SUPPLEMENTARY INFORMATION

for

Fluorescence-based pH-shift assay with wide application scope for high-throughput determination of enzymatic activity in enzyme mining and engineering

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Generation of TK_{gst} variant library

The L382F variant of TK_{gst}, previously engineered for acceptance of aliphatic aldehydes (Yi et al. 2015) was used as the starting template. Point mutations at the 264 position to specific amino acids were introduced using the Quick Change site-directed mutagenesis kit (NEB) with Q5 polymerase (primers given in Table S1). The base L382F variant and individual G264(A,V,S) mutants were further subject to random mutagenesis at S385 employing NNK codon degeneracy, following the deletion/insertion PCR strategy.

The PCR-amplified DNA was digested using 0.5 µL KLD Enzyme Mix (Kinase, Ligase, Dpn I; NEB) for 20 min at 25°C. Then 5 µL of PCR product were used to transform DH5α competent cells which were cultured in LB medium containing 50 µg/mL kanamycin. Following plasmid sequencing to evaluate the quality of the mutagenesis PCR, the clone mix was cultured on LB-kanamycin agar plates overnight. For each library 168 colonies were randomly picked into 96-well plates containing 120 µL/well LB-kanamycin medium. Glycerol (85%, 20 µL) was added into each well after 12 h incubation, and the whole plates were sealed with plastic lids and stored at -80°C.

Table S1. PCR primers used for mutagenesis

TK_{gst} variant/library	Primer sequence
G264A	5′-CGGCGTCCACGCGGCTCCGCTCG-3′ (*forward*)
	GACGTGCCCGCTTTATTTG (*reverse*)
G264V	CGGCGTCCACGTGGCTCCGCTCG (*forward*)
	GACGTGCCCGCTTTATTTG (*reverse*)
G264S	CGGCGTCCACTCGGCTCCGCTCG (*forward*)
	GACGTGCCCGCTTTATTTG (*reverse*)
S385X	Deletion primer:
	AATAAAACGCTCATCAAAGGCGGC (*forward*)
	GCTTGCGAAGTCCGCCGA (*reverse*)
	Insertion primer:
	NNKAATAAAACGCTCATCAAAGGCGGC (*forward*)
	GCTTGCGAAGTCCGCCGA (*reverse*)

Enzyme purification and assay protocols

Hexokinase from *Saccharomyces cerevisiae* (catalog no. H5000; activity stated on the bottle ≥25 U/mg protein) and acetylcholinesterase from electric eel (catalog no. C2888; stated on the bottle = 1527 U/mg protein) were procured from Merck (Sigma-Aldrich; Germany) as lyophilized powder. Fresh solutions were made in the appropriate buffer and directly used for the fluorimetric pH shift assay. For hexokinase and acetylcholinesterase, the specific activity values provided by the supplier were used as reference for comparison.

CSS from *Neisseria meningitis* was purified according to Yi et al. (2013) and desalted into the appropriate buffer before the assay. The wild-type 2,3-sialyltransferase and the A151D variant were purified according to Mertsch et al. (2020) and desalted into the appropriate buffer before the assay. Reaction mixtures for the assay were constituted as detailed in Figures S3-S7. In the case of SiaT, both the transferase (with 5 mM lactose) and hydrolase/sialidase (with water) were determined for both the wildtype enzyme and A151D variant.

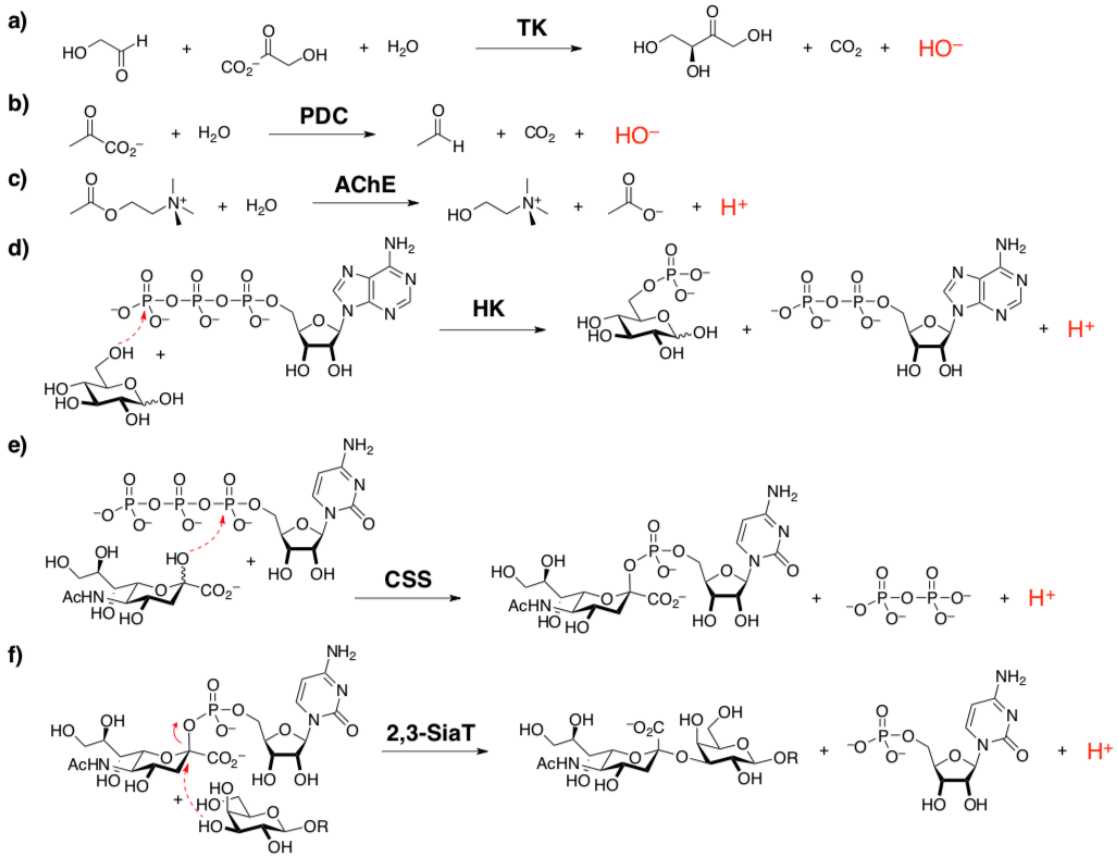


Figure S1. Exemplary enzyme reactions that are associated with a change in pH during catalysis. TK, transketolase; PDC, pyruvate decarboxylase; AchE, acetylcholine esterase; HK, hexokinase; CSS, CMP sialate synthase; SiaT, sialyltransferase.

Kinetic measurements using the fluorometric pH-shift assay principle

Table S2. Kinetic parameters for transketolase from *Geobacillus stearothermophilus* (TK_{gst}) determined using the fluorescent pH assay and comparison with reported values (Zabar et al. 2015; Saravanan et al. 2017). Values are represented as mean ± SD.

Substrate	Concentration range (mM)	K_M (mM)	v_{max} (µmol/min/mg)	Reported parameters (TK_{gst})
Glycolaldehyde	0-80 mM	4.06 ± 0.33	7.40 ± 0.15	
D-glyceraldehyde	0-80 mM	7.36 ± 1.17	6.55 ± 0.30	K_M 6 mM, v_{max} 16
Li-HPA	0-80 mM	1.80 ± 0.32	7.75 ± 0.2	K_M 2.3 mM, v_{max} 12

Application of fluorescent pH shift assay to different enzyme classes

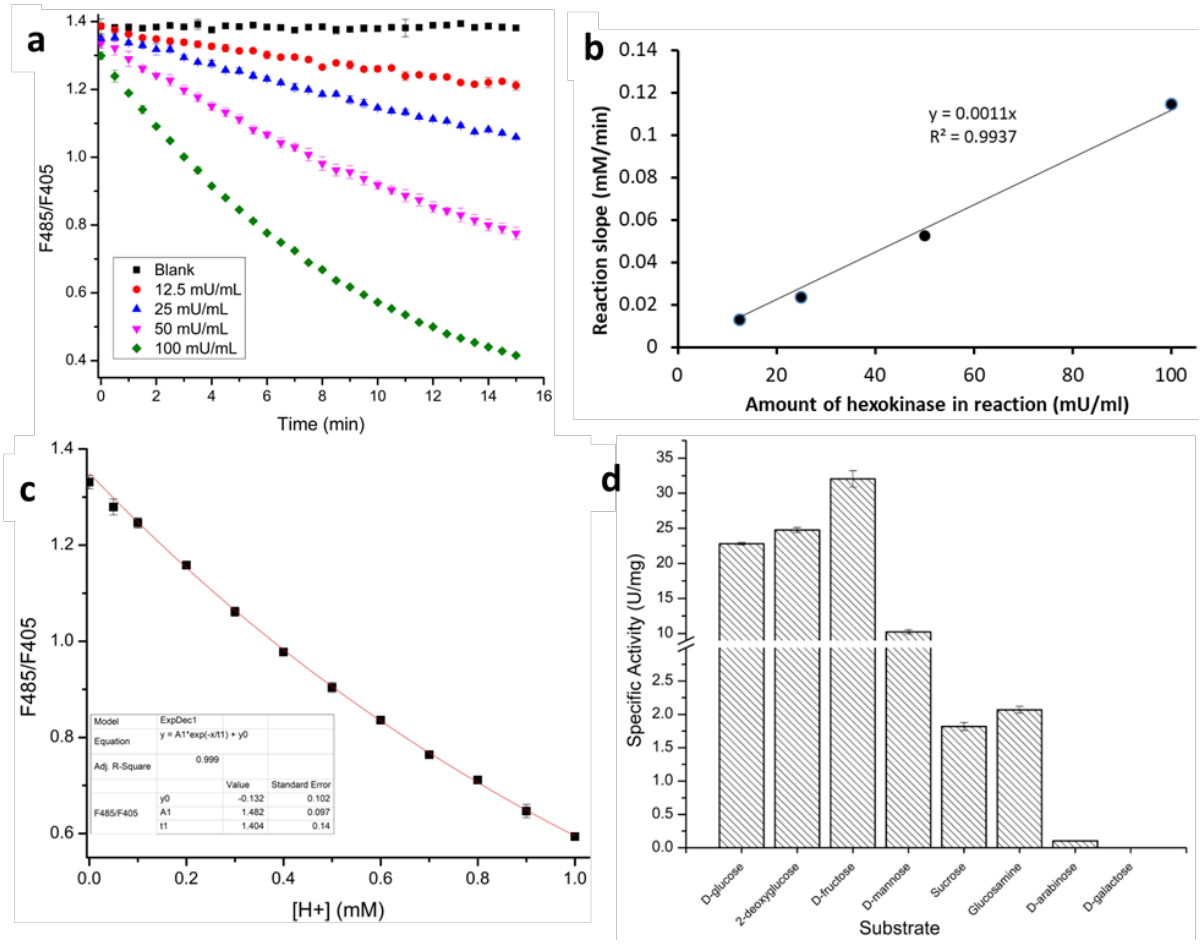


Figure S2. (a) Enzymatic activity of yeast hexokinase measured using the fluorescent pH assay. Reaction mixture (200 μL) contained 12.5–100 mU/mL hexokinase (from Sigma), 5 mM glucose, 5 mM ATP, 5 mM $MgCl_2$, 0.4 μM HPTS in 2 mM HEPES pH 8.2 at 25°C. (b) Dependence of reaction slope on the amount of hexokinase in the reaction. (c) Calibration curve measured in 2 mM HEPES buffer pH 8.2 at 25°C containing all components in the reaction mixture except glucose. (d) Activity of yeast hexokinase on different sugar substrates as measured using the fluorescent pH assay.

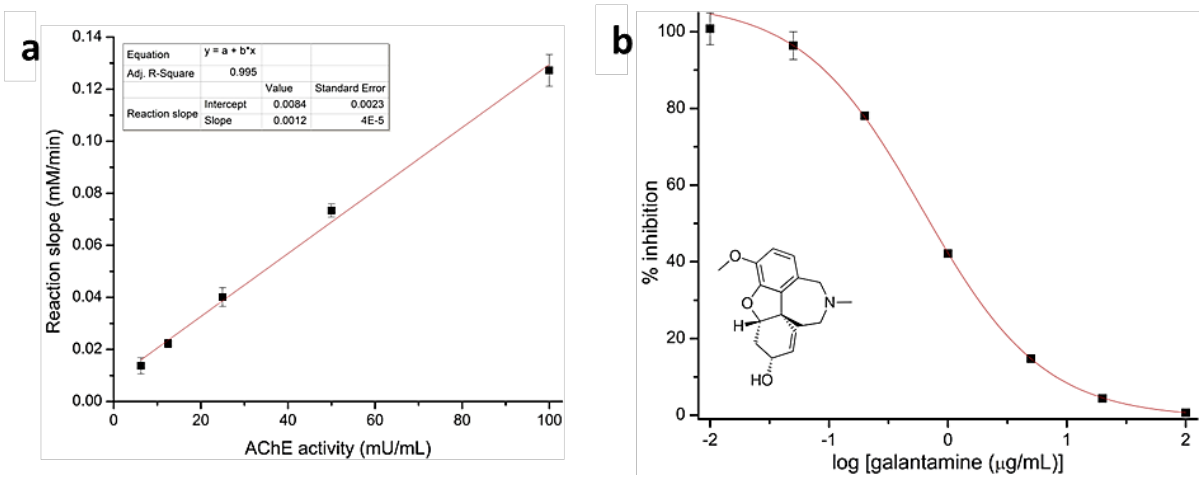


Figure S3. (a) Enzymatic activity of acetylcholinesterase (AChE, electric eel) measured using the fluorescent pH assay. Graph depicts the dependence of reaction slope on the amount of AChE in the reaction. Reaction mixture (200 μL) contained 0-100 mU/ml AChE (from Sigma), 1 mM acetylcholine chloride, 30 mM NaCl, and 0.4 μM HPTS in 2 mM HEPES buffer pH 8.0 at 37°C. (b) Inhibition plot of known cholinesterase inhibitor galantamine on AchE activity.

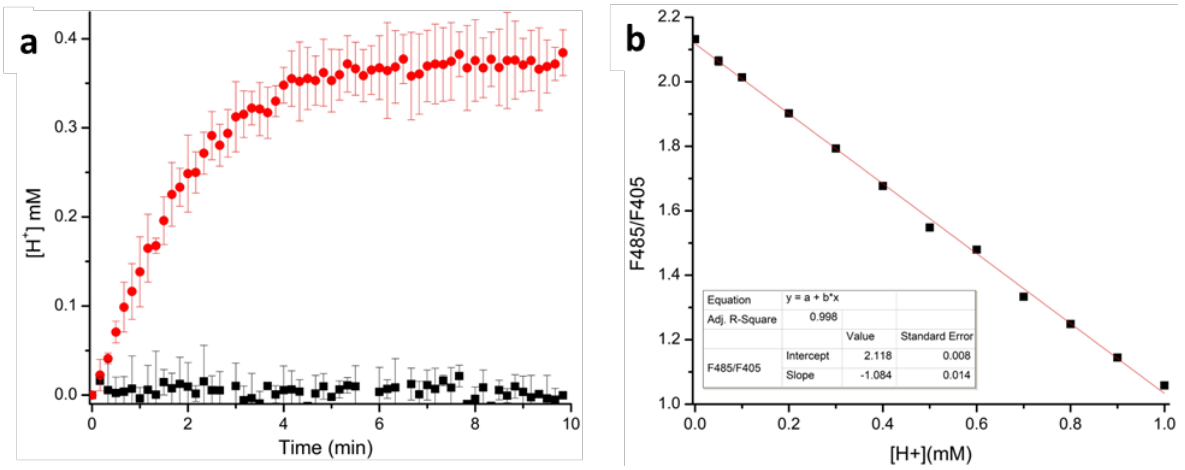


Figure S4. (a) Enzymatic activity of CMP-sialate synthetase (CSS, from *Neisseria meningitis*) measured using the fluorescent pH assay. Reaction mixture (200 μL) contained 0.63 μg CSS, 0.5 mM CTP, 2 mM sialate (Neu5Ac), 10 mM $MgCl_2$, and 0.4 μM HPTS in 2 mM HEPES pH 8.2 at 37°C. (b) Calibration using 0-1 mM HCl with all components added except Neu5Ac.

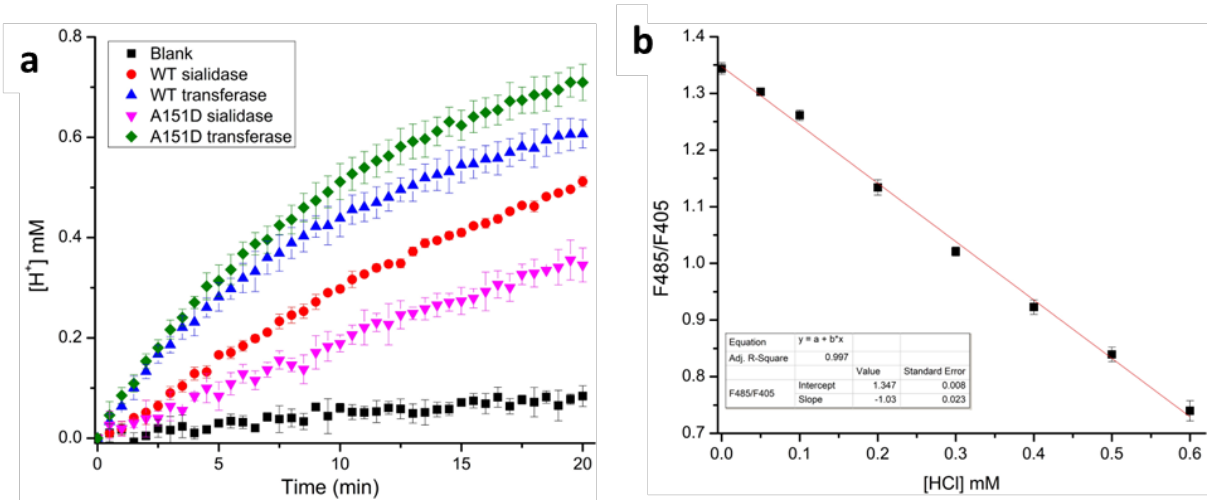


Figure S5. (a) Enzymatic activity of 2,3-sialyltransferase (SiaT, from *Photobacterium phosphoreum*) measured using the fluorescent pH assay. Activities were determined for the wildtype SiaT enzyme and A151D mutant (Mertsch et al. 2020) engineered for lower sialidase activity. Reaction mixture (200 µL) contained 2 µg wt or A151D SiaT, 1 mM CMP-Neu5Ac (CMP-activated sialic acid), 5 mM lactose, 30 mM NaCl, and 0.4 µM HPTS in 2 mM HEPES buffer pH 8.0 at 30°C. Lactose was omitted when measuring sialidase activity. (b) Calibration curve using HCl with all reaction components included except lactose.

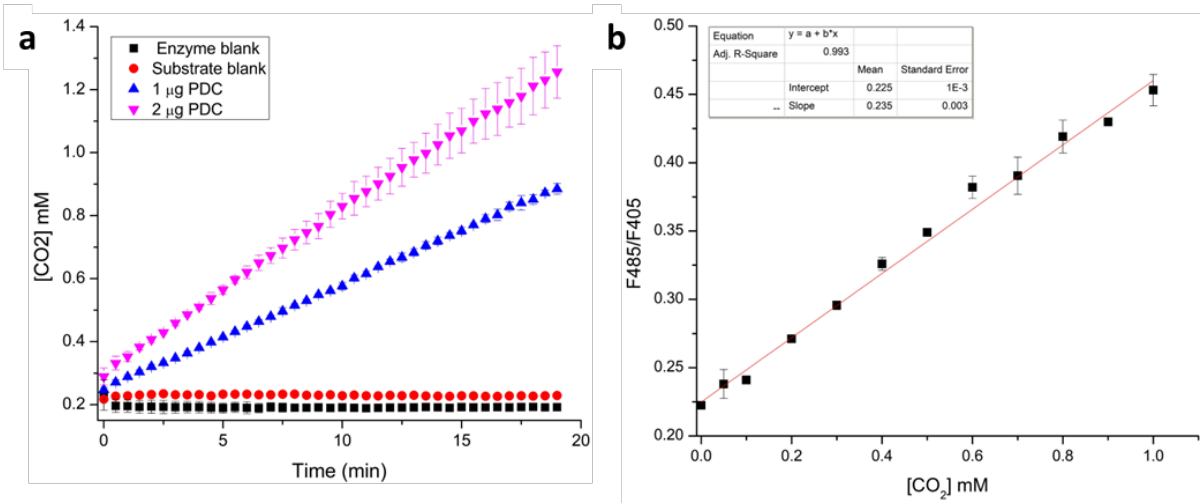


Figure S6. (a) Enzymatic activity of pyruvate decarboxylase (PDC, from *Zymobacter palmae*) measured using the fluorescent pH assay. Reaction mixture (200 µL) contained 1-2 µg PDC, 20 mM Na-pyruvate, 0.1 mM ThDP, 2.5 mM $MgCl_2$, 100 mM NaCl, and 0.4 µM HPTS in 2 mM MOPS buffer pH 6.5 at 25°C. (b) Calibration using 0-1 mM HCO_3^- with all components added except pyruvate.

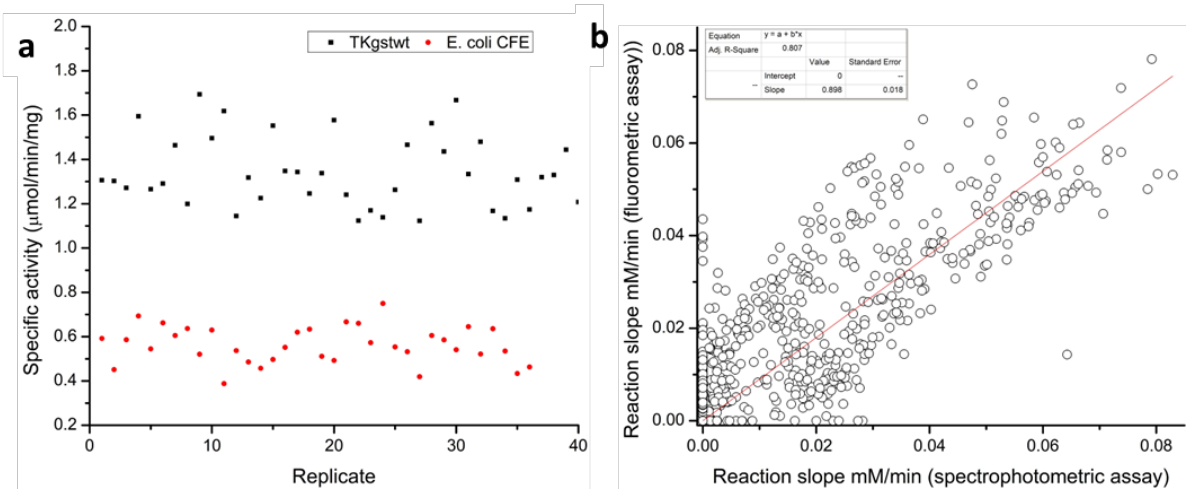


Figure S7. (a) Validation of the overall high-throughput screening system including the fluorescent pH assay by measurement of specific activity of 48 *E. coli* clones expressing the wildtype TK_{gst} (positive controls) and 48 samples of *E. coli* BL21 (DE3) pET21a (negative controls), to determine the *Z*-factor and CV. Enzyme activity assay included 5 mM glycolaldehyde, 50 mM LiHPA, 9 mM $MgCl_2$, 2.4 mM ThDP, and 0.4 mM HPTS in 2 mM HEPES buffer pH 7.0 at 25°C. (b) Correlation of specific activity measurements between fluorescence (HPTS) and spectrophotometric (phenol red) based pH shift assay data for transketolase variant library.

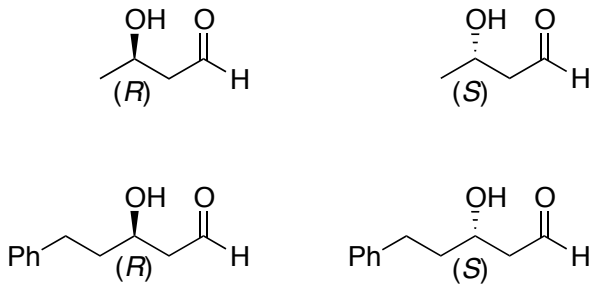
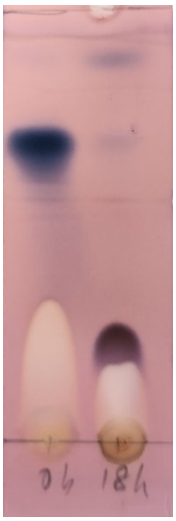


Figure S8. Chiral 3-hydroxyaldehyde substrates (3-hydroxybutanal, HPP) used in the enantioselectivity screening of transketolase libraries.

Enzymatic conversion of 3-hydroxy-5-phenylpentanal



A 10 mL preparative reaction was set up in water containing 2.4 mM ThDP, 9 mM $MgCl_2$, 50 mM Li-HPA, and 15 mM 3-HPP substrate (10% DMSO final concentration), and pH was adjusted to 7.0. Lyophilized heat-purified TK_{gst} (L382F/G264A/S385A) (20 mg) was added to start the reaction. The reaction was monitored at regular intervals on TLC and pH was adjusted by adding 0.1 M HCl as required. After 18 h at 25°C, TLC control indicated complete conversion (ethylacetate/cyclohexane 1:2; anisaldehyde stain).

References

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