction solution after a distinct reaction time.

Screening for enantiomeric excess

1a and the corresponding cyanohydrins **(*R*)-2** and **(*S*)-2** were extracted with *tert*-butylmethylether (TBME). Aliquots of the organic phase were transferred to a new 96-well PP-microplate (V-bottom, clear) and after derivatization with acetic anhydride in presence of pyridine and dichloromethane the enantiomeric purities were analyzed using a Hewlett-Packard 6890 instrument equipped with an FID and a Chirasil-DEX CB column (25 m x 0.32 mm, 0.25 µm film). The following settings were used:

Carrier gas: H_2; pressure: 1.0 bar.

Temperature program: 110°C – 0min, 10°C/min to 130°C, 20°C/min to 170°C, 170°C – 0.5 min.

FID detector: 250°C.

Retention times: **(*R*)-2**: 2.44 min; **(*S*)-2**: 2.55 min.

Improving enzyme expression I - Ligation into the *Pichia pastoris* expression vector pHILD2

*Pa*HNL5 genes of improved *Pa*HNL5 variants were amplified out of their genomic DNA by colony PCR. Genomic DNA preparation: A single colony of each mutant was resuspended in 50 µL Y-PER® from Pierce and incubated at 65°C for 10 min. Then, 60 µL of a Phenol/Chloroform/Isopropanol (25/24/1) mixture were added. After extraction and centrifugation, the water phase was separated and the DNA was precipitated with ethanol. The DNA pellet was washed twice with 70% ethanol, dried and dissolved in 50 µL water.

The colony PCR was performed in 100 µL with 0.2 mM dNTP, 40 pmol of each primer (pGAPZA484f, HNL5alp21), 2 µL of the genomic DNA preparation, 1.2 U Phusion High-Fidelity DNA Polymerase (Finnzymes) and 1x Phusion HF reaction buffer. The following program was used: 30 sec 98°C, 35 cycles (10 sec 98°C, 20 sec 60°C, 1 min 72°C) and a final extension at 72°C for 7 min. The PCR products were purified applying the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). After *Eco*RI digestion, the *Pa*HNL5 gene was ligated into the *Eco*RI linearized pHILD2 vector (behind the AOX1 promoter) and used for transformation of electrocompetent *E. coli* TOP10 F' cells from Invitrogen. Resulting clones were controlled by sequencing and correct DNA was used for *P. pastoris* GS115 transformation according to the standard protocol provided by Invitrogen. ~100 *Pichia pastoris* transformants were cultivated in 96-well deep well plates and the enzyme activity for the cleavage of mandelonitrile (benzaldehyde cyanohydrin) was assayed as described by Weis *et al.*[8] The most active clones were chosen for further characterization. Genomic DNA was isolated and the particular mutations were verified by sequencing the corresponding colony PCR products.

Enzyme preparation

Shake flask cultivation:

Enzyme was produced in 150 mL shake flask cultures: 135 mL BM0.5G medium (0.2 M potassium phosphate, pH 6.0; 13.4 g/L Yeast Nitrogen Base, 5 g/L glycerol, 0.4 mg/L biotin) were prepared in 2L baffled shake flasks and inoculated with single colonies. The cultures were shaken at 28°C, 80% humidity and 120 rpm. For induction, 15 mL BMM10 medium (0.2 M potassium phosphate, pH 6.0; 13.4 g/L Yeast Nitrogen Base, 50 mL/L methanol, 0.4 mg/L biotin) were added after ~60h. After further 10, 24 and 48h, 1.5 mL of pure methanol were added. After a total cultivation time of ~130 h, the cells were removed through centrifugation and the culture supernatant was concentrated approximately 20-fold through ultrafiltration (final protein concentration: ~ 1 mg/mL). Protein concentration was determined with the Biorad assay, using *Pa*HNL from Sigma (M-6782 Lot 41H4016) as reference.

5L-Bioreactor cultivation:

The strains *Pichia pastoris* GS115 pHILD2-*Pa*HNL5α[3] and *Pichia pastoris* GS115 pHILD2-*Pa*HNL5α–V317A, respectively, were cultivated in 5L bioreactors according to a 3-step procedure reported by Pscheidt *et al.*[9]: Briefly, biomass was first generated during exponential and second during linear growth. Finally, recombinant *Pa*HNL5 was produced during an expression phase using methanol for induction. After about 72-80 h of methanol induction, the cells were separated through centrifugation and the enzyme preparation was obtained. The culture supernatant was further concentrated through ultrafiltration (Sartorius, Vivaflow 50, 30kDa MWCO) and enzyme purification was accomplished by anion exchange chromatography using the ÄKTApurifier 10 FPLC from Amersham Biosciences UK Limited together with the anion exchanger Q Sepharose Fast Flow from GE Healthcare. The column was equilibrated with a 20 mM citrate phosphate buffer (pH 5.5), protein samples were loaded and for elution, a NaCl salt gradient was used (elution buffer: 20 mM citrate phosphate buffer + 1M NaCl, pH 5.5). Protein fractions with *Pa*HNL5 activity were collected, desalted, concentrated and used for further examination. Protein concentration was determined with the Biorad assay, using *Pa*HNL from Sigma (M-6782 Lot 41H4016) as reference.

Determination of enzyme activity and biocatalytic conversions - Materials

All solvents and materials except for **1a** and HCN were commercially available and purified if necessary.

1a was prepared according to [9]. Shortly, 78.0 g of a 37% aqueous solution of formaldehyde (0.961 mol) and 61.5 g (0.853 mol) of isobutyraldehyde were mixed and cooled at 0°C. Then, small portions of solid K_2CO_3 (122 g, 0.883 mol) were added so that the temperature of the reaction mixture did not exceed 20°C. After another 1.5 h, the mixture was brought to room temperature. Then, the viscous liquid was extracted with ether, dried over Na_2SO_4, concentrated and yielded in 87 g of crude product (99% yield), which slowly crystallized forming the dimer. The product was used without any further purification. NMR analysis showed a monomer/dimer mixture and the recorded data was consistent with the data reported by Upadhya *et al.*[10] and Toermaekangas *et al.*[11] Before application, the dimer was monomerized, heating it gently for 15 min at 80°C.

HCN formation:

The required amount of HCN was freshly prepared, dropping a saturated NaCN solution into aqueous sulfuric acid (60%) at 80°C. HCN was trapped at -12°C using a cooling trap. For continuous warning, an electrochemical HCN sensor was applied. All reactions with HCN or cyanides were performed under a well-ventilated hood.

General procedure for the synthesis of racemic cyanohydrins:

Weakly basic ion-exchange resin (Amberlyst A-21) and freshly generated HCN (5 equiv.) were added to a solution of aldehyde (5%) in TBME. The reaction mixture was stirred at room temperature. After quantitative conversion, the mixture was filtered over Na_2SO_4 and the solvent was evaporated yielding in the crude cyanohydrins.

Substrate monomerization

In order to test the reversibility of the substrate's dissociation process at different pH, 180 mg of gently liquefied **1b** were dissolved in 5 mL of a 30 mM potassium phosphate buffer (pH 2.0 – 7.0) and stirred at 250 rpm and 26°C. At scheduled time, samples were taken, acetylated and examined by chiral GC. During the first 4 h, higher amounts of monomer were present at pH = 2.5 and pH = 6.5. After 28 h, approximately 90% of **1** were present as **1a** at pH = 2.5. With increasing pH, the degree of monomerization dropped again. Regarding the time scale necessary for equilibration, it was again[12] shown that it takes quite long, shifting the more stable dimer to the monomeric form (**1a**). As in aqueous solution cyanohydrins are stable at acidic pH but decompose to HCN and carbonyls at neutral or basic pH-values,[13] the enzymatic production of **(*R*)-2** has to be performed at low pH.

5 mL reaction scale for the conversion of 1a – buffered aqueous phase:

Varying amounts of enzyme were diluted to 5 mL using a 3 M citrate phosphate buffer (pH 2.4) or a 2 M potassium phosphate buffer at different pH values (pH 1.5 – 8.0). Then, *i.e.* 120 mg of **1a** and 100 µL of anhydrous HCN were added. The reactions were performed at 4°C and 600 rpm applying the magnetic stirrer Variomag Electronicrührer Poly 15. After the scheduled time, 300 µL of the samples were taken, mixed with 400 µL of *tert*-butylmethylether (TBME) and centrifuged. 200 µL of the organic phase were used for derivatization with 100 µL acetic anhydride in presence of 100 µL pyridine and 800 µL dichloromethane.

The enantiomeric purities and conversions were analyzed using a Hewlett-Packard 6890 instrument equipped with an FID and a Chirasil-DEX CB column (25 m x 0.32 mm, 0.25 µm film). The following settings were used:

Carrier gas: H_2; pressure: 1.0 bar.

Temperature program: 90°C – 2 min, 30°C/min to 130 °C, 10°C/min to 140°C, 20°C/min to 170°C, 170°C – 1 min.

FID detector: 250°C.

Retention times: **1a** (monomer): 2.25 min; **1b** (dimer): 5.98 min, 6.08 min, 6.14 min, 6.20 min; **(*R*)-2**: 4.31 min; **(*S*)-2**: 4.44 min.

Comparison between pure aqueous and biphasic reaction system (H_2O/TBME):

Aqueous system: 170 U of mutein V317A (= 2 mg) were diluted to 5 mL using a 50 mM potassium phosphate buffer (pH 3.4). Then, 180 mg of **1a** and 100 µL of anhydrous HCN were added. The reactions were performed at 4°C and 600 rpm applying the magnetic stirrer Variomag Electronicrührer Poly 15. After the scheduled time, samples were taken and derivatized for chiral GC analysis.

Biphasic system: 170 U of mutein V317A (= 2 mg) were diluted to 2.5 mL using a 50 mM potassium phosphate buffer (pH 3.4). Then, 2.5 mL TBME, 180 mg of **1a** and 100 µL of anhydrous HCN were added. The reactions were performed at 4°C and 600 rpm applying the magnetic stirrer Variomag Electronicrührer Poly 15. After the scheduled time, samples were taken and derivatized for chiral GC analysis.

Reaction in micro-aqueous, organic solvent system with immobilized *Pa*HNL5-V317A

For subsequent use in organic solvent, 120 U of V317A (= 1.41 mg) were colyophilized onto 140 mg of Celite® in 8.6 mL of a 30 mM potassium phosphate buffer (pH 3.4). Then, 2.97 mL of diisopropyl ether (DIPE), 24.8 µL of a 30 mM potassium phosphate buffer (pH 3.4), 120 mg of **1a** and 100 µL of anhydrous HCN were added and the reaction mixture was stirred at room temperature. After the scheduled time, samples were taken and prepared for chiral GC analysis.

Preparative reactions:

Reactions were performed in a 100 mL Schmizo KPG reactor. 2560 U of the V317A enzyme preparation (= 30 mg enzyme) were diluted to 50 mL using a 2 M potassium phosphate buffer (pH 2.5) and set to pH 2.5 again. 1.2 g of **1a** were added and the mixture was stirred vigorously keeping the temperature constant at 4°C. Finally, the reaction was started adding 1 mL of anhydrous HCN. After a reaction time of 20 h, the aqueous reaction mixture was extracted three times with TBME. The organic phases were pooled, dried over Na_2SO_4 and TBME was evaporated. After derivatization, the sample was analyzed by GC as described above.

Reactions for the conversion of pivalaldehyde (PA):

2 mg of the corresponding enzyme preparation (= V317A: 170U and *Pa*HNL5: 545U, respectively) were diluted in 5 mL of a 1 M potassium phosphate buffer (pH 3.0). Then, 200 µL of liquid PA (T71501 from Sigma-Aldrich) were added. The reactions were performed at 9°C and 600 rpm applying the magnetic stirrer Variomag Electronicrührer Poly 15. To start the reaction, 100 µL of anhydrous HCN were added. After the scheduled time, samples were taken, derivatized with acetic anhydride in presence of pyridine and dichloromethane. The enantiomeric purities were analyzed using a Hewlett-Packard 6890 instrument equipped with an FID and a Chirasil-DEX CB column (25 m x 0.32 mm, 0.25 µm film). The following settings were used:

Carrier gas: H_2; pressure: 1.0 bar.

Temperature program: 40°C – 2 min, 15°C/min to 130°C.

FID detector: 250°C.

Retention times: **pivalaldehyde**: 1.10 min; **(*R*)-pivalaldehyde cyanohydrin**: 5.85 min; **(*S*)-pivalaldehyde cyanohydrin**: 6.42 min.

Tabelle 19: Comparison of V317A with unmodified *Pa*HNL5 for the hydrocyanation of pivalaldehyde

reaction time		Blank	*Pa*HNL5	V317A
1 h	%ee	*rac*	82.5	97.3
	%conv	33.8	77.8	99.9
2 h	%ee	*rac*	85.0	97.2
	%conv	46.7	94.2	100
6 h 10 min	%ee	*rac*	86.3	97.2
	%conv	72.8	100	100

Improving enzyme expression II - Combining the expression of a codon-optimized, synthetic *Pahnl5α* gene using improved *AOX1* promoter variants with *Pichia pastoris* PDI co-expression:

The *Pahnl5α* gene (*Pahnl5* gene plus signal sequence of the *Saccharomyces cerevisiae* alpha mating factor)[7] was codon-optimized using the DNA-optimizing software Leto 1.0 provided by Entelechon (Regensburg, Germany). The optimization was carried out applying the codon usage of highly expressed genes from *Pichia pastoris* (*i.e. AOX1*). The resulting, optimized nucleotide sequence is depicted in Figure S2.

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atgagattcccatctattttcaccgctgtcttgttcgctgcatcttccgcattagctgcacctgttaacactaccactgaagacga
gactgctcaaattccagctgaagcagttatcggttactctgaccttgagggtgatttcgacgtcgctgttttgcctttctctaact
ccactaacaacggtttgctgttcattaacaccactatcgcttccattgctgcaaaggaagagggtgtctctcttgagaagagagag
gccgaagctttggctaacacttctgctcacgacttctcttacctgaagttcgtttacaacgctactgacacttcttccgaaggttc
ttacgactacatcgtcattggtggaggtacttctggttgtccattagctgcaactttgtctgagaagtacaaggttctcttgcttg
agagaggtaccattgctactgagtatccaaacaccttaactgcagacggatttgcctacaacttgcaacaacaagacgatggaaaa
actcctgttgagagattcgtttctgaagatggtatcgacaacgttagagcacgtattttgggtggaactaccatcattaacgcagg
tgtttatgccagagctaacatctccttctactctcaaactggcattgaatgggacttagacttggtcaacaagacctatgaatggg
ttgaagatgccatcgttgtgaagccaaacaaccaatcttggcaatccgttattggtgaaggattccttgaagcaggtattttgcca
gacaatggattctctcttgaccacgaagcaggtactagactgactggttctaccttcgacaacaacggtactagacatgctgcaga
cgaattgttgaacaaaggagatccaaacaacctgttggttgcagtccaagcttctgtcgagaagattctgttctcctctaacactt
ccaacctttctgctatcggagtgatctacacagactctgatggtaactctcaccaagcattcgttagaggtaacggtgaggtcatt
gtttccgcaggaactattggtactccacaactgttgcttttgtctggtgttggacctgaatcctacctgtcttccttgaacatcac
tgttgtccaacctaacccttatgtcggacaattcgtgtacgataaccctagaaacttcatcaacatccttccacctaatcctattg
aagcatctgttgtcactgttttgggaatcagatccgattactaccaagtctctctgtcttccttgccattctctactccaccttc
tctcttttcccaaccacttcttacccacttcctaactccactttcgctcacattgtgtctcaagttcctggaccattgtcacacgg
ttctgtgaccttgaactcttcctctgacgttcgtattgcaccaaacatcaagttcaactactactctaactctactgacttggcta
attgcgtttctggtatgaaaaagctgggtgacttgttgcgtactaaggctcttgaaccatacaaagctagagacgttttgggaatt
gacggtttcaactaccttggtgttcctttaccagagaaccaaactgatgacgcatctttcgagactttctgtcttgacaatgttgc
ctcttactggcactaccatggtggttctttggttggtaaggttttggacgattccttcagagtcatgggtatcaaggctttgagag
tcgttgatgcttctaccttcccttatgagccaaattctcaccctcaaggtttctacctcatgttgggaagatacgttggtttgcag
attttgcaggagagatccatcagattggaagctatccacaacattcaggagtctatgtaa
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Figure S2: Codon-optimized *Pahnl5a* nucleotide sequence

Based on the optimized *Pahnl5a* gene, alternating forward and reverse primers were designed with a maximum length of 60 nucleotides and 18-30 overlapping nucleotides between consecutive primers. The primers were purchased from Invitrogen and the *Pahnl5a* gene was subsequently synthesized as described by Xiong *et al.*[14] The resulting synthetic gene was cloned into electrocompetent *E. coli* TOP10F' cells using the GeneJet™ PCR Cloning Kit purchased from Fermentas. Plasmid DNA was amplified and controlled by sequencing. Then, site-directed mutagenesis was performed to introduce the mutation V317A (GTC → GCT). This was done employing the QuikChange XL Site Directed Mutagenesis Kit from Stratagene, template plasmid pJET1-PaHNL5α_syn and the following two mutagenesis primers:

V317A_synfw: 5'-ccacctaatcctattgaagcatctgttgctactgttttgggaatcagatccgattactac-3'

V317A_synrv: 5'-taatcggatctgattcccaaaacagtagcaacagatgcttcaataggattaggtggaagg-3'

After *Dpn*I digestion, 10 µL were used for *E. coli* TOP10 F' (Invitrogen) transformation. Then, plasmid DNA was prepared and sent for sequencing. After *Eco*RI/*Not*I digestion, the correct synthetic *Pahnl5a-V317A* gene was ligated into the *Eco*RI/*Not*I linearized pPICZ B AOX1-Δ1[15] vector which is based on the pPICZ B vector from Invitrogen but contains a modified *AOX1* promoter variant designed by Hartner *et al.*[15] The resulting plasmid was again controlled by sequencing and subsequently, *Pichia pastoris* KM71H was transformed according to the condensed protocol for competent cell preparation and *Pichia pastoris* transformation.[16] Approximately 150 transformants were cultivated in 96-well deep well plates and assayed for the cleavage of mandelonitrile as described by Weis *et al.*[8]

Pichia pastoris protein disulfide isomerase (PDI) coexpression

The *Pichia pastoris* PDI gene[17] was amplified out of the *Pichia pastoris* CBS7435 genome. The genomic DNA preparation was performed as described before, and 5 µL thereof were employed for a colony PCR using the primers PDI-NotI-R (5'-GGCGGCCGCTTAAAGCTCGTCGTGAGCGTC-3') and PDI-EcoRI-F (5'-GGAATTCGAAACGATGCAATTCAACTGGGATATTAAAAC-3'). The colony PCR was done in 50 µL with 0.2 mM dNTP, 0.2 µM of each primer, 0.6 U Phusion High-Fidelity DNA Polymerase (Finnzymes) and 1x Phusion HF reaction buffer. The following program was used: 30 sec 98°C, 30 cycles (10 sec 98°C, 15 sec 55°C, 45 sec 72°C) and a final extension at 72°C for 5 min. The PCR product was purified applying the Wizard® SV Gel and PCR Clean-Up System from Promega.

The Zero Blunt® TOPO® PCR Cloning Kit from Invitrogen was used for cloning the *P. pastoris* PDI gene into electrocompetent *E. coli* TOP10 F' cells from Invitrogen. After *Eco*RI/*Not*I digestion, the *P. pastoris* PDI gene was ligated into the *Eco*RI/*Not*I linearized pPICZ B vector from Invitrogen resulting in the vector pPICZ-PDI. In addition, the AOX1 promoter region was exchanged for modified *AOX1* promoter sequences[15] like AOX1-Δ1 and AOX1- Δ6*, respectively, and the zeocin resistance cassette was replaced by the kanamycin resistance cassette. All newly constructed plasmids were controlled by sequencing. The resulting pPICK AOX1- Δ1 PDI and pPICK AOX1- Δ6* PDI plasmids were used for transforming a mixture of active *Pichia pastoris* KM71H pPICZ B AOX1- Δ1 *Pahnl5a-V317A* strains according to the condensed protocol for competent cell preparation and *Pichia pastoris* transformation.[16] Again, genomic DNA of the resulting variants was isolated and the integration of the *Pahnl5a-V317A* and the PDI gene were controlled by colony PCR.

Approximately 350 PDI- Δ1 and PDI- Δ6* co-transformants were cultivated in 96-well deep well plates and assayed for the cleavage of mandelonitrile as described by Weis *et al*.[8] The 10 best variants were chosen for a rescreening experiment and the very best variant was cultivated in baffled shake flasks as described above (see Enzyme preparation - Shake flask cultivation), to confirm improved expression.

References

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