Electronic Supplementary Material (ESI) for Reaction Chemistry & Engineering.

Supplementary Information

Directed Evolution of *Rhodotorula gracilis* D-Amino Acid Oxidase using Single-cell Hydrogel Encapsulation and Ultrahigh-throughput Screening

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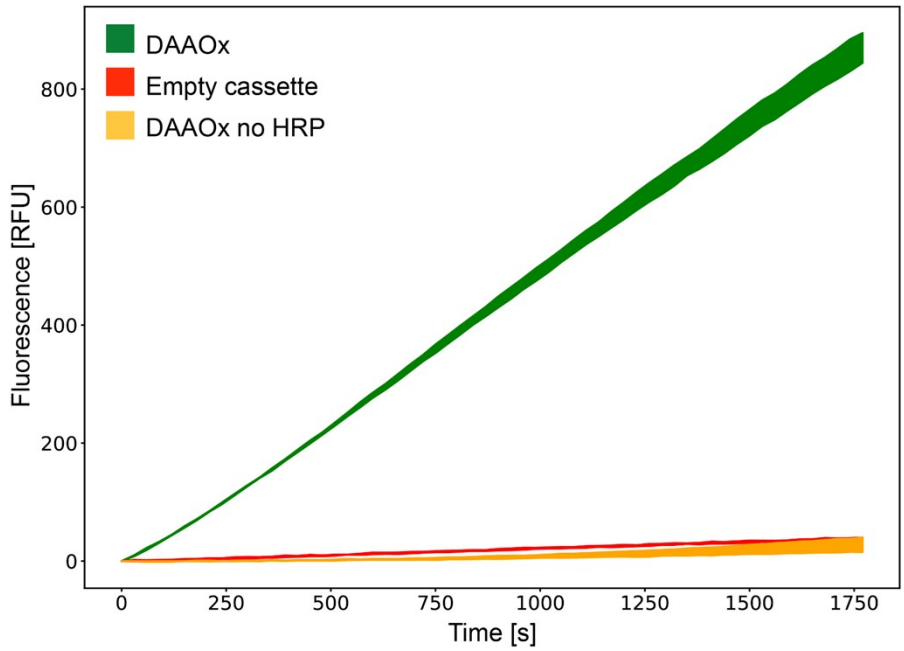
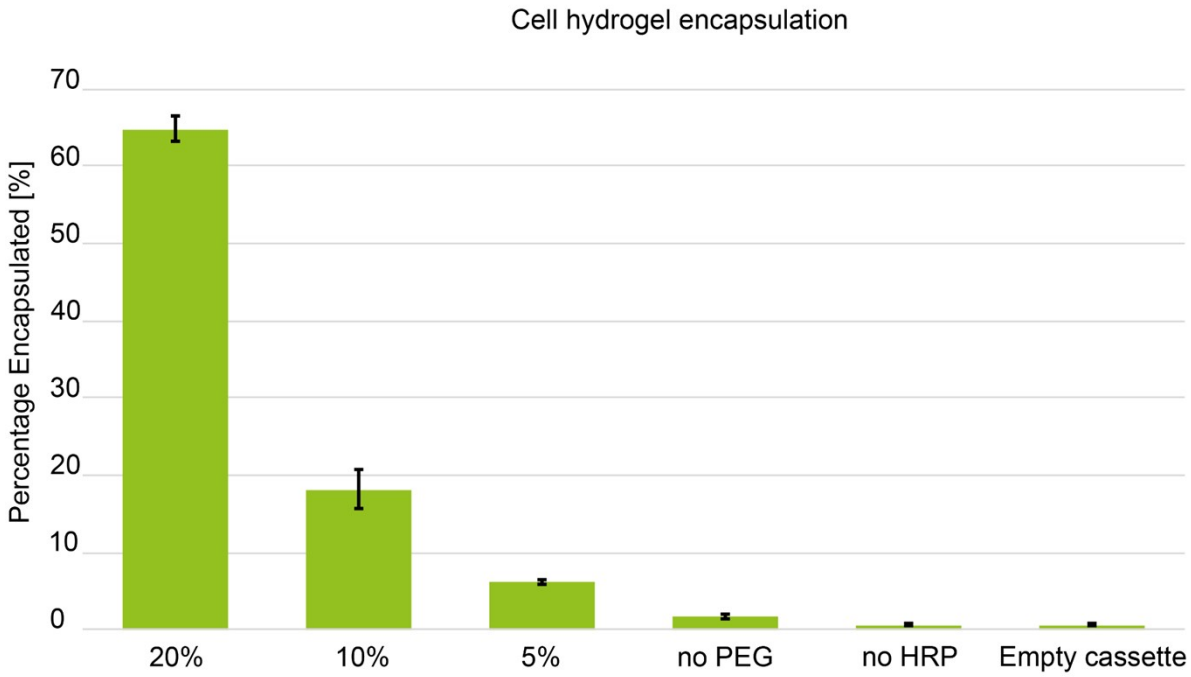
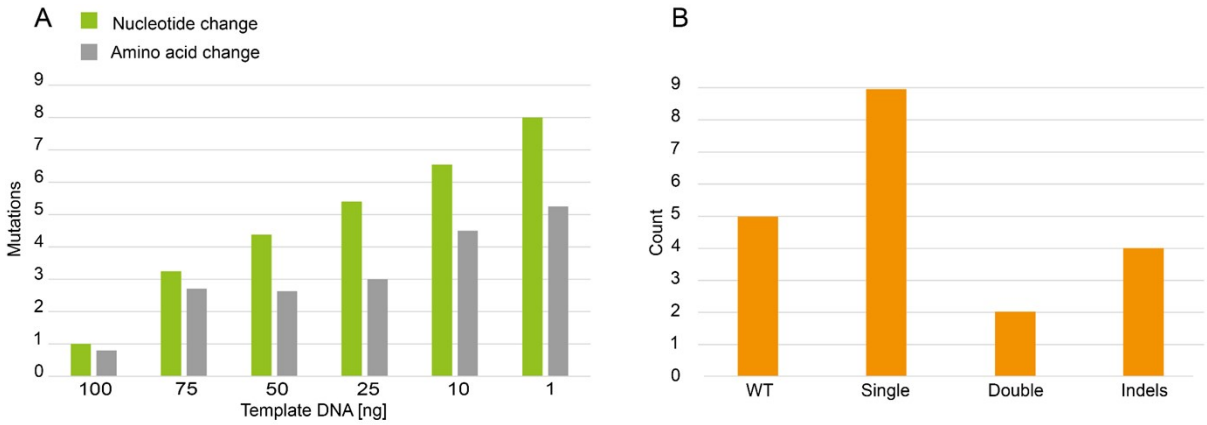


Supplementary Figure 1

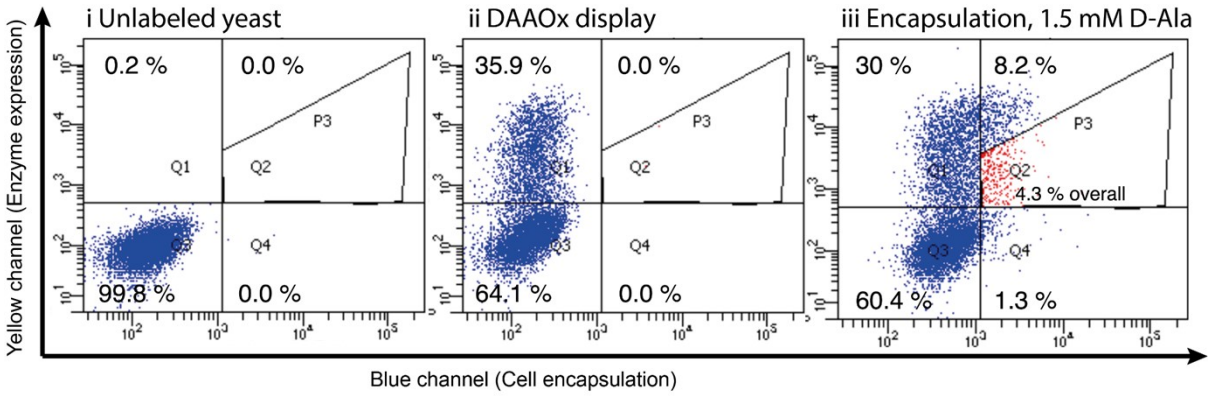
Activity of yeast surface displayed D-Amino Acid Oxidase measured in an Amplex Red™ assay. Enzyme (green), empty cassette (red) and enzyme w/o HRP (orange) were each measured in triplicates. Error bands show standard deviations.



Supplementary Figure 2
Barplot showing impact of addition of PEG on percentage of encapsulated population of yeast cells. Negative controls are reaction mixtures without HRP and reaction mixture with cells displaying the empty cassette thus lacking the enzyme. Experiment was conducted in triplicates and error bars show standard deviation.

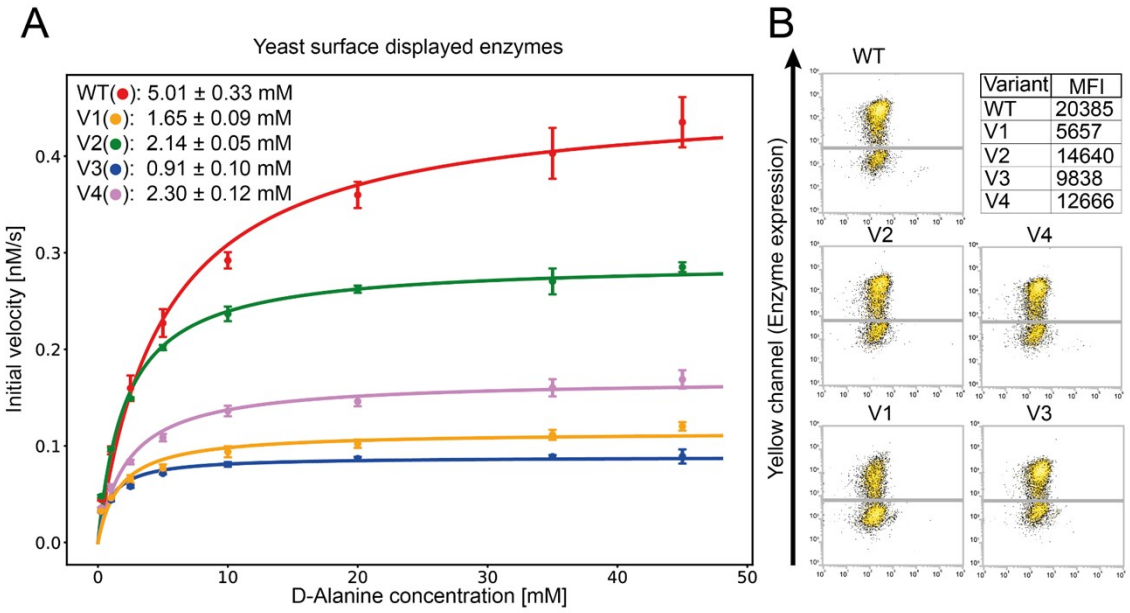


Supplementary Figure 3 A) Several conditions for error prone PCR were experimented with, producing libraries with mutational loads. Depending on the amounts of template genetic material different numbers of mismatches were achieved. For the mutant screening, we used 75 ng of starting material yielding a library with an average of 3 mutations, which was considered a good complement to the saturation library mainly focusing on single mutants. B) Overview of the one pot mutagenesis library. 20 sequences were used to characterize the library.



Supplementary Figure 4

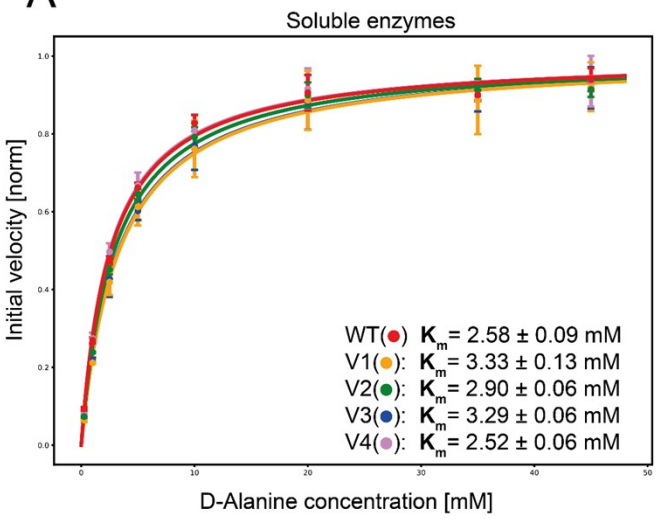
Library on the fluorescent activated cell sorter (FACS). i) shows the unstained cells, ii) the yeast cells labeled with Alexa Fluor™ 594 fluorophore and iii) shows the cells after the encapsulation reaction. The gate labeled P3 (red) was used for cell sorting. Analyzing $5*10^6$ cells resulted in 200'000 sorted cells. Numbers in corners represent percentages of parental gate sorting for single cells.



Supplementary Figure 5

A) Michaelis Menten curves showing initial enzymatic velocities over substrate concentration. **B)** Flow cytometry dot plots indicate different expression levels. Table on the top right indicates Median Fluorescence Intensity (MFI) of the upper populations (Expressing cells).

A



B

Variants	Mutations	Km [mM] YSD	Km [mM] Soluble
WT	-	5.01 (± 0.33)	2.58 (± 0.09)
V1	Thr161Ile	1.65 (± 0.09)	3.33 (± 0.13)
V2	Cys234Val	2.14 (± 0.05)	2.90 (± 0.06)
V3	His138Arg	0.91 (± 0.10)	3.29 (± 0.06)
V4	Gly290Arg	2.30 (± 0.12)	2.52 (± 0.06)

Supplementary Figure 6

A) Normalized initial velocities of Amplex Red activity assay. Experiment was conducted in triplicates and Michaelis-Menten equation was fit for determination of K_m. **B)** Mutations of variants and Michaelis-Menten constants for yeast surface displayed and soluble enzymes of wild type and DAAOx-variants.