Supplemental Material

Synthesis of chiral cyanohydrins with recombinant *Escherichia coli* cells in a micro-aqueous reaction system

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Supplementary Materials and Methods

Construction of the translation fusion construct

The gene fragment *nFbFP* encoding for the respective YtvA-LOV domain region (nFbFP: residue 25 - 147) was PCR amplified from a pET28a vector containing the full-length *ytvA* gene bearing a single codon substitution which results in the amino acid exchange C62A (1). This substitution renders the LOV domain autofluorescent by preventing light-dependent cysteinyl-thiol adduct formation. The *nFbFP* gene fragment was amplified using the oligonucleotides nFbFP_NdeI_fw (5'-ACAGACATATGGTCGGTGTGGTAATTACAGATCCCG -3') and nFbFP-Xa_SacI_rev (5'-CTGTGAGCTCACGACCTTCGATTGAAAGTGCAGTAATTTC -3'). The fragment was digested using *Nde*I, and *Sac*I restriction enzymes and were subsequently ligated into a similarly hydrolyzed pET28a vector (Novagen, Darmstadt, Germany). This results in the construct called pETnFbFP which was used for all subsequent cloning steps. Oligonucleotides used for amplification and cloning of the *At*HNL encoding gene were as follows nHNL_blunt_fw (5'- ATG GAG AGG AAA CAT CAC TTC GTG TTA GTT CA -3') and nHNL_blunt_rev (5'- TTA CAT ATA ATC GGT

GGC AAT AGC AGA GAG AGA G -3'). The gene fragment was PCR amplified from an existing expression plasmid described previously (2). The amplified fragment was ligated into *Ecl136*II digested pETnFbFP. *Ecl136*II is an isoschizomer of the restriction enzyme *Sac*I, which was used for directional cloning of the nFbFP encoding gene fragment into pET28a (see above). This results in an in-frame translational fusion of the nFbFP and *At*HNL encoding genes. All constructs were sequenced (Seqlab GmbH, Göttingen, Germany) prior to transformation of *E. coli* BL21(DE3).

Protein expression

All proteins were expressed in modified auto induction media (4). The media contained 12 g/L casein hydrolysate, 24 g/L yeast extract and 5 g/L glycerol. Media components were dissolved in 100 mM potassium phosphate buffer pH 7. After autoclaving for 20 min at 120 °C and 200 kPa the media was supplemented with 5 % (w/v) glucose and 2% (w/v) lactose. Overexpression was carried out in 1 L autoinduction media using *E. coli* BL21(DE3) cells inoculated to a starting OD_{580nm} of 0.025 – 0.05. Initially, the culture was incubated at 37 °C and 120 rpm. After three hours the temperature was reduced to 15 °C, followed by incubation for 72 h at constant agitation (120 rpm). For protein purification, cells were harvested by centrifugation at 6000xg (30 min, 4 °C).

Enzymatic activity assays

Synthesis of (R)-mandelonitrile by HNL expressing whole E. coli *cells*

Synthesis of (*R*)-mandelonitrile by whole *E. coli* cells expressing the *At*HNL (wild-type) or the respective fusion constructs was carried out as described previously for

HNL immobilizates (3). All reactions were carried out at 20°C. The reaction system consisted of a 1 ml 2 M HCN solution in buffer saturated methyl *tert*-butyl ether (MTBE) containing 0.5 mmol of the respective aldehyde. 350 mg of wet cells (or the corresponding amount of lyophilized cells) were mixed with the HCN solution (1 ml, in MTBE) under argon atmosphere. The reaction was started by addition of the respective aldehyde. Synthesis reactions were followed using chiral gas chromatography (GC) as previously described (3). For recycling experiments, 350 mg of cells were washed three times with buffer saturated (50 mM citrate phosphate pH 5.5) methyl *tert*-butyl ether (MTBE). Cells were subsequently transferred to a sealed nylon mesh bag (nylon-net pore size 0.4 µm). The reactions were monitored over 60 minutes using chiral GC. Up to five consecutive hydrocyanation reactions were performed. Between each reaction cycle the cells were washed with buffer saturated MTBE. For synthesis reactions with lyophilized cells expressing the *At*HNL, 80 mg lyophilized cells were transferred to the reaction vessel. The synthesis reaction was carried out for 60 minutes and product formation was monitored by chiral GC.

Spectrofluorimetry, fluorescence microscopy and image analysis

Spectrofluorimetric analyses

All fluorescence measurements were carried out using thermostated (25 °C) T-format Fluorolog-3 spectrofluorimeter (Horiba, Kyoto, Japan) equipped with double-grating excitation and emission monochromators and a 450 W Xenon CW lamp as excitation source. FbFP fluorescence emission spectra were recorded from diluted whole-cell samples. *E. coli* BL21(DE3) cells expressing the respective fluorescent fusion

construct were diluted in 50 mM sodium phosphate buffer to an OD_{600nm} of 0.1. FbFP emission spectra (470 nm to 650 nm) were recorded after excited at 450 nm. To account for changes in sample scattering due to changes in the cell size during incubation in MTBE, the scattering signal was detected at 600 nm using the same set-up. Scattering data was used to correct reporter protein fluorescence spectra and emission intensities for changes in light scattering.

Fluorescence microscopy

To mimic synthesis reaction conditions, nFbFP-HNL expressing *E. coli* BL21(DE3) cells were incubated in buffer-saturated MTBE, without addition of the substrates benzaldehyde and hydrogen cyanide (HCN), for up to 4.5 hours. Experimental conditions (temperature, solvent, reaction vessel) were otherwise identical to the above described whole-cell biotransformation set-up. Modification of the reaction conditions for microscopic analyses was necessary, because it is impossible to work with highly toxic HCN (2 M in the assay) using a standard fluorescence microscope set-up. At fixed time points 3 µl of the diluted (OD_{600nm}=1) cell suspension was immobilized on a glass slide covered with a 1% agarose pad of uniform thickness. The sample was covered with a cover glass slide and placed on a fully motorized inverted microscope (Nikon Eclipse Ti, Nikon Instruments Inc., Melville, NY, USA) suitable for time lapse live cell imaging. The setup is equipped with a focus assistant (Nikon PFS) compensating for thermal drift during long term microscopy, Apo TIRF 100x Oil DIC N objective, ANDOR LUCA R DL604 camera, Xenon fluorescence light source for fluorescence excitation. Standard filters for the proper excitation and detection of the FbFP reporter (eCFP excitation: 426 nm - 446 nm and emission lines: 460 nm - 500 nm) were used. DIC microscopy images as well as fluorescence

images were captured and analyzed using the Nikon NIS Elements AR software package.

Image analysis and statistical data evaluation

For each time point 10 X 10 images (stitched images), each containing between 10 and 100 bacterial cells, were generated and analyzed for morphological changes. The automated cell count tool was used to recognize the number of cells in the sample. Using the automated threshold picking tool on the image the intensity/part of the histogram can be adjusted to determine which areas/cells are considered as accepted. Unwanted objects such as aggregates or small particles were removed manually from the object count. All accepted objects were used to calculate the elongation of the bacteria. The elongation is defined as the Max Feret's diameter divided through the Min Ferets's diameter. The results were exported and post-processed using Origin7G (OriginLab, Northampton, MA, USA) to generate the graphs illustrated in Supplementary Figure 1. Values plotted in Figure 2 D represent the mean cell perimeter, determined from at least 1000 - 10,000 cells per time point.

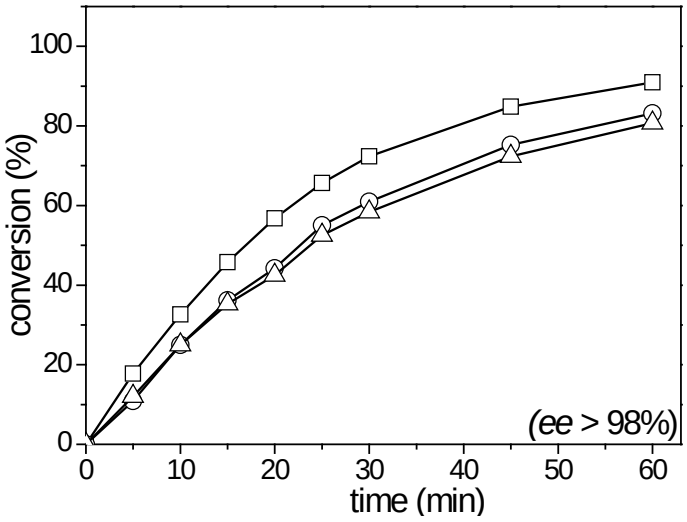
Supplementary Results

Supplementary Figure 1 depicts recycling of frozen *E. coli* BL21(DE3) cells expressing wild-type *At*HNL. The frozen cells exhibit very similar conversion rates and enantioselectivities over several reaction cycles, compared to freshly prepared cells.

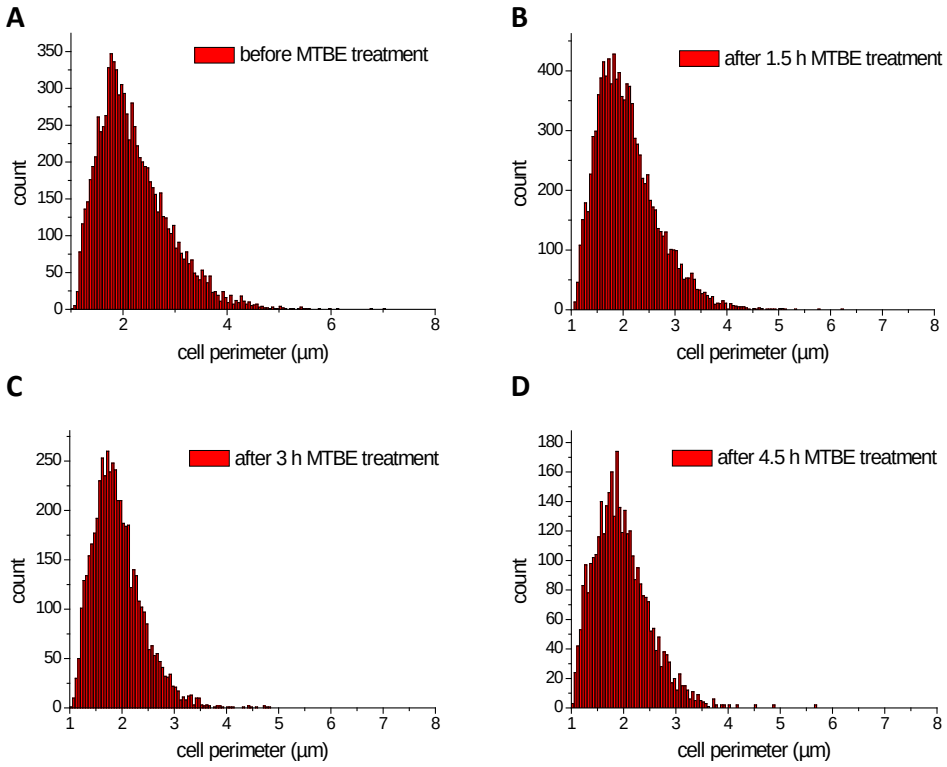
Supplementary Figure 2 depicts the development of the cell perimeter distribution during incubation in MTBE. To obtain a reliable estimate for the mean cell size at each time point, 1000 - 10,000 cells were taken into account. As described above, the cell perimeter was measured automatically using the microscope image analysis software. To generate the graph depicted in Figure 2 D, the mean cell perimeter was calculated from all measured cell perimeters at a given time point.

In order to evaluate fusion protein integrity during incubation of the whole cells in MTBE, we recorded FbFP fluorescence emission spectra from diluted whole-cell samples. Surprisingly, during the incubation in MTBE, FbFP fluorescence emission increased over time. This effect can be attributed to a change in light scattering of the samples probably caused be the microscopically observed changes in cell size. To account for this effect, the static light scattering signal was recorded for each whole cell sample and the respective FbFP emission spectra were corrected correspondingly. Supplementary Figure 3 depicts the development of the respectively normalized FbFP fluorescence signal (emission maximum at 495 nm). Thus, when taking the changes in light scattering into account, almost no change in FbFP fluorescence intensity occurs during incubation in MTBE. This indicates proper FbFP reporter protein integrity within the cell.

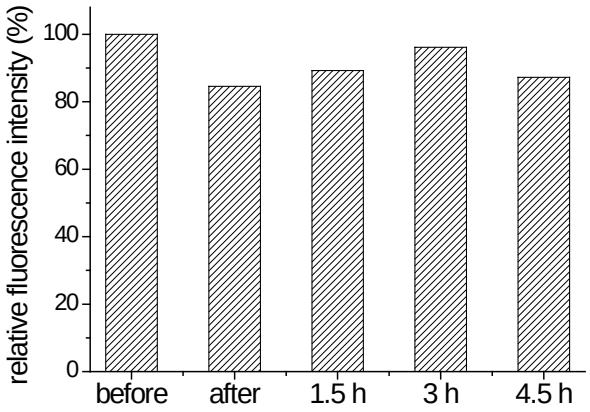
Supplementary Figures



Supplementary Figure 1: Recycling of 350 mg of frozen *E. coli* BL21(DE3) cells expressing wild-type *At*HNL using benzaldehyde and HCN as substrates. All reactions were performed using in buffer-saturated MTBE cells are placed in a nylon mesh (pore-size 0.4 µm). After each conversion round the cells in the nylon mesh were washed with MTBE, placed in a fresh reaction vessel, and the subsequent conversion was started by the addition of new substrates. I. conversion (□), II.conversion (○), and III. conversion (Δ).



Supplementary Figure 2: Changes in distribution of the cell perimeter during incubation in MTBE. Mean cell perimeter and correspondingly the overall cell size decreases during incubation in MTBE. *E. coli*BL21(DE3) cells expressing the respective HNL construct. (A) Cells resuspended in 10 mM potassium phosphate pH 7.5; before incubation in MTBE. (B) Cells after 1.5 hours in MTBE (C), after 3 h in MTBE and (D) after 4.5 h in MTBE.



Supplementary Figure 3: Development of the fluorescence signal (FbFP emission maximum at 495 nm) during MTBE treatment of *E. coli* BL21(DE3) cells, expressing nFbFP-*At*HNL. FbFP fluorescence emission spectra were recorded at the indicated time points using whole *E. coli* cell diluted to an OD_{600nm} of 0.1. Probably, due to the microscopically observed decrease in cell size, light scattering increases significantly with prolonged incubation times. Therefore the fluorescence signal was normalized to account for the changes in light scattering.

Supplementary References

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