Supplementary Information (SI) for Green Chemistry.

Sequential single-enzyme oxidation of 5-hydroxymethylfurfural to 2,5-furandicarboxylicacid by an engineered lanthanide-dependent alcohol dehydrogenase

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Supplementary Figures

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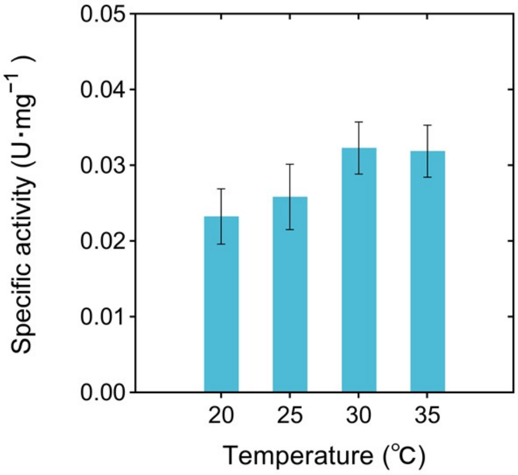

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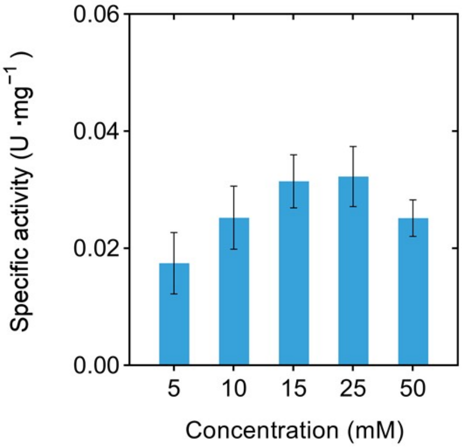
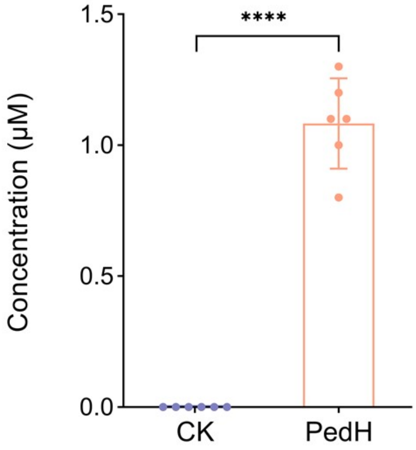
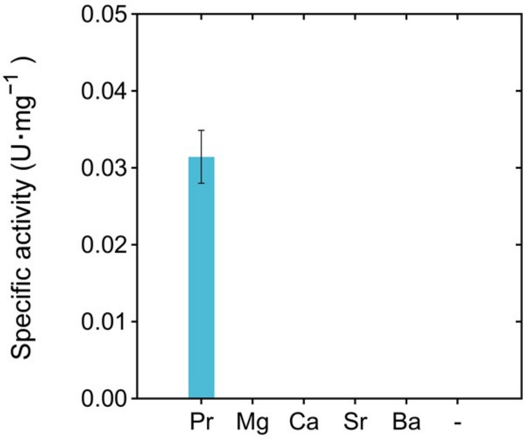


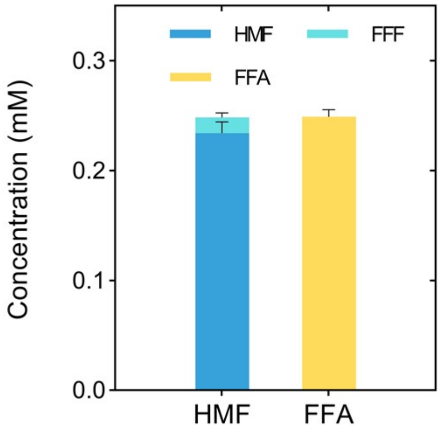
Supplementary Figure 1: The effect of temperature (A) and substrate concentration (B) on the specific activity of PedH toward HMF.



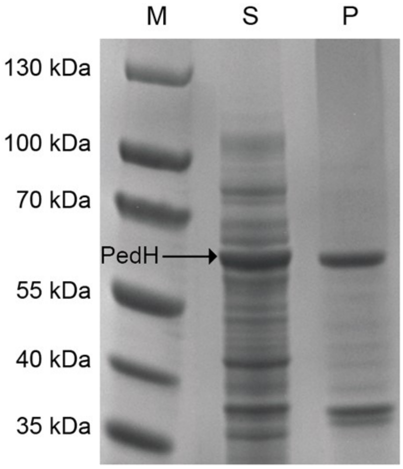
Supplementary Figure 2: FDCA production from HMF catalyzed by PedH. Conversion was performed in 100 mM Tris-HCl buffer (pH 8.0), HMF 0.25 mM, PedH 1 μM, at 30 °C.



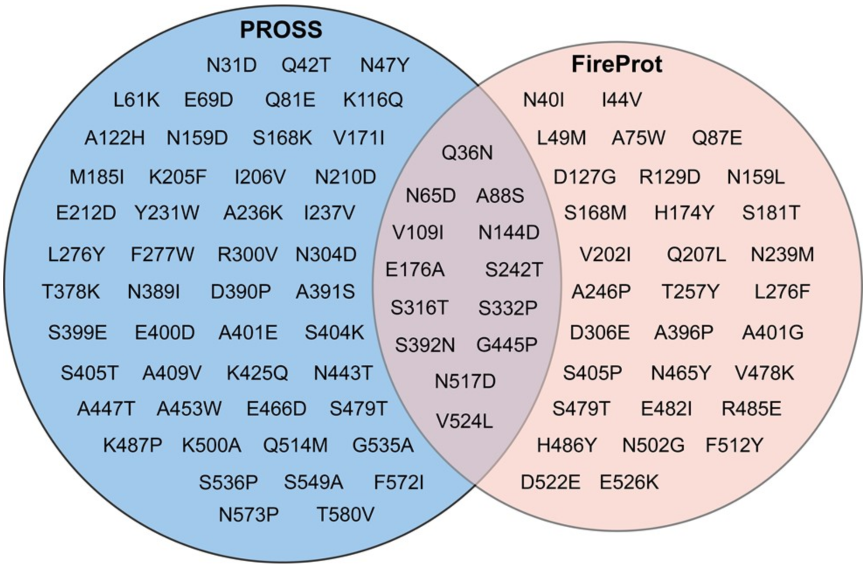
Supplementary Figure 3: The specific activity of PedH toward HMF in the presence of different metal ions. "-" indicates that no metal ions were added.



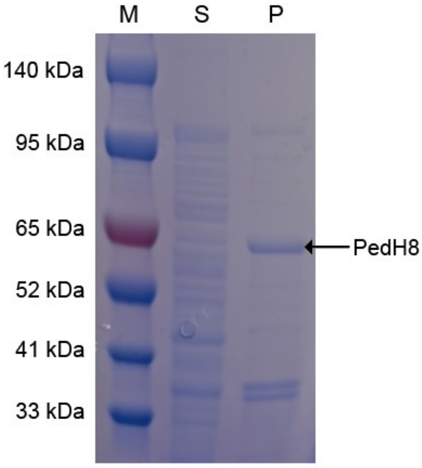
Supplementary Figure 4: Biocatalysis of HMF and FFA using PedE. Conversions were performed in triplicate in 100 mM Tris-HCl buffer (pH 8.0), PedE 1 μM, 0.25 mM of substrates, at 30 °C, for 24 h.



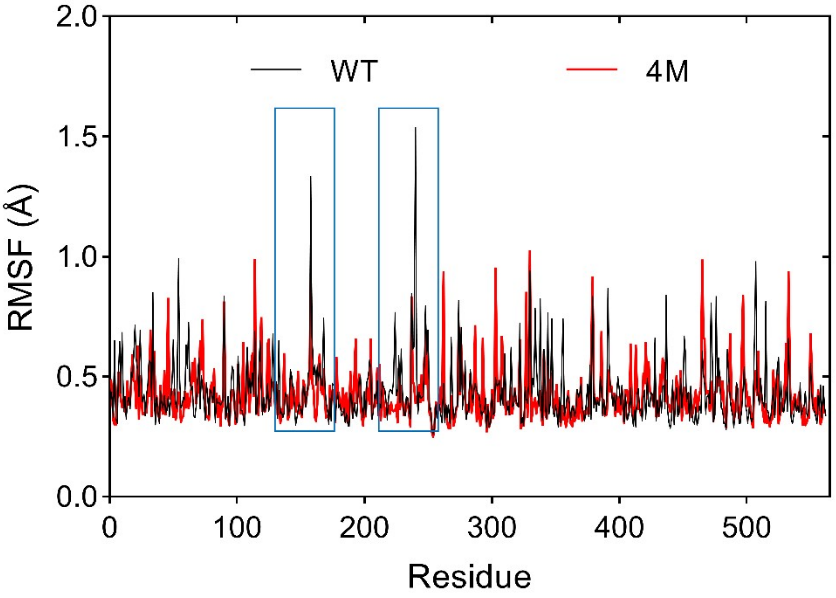
Supplementary Figure 5: SDS-PAGE analysis of PedH wild type. Lane M, protein marker; lane 1, supernatant of cell lysates by sonication of recombinant *E. coli*; lane 2, precipitate of cell lysates by sonication of recombinant *E. coli*.



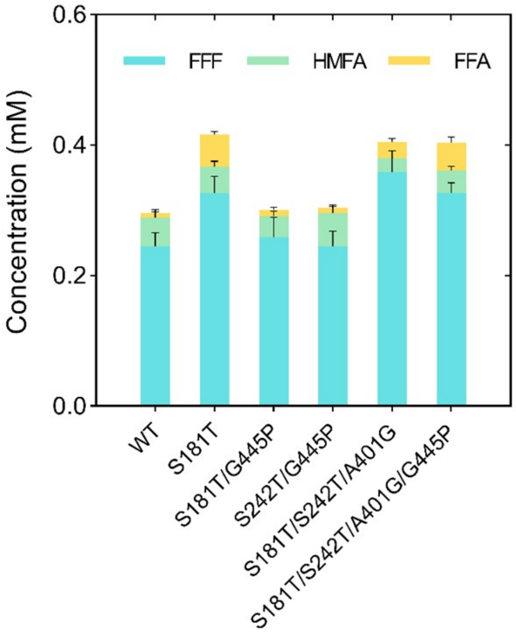
Supplementary Figure 6: The thermostable single mutants predicted by PROSS and FireProt.



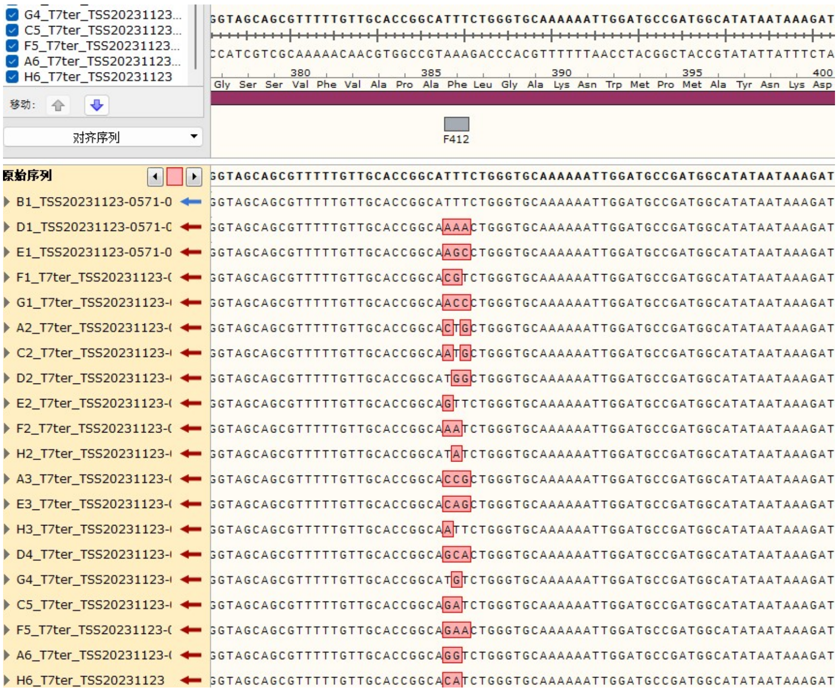
Supplementary Figure 7: SDS-PAGE analysis of variant PedH8.



Supplementary Figure 8: Root-mean-square fluctuations (RMSF) values of backbone atoms for WT and 4M.



Supplementary Figure 9: Transformation of HMF using thermostable variants. Conversions were performed in triplicate in 100 mM Tris-HCl buffer (pH 8.0), HMF 10 mM, PedH 1 μM, 30 °C.



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☑ G4_T7ter_TSS20231123...
☑ C5_T7ter_TSS20231123...
☑ F5_T7ter_TSS20231123...
☑ A6_T7ter_TSS20231123...
☑ H6_T7ter_TSS20231123

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Gly Ser Ser Val Phe Val Ala Pro Ala Phe Leu Gly Ala Lys Asn Trp Met Pro Met Ala Tyr Asn Lys Asp

F412

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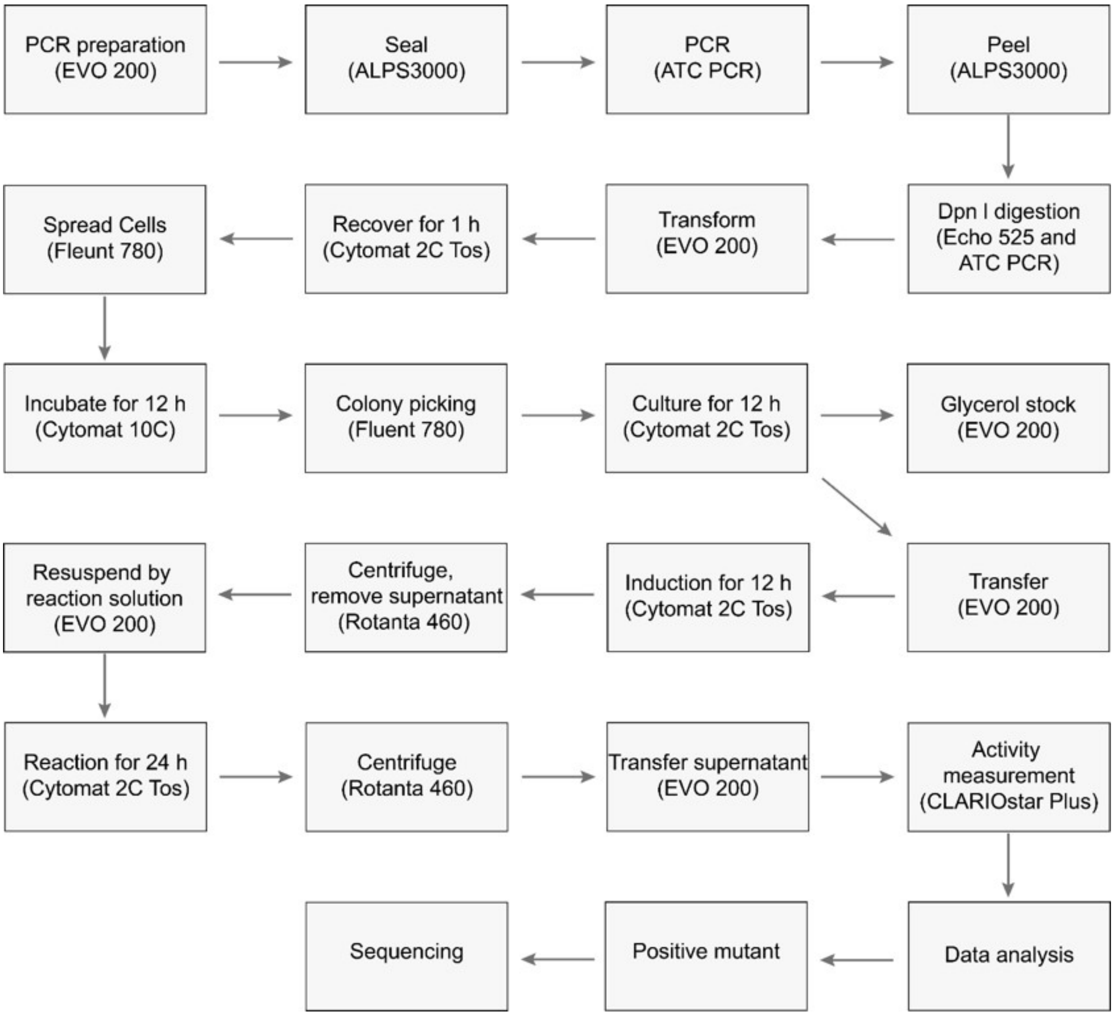
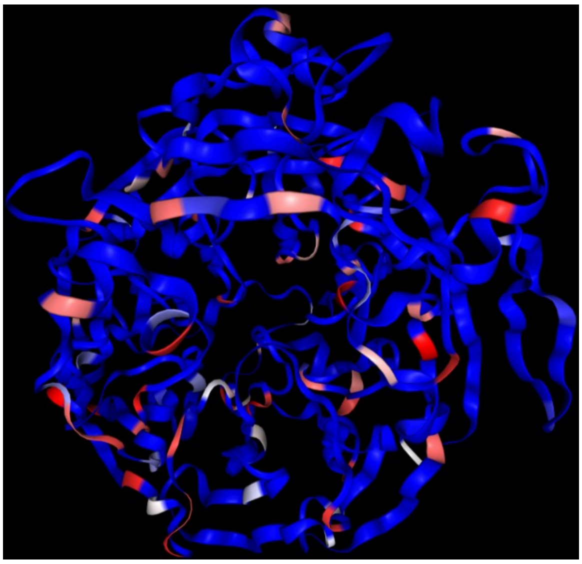
Supplementary Figure 10: Quality validation of site-saturation mutagenesis libraries with NNK randomization.

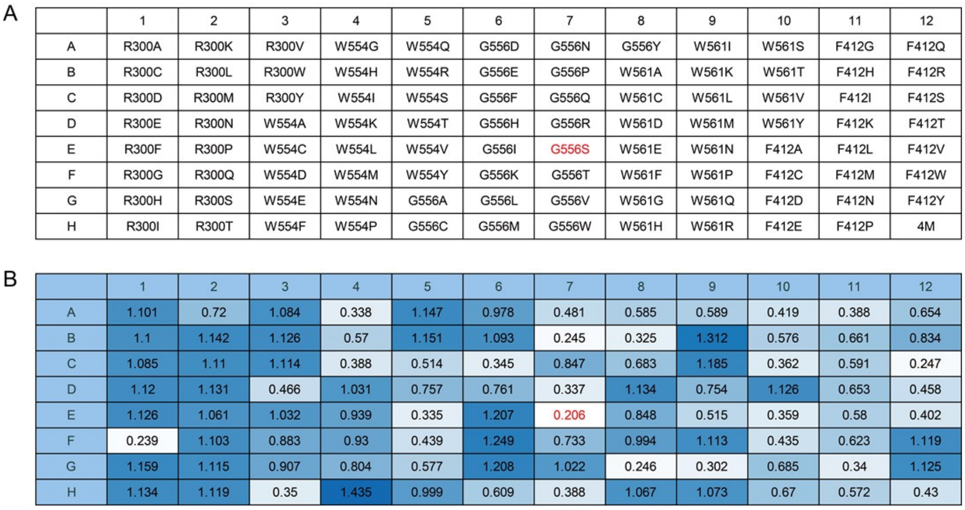
Supplementary Figure 11: Site-directed mutations on agar plates that were carried out by iBiofoundry.



Supplementary Figure 12: Process flow diagram for automatic screening of PedH variants.



Supplementary Figure 13: Heatmap of mutation predictions for PedH by MutCompute. Disfavored residues are marked in red. Disfavored residues refer to those where the predicted amino acid with the highest probability differs from the wild-type amino acid.



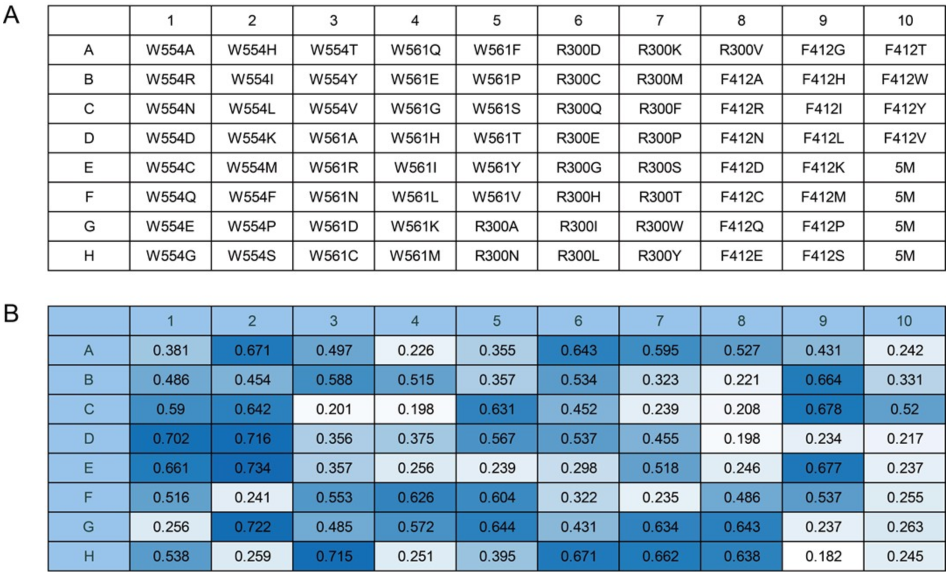
A

	1	2	3	4	5	6	7	8	9	10	11	12
A	R300A	R300K	R300V	W554G	W554Q	G556D	G556N	G556Y	W561I	W561S	F412G	F412Q
B	R300C	R300L	R300W	W554H	W554R	G556E	G556P	W561A	W561K	W561T	F412H	F412R
C	R300D	R300M	R300Y	W554I	W554S	G556F	G556Q	W561C	W561L	W561V	F412I	F412S
D	R300E	R300N	W554A	W554K	W554T	G556H	G556R	W561D	W561M	W561Y	F412K	F412T
E	R300F	R300P	W554C	W554L	W554V	G556I	G556S	W561E	W561N	F412A	F412L	F412V
F	R300G	R300Q	W554D	W554M	W554Y	G556K	G556T	W561F	W561P	F412C	F412M	F412W
G	R300H	R300S	W554E	W554N	G556A	G556L	G556V	W561G	W561Q	F412D	F412N	F412Y
H	R300I	R300T	W554F	W554P	G556C	G556M	G556W	W561H	W561R	F412E	F412P	4M

B

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.101	0.72	1.084	0.338	1.147	0.978	0.481	0.585	0.589	0.419	0.388	0.654
B	1.1	1.142	1.126	0.57	1.151	1.093	0.245	0.325	1.312	0.576	0.661	0.834
C	1.085	1.11	1.114	0.388	0.514	0.345	0.847	0.683	1.185	0.362	0.591	0.247
D	1.12	1.131	0.466	1.031	0.757	0.761	0.337	1.134	0.754	1.126	0.653	0.458
E	1.126	1.061	1.032	0.939	0.335	1.207	0.206	0.848	0.515	0.359	0.58	0.402
F	0.239	1.103	0.883	0.93	0.439	1.249	0.733	0.994	1.113	0.435	0.623	1.119
G	1.159	1.115	0.907	0.804	0.577	1.208	1.022	0.246	0.302	0.685	0.34	1.125
H	1.134	1.119	0.35	1.435	0.999	0.609	0.388	1.067	1.073	0.67	0.572	0.43

Supplementary Figure 14: Plate reader data of first-round iterative saturation mutagenesis (ISM).



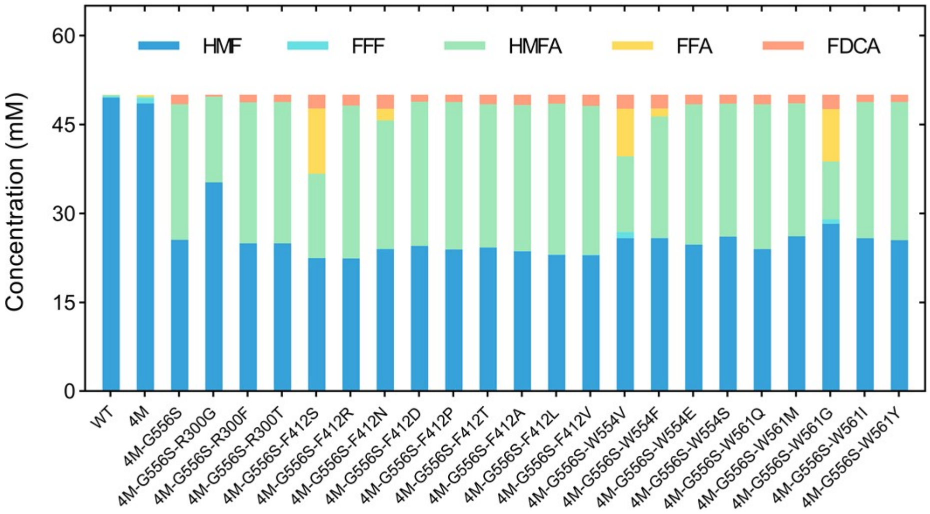
A

	1	2	3	4	5	6	7	8	9	10
A	W554A	W554H	W554T	W561Q	W561F	R300D	R300K	R300V	F412G	F412T
B	W554R	W554I	W554Y	W561E	W561P	R300C	R300M	F412A	F412H	F412W
C	W554N	W554L	W554V	W561G	W561S	R300Q	R300F	F412R	F412I	F412Y
D	W554D	W554K	W561A	W561H	W561T	R300E	R300P	F412N	F412L	F412V
E	W554C	W554M	W561R	W561I	W561Y	R300G	R300S	F412D	F412K	5M
F	W554Q	W554F	W561N	W561L	W561V	R300H	R300T	F412C	F412M	5M
G	W554E	W554P	W561D	W561K	R300A	R300I	R300W	F412Q	F412P	5M
H	W554G	W554S	W561C	W561M	R300N	R300L	R300Y	F412E	F412S	5M

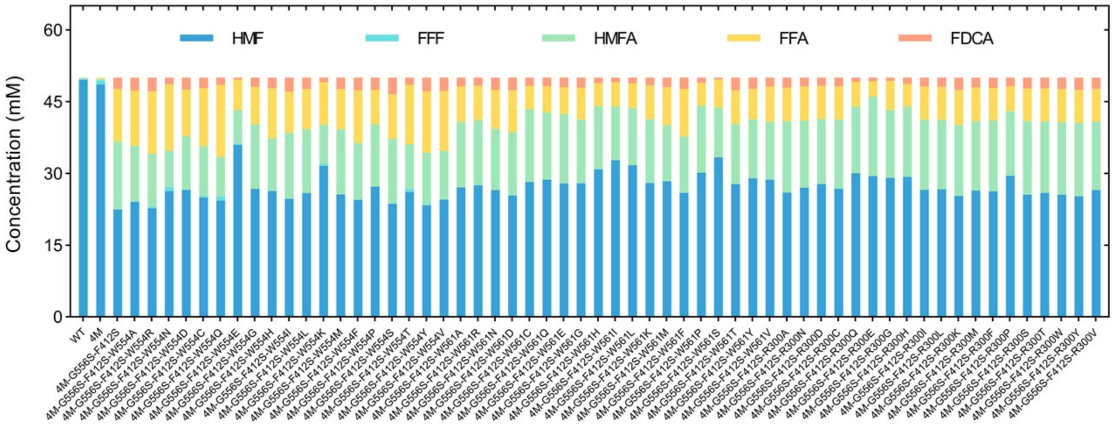
B

	1	2	3	4	5	6	7	8	9	10
A	0.381	0.671	0.497	0.226	0.355	0.643	0.595	0.527	0.431	0.242
B	0.486	0.454	0.588	0.515	0.357	0.534	0.323	0.221	0.664	0.331
C	0.59	0.642	0.201	0.198	0.631	0.452	0.239	0.208	0.678	0.52
D	0.702	0.716	0.356	0.375	0.567	0.537	0.455	0.198	0.234	0.217
E	0.661	0.734	0.357	0.256	0.239	0.298	0.518	0.246	0.677	0.237
F	0.516	0.241	0.553	0.626	0.604	0.322	0.235	0.486	0.537	0.255
G	0.256	0.722	0.485	0.572	0.644	0.431	0.634	0.643	0.237	0.263
H	0.538	0.259	0.715	0.251	0.395	0.671	0.662	0.638	0.182	0.245

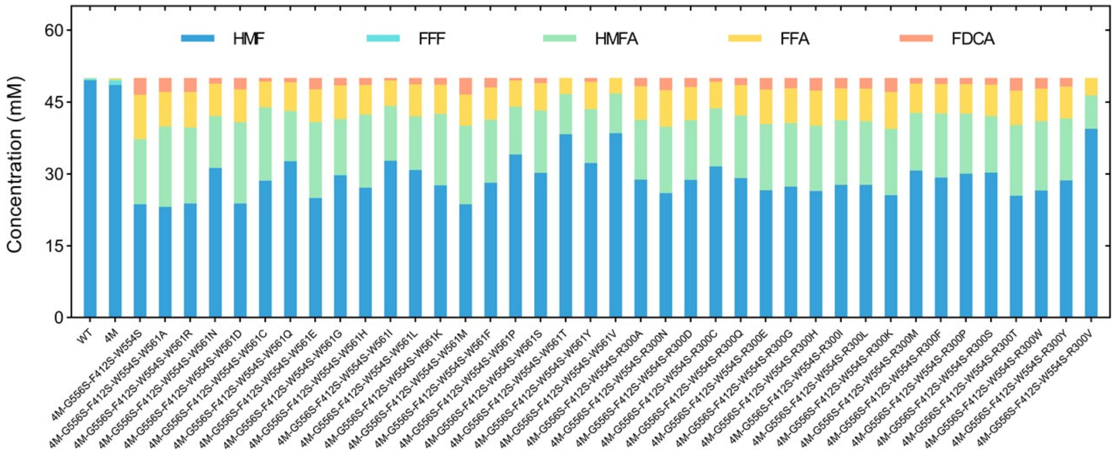
Supplementary Figure 15: Plate reader data of second-round perfect iterative saturation mutagenesis.



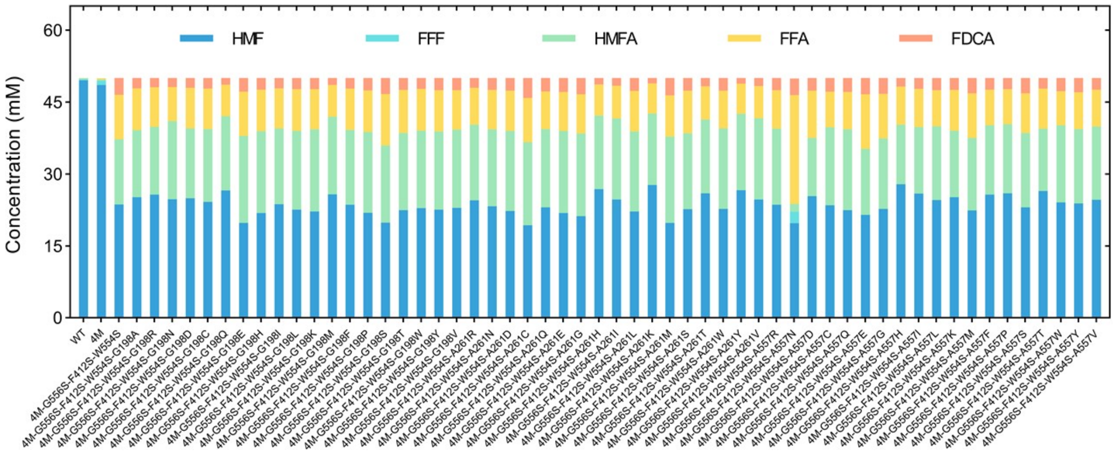
Supplementary Figure 16: Whole-cell conversion results of selected mutants from the second round of evolution.



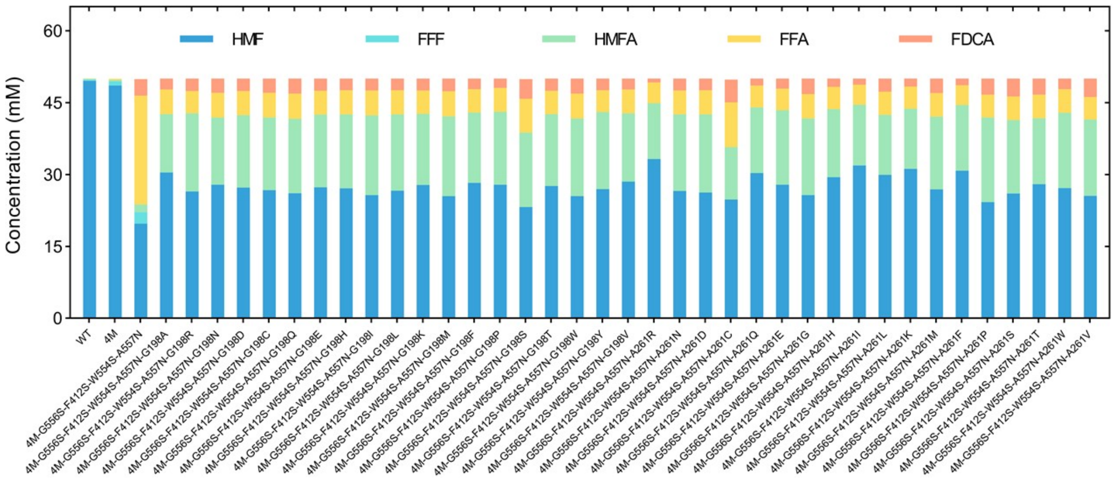
Supplementary Figure 17: Whole-cell conversion results of mutants from the third round of evolution.



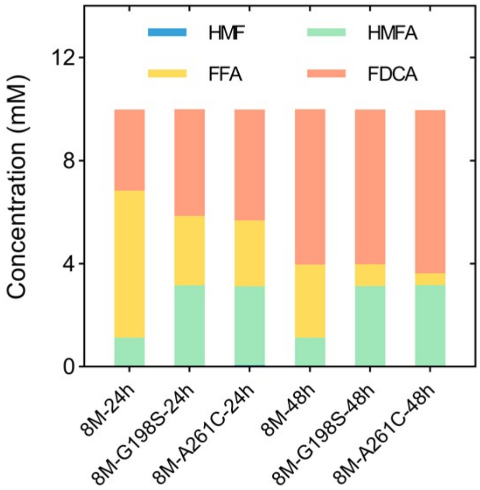
Supplementary Figure 18: Whole-cell conversion results of mutants from the fourth round of evolution.



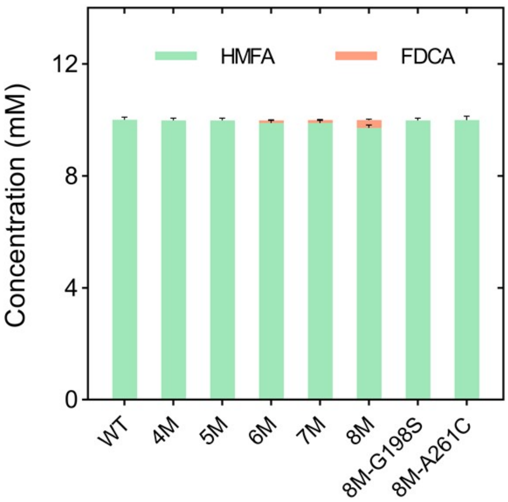
Supplementary Figure 19: Whole-cell conversion results of additional mutants from the fourth round of evolution.



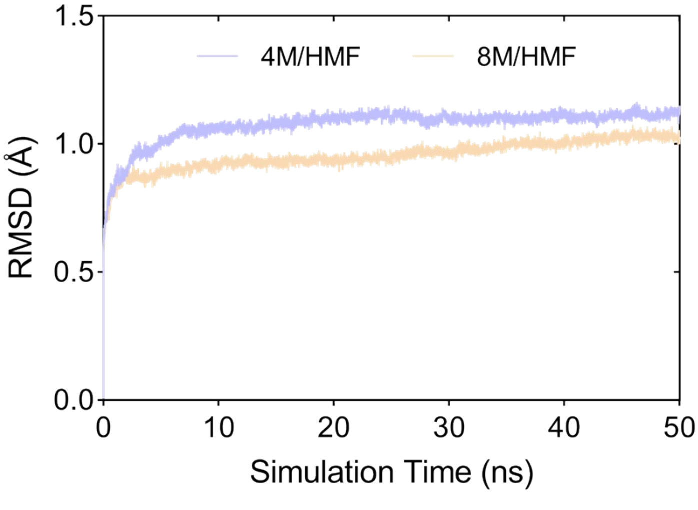
Supplementary Figure 20: Whole-cell conversion results of mutants from the fifth round of evolution.



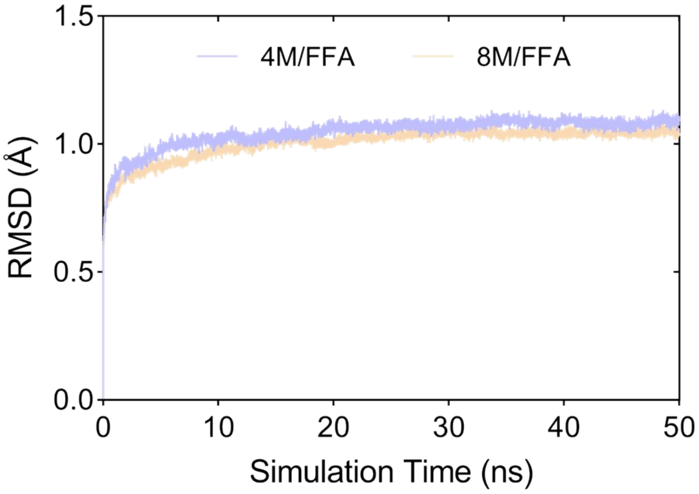
Supplementary Figure 21: Transformation of HMF using 8M, 8M-G198S and 8M-A261C. Conversions were performed in triplicate in 100 mM Tris-HCl buffer (pH 8.0), HMF 10 mM, cells OD_{600}=10, 30 ^{o}C.



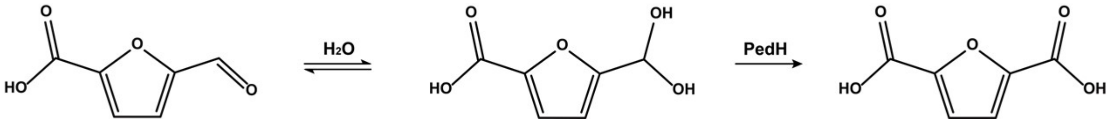
Supplementary Figure 22: Conversion of HMFA by different mutants. Conversions were performed in triplicate in 100 mM Tris-HCl buffer (pH 8.0), HMFA 10 mM, cells OD_{600}=10, 30 °C.



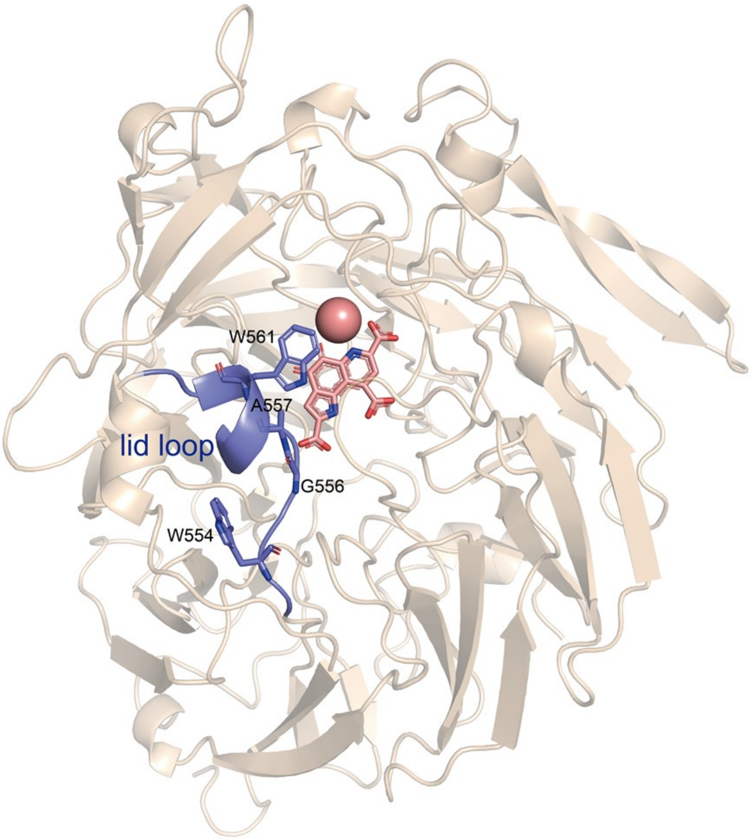
Supplementary Figure 23: RMSD values of 4M/HMF and 8M/HMF calculated for protein backbone atoms with the initial structure as reference. RMSD: root mean squared deviation.



Supplementary Figure 24: RMSD values of 4M/FFA and 8M/FFA calculated for protein backbone atoms with the initial structure as reference.



Supplementary Figure 25: Hydration and oxidation of FFA. The formed *gem*-diol is oxidized by the PedH to form the FDCA.



Supplementary Figure 26: Schematic representation of the lid loop and the relative positions of the W554, G556, A557, and W561 sites. The lid loop is shown in blue.

Supplementary Tables

Supplementary Table 1: Amino acid sequences of PedH5, PedH6, PedH7 and PedH8 predicted by PROSS. The mutation sites are highlighted in yellow.

PedH5	MAVSDEEILNDPKNPQQIVTYGLGVQGQRYSPLDKLNVNNVKELRPVWAFSFGGEKQRGQQAQPLIKDGVMYLTASYSRVFAVDARTGKKLWQYDARLPDDIRPCCDVINRGVALYGDLVFFGTLDAKLVALNKDTGKVVWSKKVAD

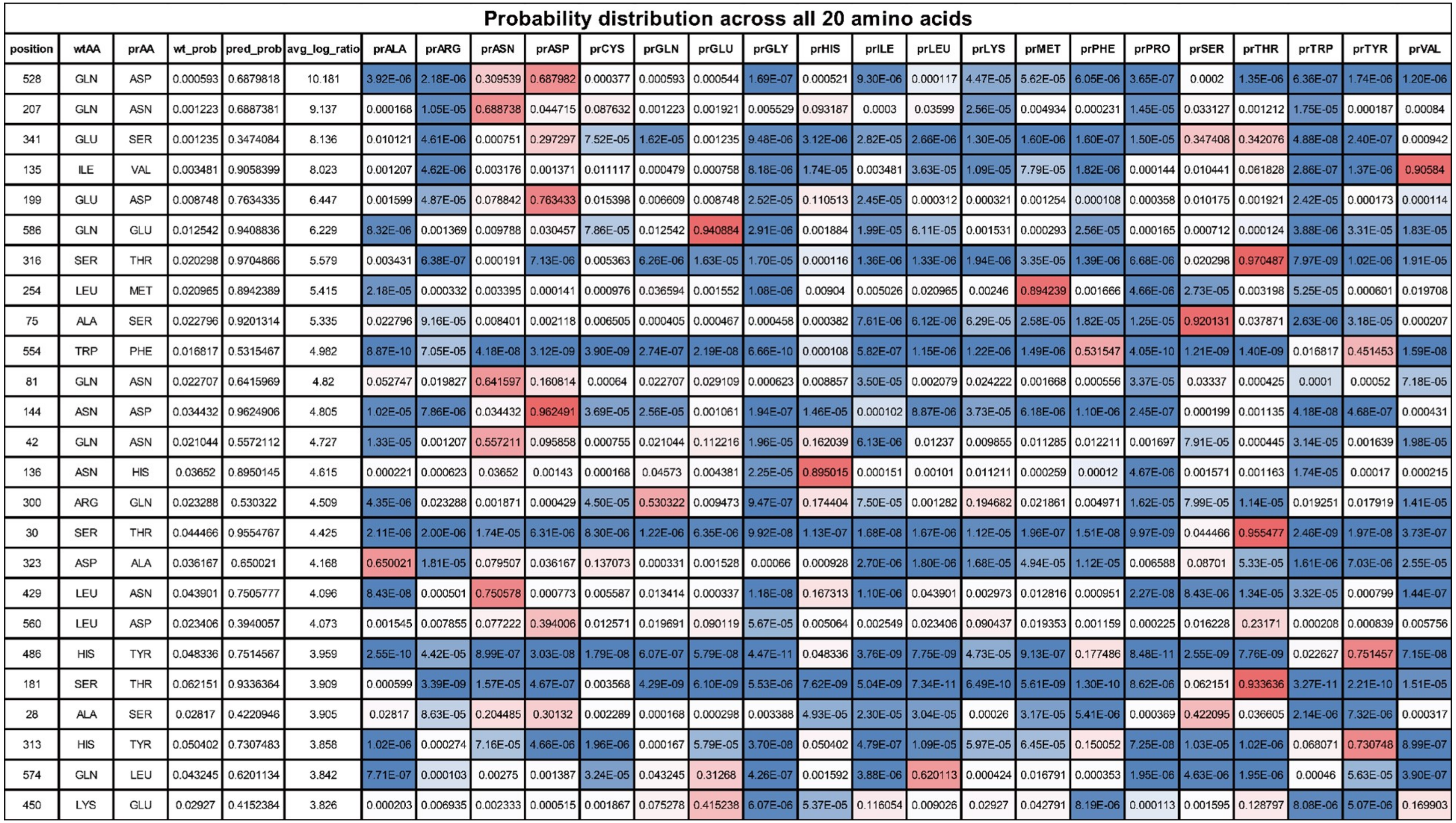
	HKEGYSISAAPMIVNGKLITGVAGGEFGVVGFIQAYNPENGELLWMRPTVEGHMGYVVYKDGKAIENGISGGEPGKTWPGDLWKTGGAAPWLGGYYDPETNLILWGTGNPAPWNSHLRPGDNLYSSSLLALNPDDGKIKWHFQHTPHDGWDFDGVNELIPFNYKDGGKEVKAAATADRNGFFYVLDRTNGKFIRGFPFVDKITWATGLDKDGRPIYNDANRPGDPGSEEKGSSVFVVPAFLGAKNWMPMAYNQDTGLFYVPSNEWGMDIWNEPITYKKGAAFLGAGFTIKPLNEDYIGVLRAIDPVSGKEVWRHPNYAPLWGGVLTTKGNLVFTGTPEGYLMAFNAKTGDKVWEFQTGSGVLGPPVTWEMDGEQYVAVVSGWGGAVPLWGGEVAKRVKDINQGGMLWVFKLPKQLQQTASVKPHHHHHH
PedH6	MAVSDEEILNDPKNPQQIVTYGLGVQGQRYSPLDKLNVDNVKELRPVWAFSFGGEKQRGQQAQPLIKDGVMYLTASYSRVFAVDARTGKKLWQYDHRLPDDIRPCCDVINRGVALYGDLVFFGTLDAKLVALNKDTGKVVWSKKVADHKEGYSISAAPMIVNGKLITGVAGGEFGVVGFIQAYNPENGELLWMRPTVEGHMGYVVYKDGKAIENGISGGEPGKTWPGDLWKTGGAAPWLGGYYDPETNLILWGTGNPAPWNSHLRPGDNLYSSSLLALNPDDGKIKWHFQHTPNDAWDFDGVNELIPFNYKDGGKEVKAAATADRNGFFYVLDRTNGKFIRGFPFVDKITWATGLDKDGRPIYNDANRPGDPGSEEKGSSVFVVPAFLGAKNWMPMAYNQDTGLFYVPSNEWGMDIWNEPITYKKGAPYLGAGFTIKPLNEDYIGVLRAIDPVSGKEVWRHPNYAPLWGGVLTTKGNLVFTGTPEGYLMAFNAKTGDKVWEFQTGSGVLGPPVTWEMDGEQYVAVVSGWGGAVPLWGGEVAKRVKDINQGGMLWVFKLPKQLQQTASVKPHHHHHH
PedH7	MAVSDEEILNDPKNPTQIVTYGLGVQGQRYSPLDKLNVDNVKELRPVWAFSFGGEKQRGQQAQPLIKDGVMYLTASYSRVFAVDARTGKKLWQYDHRLPDDIRPCCDVINRGVALYGDLVFFGTLDAKLVALNKDTGKVVWSKKVADHKAGYSISAAPMIVNGKLITGVAGGEFGVVGFIQAYDPENGELLWMRPTVEGHMGYVVYKDGKAIENGISGGEPGKTWPGDLWKTGGAAPWLGGYYDPETNLIFWGTGNPAPWNSHLRPGDNLYSSSVLALNPDDGKIKWHFQYTPNDAWDFDGVNELIPFNYKDGGKEVKAAATADRNGFFYVLDRTNGKFIRGFPFVDKITWATGLDKDGRPIYNDSSRPGDPGSEEKGKTVFVVPAFLGAKNWMPMAYNQDTGLFYVPSNEWGMDIWNEPITYKKGAPYLGAGFTIKPLNEDYIGVLRAIDPVSGKEVWRHPNYAPLWGGVLTTAGNLVFTGTPEGYLMAFDAKTGKKVWEFQTGSGVLGPPVTWEMDGEQYVAVVSGWGGAVPLWGGEVAKRVKDINQGGMLWVFKLPKQLQQTASVKPHHHHHH
PedH8	MAVSDEEILNDPKNPTQIVTYGLGVQGQRYSPLDKLNVDNVKELRPVWAFSFGGEKQRGQQAQPLIKDGVMYLTASYSRVFAIDARTGKKLWQYDHRLPDDIRPCCDVINRGVALYGDLVFFGTLDAKLVALNKDTGKVVWKKKVADHKAGYSISAAPMIVNGKLITGVAGGEFGVVGFVQAYDPENGELLWMRPTVEGHMGYVVYKDGKAIENGISGGEPGKTWPGDLWKTGGAAPWLGGYYDPETNLIFWGTGNPAPWNSHLRPGDNLYSCSVLALNPDDGKIKWHFQYTPNDAWDFDGVNELIPFNYKDGGKEVKAAATADRNGFFYVLDRTNGKFIRGFPFVDKITWATGLDKDGRPIYIPDNRPGDPGSDEKGKTVFVVPAFLGAKNWMPMAYNQDTGLFYVPSNEWGMDIWNEPITYKKGAPYLGAGFTIKPLNEDYIG

	VLRAIDPVSGKEVWRHPNYAPLWGGVLTTAGNLVFTGTPEGYLMAFDAK TGKKVWEFQTGSGVLGPPVTWEMDGEQYVAVVSGWGGAVPLWGGEVA KRVKDIPQGGMLWVFKLPKQLQQTASVKPHHHHHH

Supplementary Table 2: Hydrogen bond occurrence analysis of thermostable mutant.

	WT	4M
S/T242.OG1 - P221.O	68.6%	88.6%
A/G374.O - WAT561.O	0	93%

Supplementary Table 3: Disfavored PedH residues predicted by MutCompute. MutCompute outputs a probability distribution that describes the likelihood of each of the 20 standard amino acids occurring as the wild-type amino acid in the surrounding chemical environment. Avg_log_ratio refers to the fold difference in probability between the predicted amino acid and the wild-type amino acid. Here, a cutoff of avg_log_ratio >1 was used to down select disfavored residues.



Probability distribution across all 20 amino acids																									
position	**wtAA**	**prAA**	**wt_prob**	**pred_prob**	**avg_log_ratio**	**prALA**	**prARG**	**prASN**	**prASP**	**prCYS**	**prGLN**	**prGLU**	**prGLY**	**prHIS**	**prILE**	**prLEU**	**prLYS**	**prMET**	**prPHE**	**prPRO**	**prSER**	**prTHR**	**prTRP**	**prTYR**	**prVAL**
528	GLN	ASP	0.000593	0.6879818	10.181	3.92E-06	2.18E-06	0.309539	0.687982	0.000377	0.000593	0.000544	1.69E-07	0.000521	9.30E-06	0.000117	4.47E-05	5.62E-05	6.05E-06	3.65E-07	0.0002	1.35E-06	6.36E-07	1.74E-06	1.20E-06
207	GLN	ASN	0.001223	0.6887381	9.137	0.000168	1.05E-05	0.688738	0.044715	0.087632	0.001223	0.001921	0.005529	0.093187	0.0003	0.03599	2.56E-05	0.004934	0.000231	1.45E-05	0.033127	0.001212	1.75E-05	0.000187	0.00084
341	GLU	SER	0.001235	0.3474084	8.136	0.010121	4.61E-06	0.000751	0.297297	7.52E-05	1.62E-05	0.001235	9.48E-06	3.12E-06	2.82E-05	2.66E-06	1.30E-05	1.60E-06	1.60E-07	1.50E-05	0.347408	0.342076	4.88E-08	2.40E-07	0.000942
135	ILE	VAL	0.003481	0.9058399	8.023	0.001207	4.62E-06	0.003176	0.001371	0.011117	0.000479	0.000758	8.18E-06	1.74E-05	0.003481	3.63E-05	1.09E-05	7.79E-05	1.82E-06	0.000144	0.010441	0.061828	2.86E-07	1.37E-06	0.90584
199	GLU	ASP	0.008748	0.7634335	6.447	0.001599	4.87E-05	0.078842	0.763433	0.015398	0.006609	0.008748	2.52E-05	0.110513	2.45E-05	0.000312	0.000321	0.001254	0.000108	0.000358	0.010175	0.001921	2.42E-05	0.000173	0.000114
586	GLN	GLU	0.012542	0.9408836	6.229	8.32E-06	0.001369	0.009788	0.030457	7.86E-05	0.012542	0.940884	2.91E-06	0.001884	1.99E-05	6.11E-05	0.001531	0.000293	2.56E-05	0.000165	0.000712	0.000124	3.88E-06	3.31E-05	1.83E-05
316	SER	THR	0.020298	0.9704866	5.579	0.003431	6.38E-07	0.000191	7.13E-06	0.005363	6.26E-06	1.63E-05	1.70E-05	0.000116	1.36E-06	1.33E-06	1.94E-06	3.35E-05	1.39E-06	6.68E-06	0.020298	0.970487	7.97E-09	1.02E-06	1.91E-05
254	LEU	MET	0.020965	0.8942389	5.415	2.18E-05	0.000332	0.003395	0.000141	0.000976	0.036594	0.001552	1.08E-06	0.00904	0.005026	0.020965	0.00246	0.894239	0.001666	4.66E-06	2.73E-05	0.003198	5.25E-05	0.000601	0.019708
75	ALA	SER	0.022796	0.9201314	5.335	0.022796	9.16E-05	0.008401	0.002118	0.006505	0.000405	0.000467	0.000458	0.000382	7.61E-06	6.12E-06	6.29E-05	2.58E-05	1.82E-05	1.25E-05	0.920131	0.037871	2.63E-06	3.18E-05	0.000207
554	TRP	PHE	0.016817	0.5315467	4.982	8.87E-10	7.05E-05	4.18E-08	3.12E-09	3.90E-09	2.74E-07	2.19E-08	6.66E-10	0.000108	5.82E-07	1.15E-06	1.22E-06	1.49E-06	0.531547	4.05E-10	1.21E-09	1.40E-09	0.016817	0.451453	1.59E-08
81	GLN	ASN	0.022707	0.6415969	4.82	0.052747	0.019827	0.641597	0.160814	0.00064	0.022707	0.029109	0.000623	0.008857	3.50E-05	0.002079	0.024222	0.001668	0.000556	3.37E-05	0.03337	0.000425	0.0001	0.00052	7.18E-05
144	ASN	ASP	0.034432	0.9624906	4.805	1.02E-05	7.86E-06	0.034432	0.962491	3.69E-05	2.56E-05	0.001061	1.94E-07	1.46E-05	0.000102	8.87E-06	3.73E-05	6.18E-06	1.10E-06	2.45E-07	0.000199	0.001135	4.18E-08	4.68E-07	0.000431
42	GLN	ASN	0.021044	0.5572112	4.727	1.33E-05	0.001207	0.557211	0.095858	0.000755	0.021044	0.112216	1.96E-05	0.162039	6.13E-06	0.01237	0.009855	0.011285	0.012211	0.001697	7.91E-05	0.000445	3.14E-05	0.001639	1.98E-05
136	ASN	HIS	0.03652	0.8950145	4.615	0.000221	0.000623	0.03652	0.00143	0.000168	0.04573	0.004381	2.25E-05	0.895015	0.000151	0.00101	0.011211	0.000259	0.00012	4.67E-06	0.001571	0.001163	1.74E-05	0.00017	0.000215
300	ARG	GLN	0.023288	0.530322	4.509	4.35E-06	0.023288	0.001871	0.000429	4.50E-05	0.530322	0.009473	9.47E-07	0.174404	7.50E-05	0.001282	0.194682	0.021861	0.004971	1.62E-05	7.99E-05	1.14E-05	0.019251	0.017919	1.41E-05
30	SER	THR	0.044466	0.9554767	4.425	2.11E-06	2.00E-06	1.74E-05	6.31E-06	8.30E-06	1.22E-06	6.35E-06	9.92E-08	1.13E-07	1.68E-08	1.67E-06	1.12E-05	1.96E-07	1.51E-08	9.97E-09	0.044466	0.955477	2.46E-09	1.97E-08	3.73E-07
323	ASP	ALA	0.036167	0.650021	4.168	0.650021	1.81E-05	0.079507	0.036167	0.137073	0.000331	0.001528	0.00066	0.000928	2.70E-06	1.80E-06	1.68E-05	4.94E-05	1.12E-05	0.006588	0.08701	5.33E-05	1.61E-06	7.03E-06	2.55E-05
429	LEU	ASN	0.043901	0.7505777	4.096	8.43E-08	0.000501	0.750578	0.000773	0.005587	0.013414	0.000337	1.18E-08	0.167313	1.10E-06	0.043901	0.002973	0.012816	0.000951	2.27E-08	8.43E-06	1.34E-05	3.32E-05	0.000799	1.44E-07
560	LEU	ASP	0.023406	0.3940057	4.073	0.001545	0.007855	0.077222	0.394006	0.012571	0.019691	0.090119	5.67E-05	0.005064	0.002549	0.023406	0.090437	0.019353	0.001159	0.000225	0.016228	0.23171	0.000208	0.000839	0.005756
486	HIS	TYR	0.048336	0.7514567	3.959	2.55E-10	4.42E-05	8.99E-07	3.03E-08	1.79E-08	6.07E-07	5.79E-08	4.47E-11	0.048336	3.76E-09	7.75E-09	4.73E-05	9.13E-07	0.177486	8.48E-11	2.55E-09	7.76E-09	0.022627	0.751457	7.15E-08
181	SER	THR	0.062151	0.9336364	3.909	0.000599	3.39E-09	1.57E-05	4.67E-07	0.003568	4.29E-09	6.10E-09	5.53E-06	7.62E-09	5.04E-09	7.34E-11	6.49E-10	5.61E-09	1.30E-10	8.62E-06	0.062151	0.933636	3.27E-11	2.21E-10	1.51E-05
28	ALA	SER	0.02817	0.4220946	3.905	0.02817	8.63E-05	0.204485	0.30132	0.002289	0.000168	0.000298	0.003388	4.93E-05	2.30E-05	3.04E-05	0.00026	3.17E-05	5.41E-06	0.000369	0.422095	0.036605	2.14E-06	7.32E-06	0.000317
313	HIS	TYR	0.050402	0.7307483	3.858	1.02E-06	0.000274	7.16E-05	4.66E-06	1.96E-06	0.000167	5.79E-05	3.70E-08	0.050402	4.79E-07	1.09E-05	5.97E-05	6.45E-05	0.150052	7.25E-08	1.03E-05	1.02E-06	0.068071	0.730748	8.99E-07
574	GLN	LEU	0.043245	0.6201134	3.842	7.71E-07	0.000103	0.00275	0.001387	3.24E-05	0.043245	0.31268	4.26E-07	0.001592	3.88E-06	0.620113	0.000424	0.016791	0.000353	1.95E-06	4.63E-06	1.95E-06	0.00046	5.63E-05	3.90E-07
450	LYS	GLU	0.02927	0.4152384	3.826	0.000203	0.006935	0.002333	0.000515	0.001867	0.075278	0.415238	6.07E-06	5.37E-05	0.116054	0.009026	0.02927	0.042791	8.19E-06	0.000113	0.001595	0.128797	8.08E-06	5.07E-06	0.169903

419	MET	GLN	0.026211	0.3563406	3.765	0.000647	0.011	0.018498	0.000105	0.000202	0.356341	0.014837	2.09E-05	0.207114	4.33E-07	0.000258	0.000265	0.026211	0.018108	0.000117	0.000688	2.43E-06	0.020402	0.325183	3.43E-07
82	LYS	GLN	0.053764	0.6969547	3.696	1.94E-09	0.000329	0.000433	7.35E-06	1.09E-06	0.696955	0.053669	8.12E-10	0.000258	1.96E-06	0.028861	0.053764	0.165164	0.00012	1.84E-09	1.06E-08	2.77E-08	0.000289	0.000148	5.87E-09
126	ASP	SER	0.034285	0.4366157	3.671	0.06801	0.029967	0.008976	0.034285	0.00214	0.035362	0.235166	0.005675	0.002407	0.005398	0.003937	0.089013	0.002274	0.000683	0.009788	0.436616	0.023174	0.000214	0.000564	0.006349
329	GLU	PRO	0.06281	0.7932398	3.659	0.006554	8.75E-06	0.001503	0.00014	0.000228	0.115848	0.06281	8.21E-05	7.15E-07	9.54E-07	1.51E-06	5.31E-06	1.38E-05	1.24E-08	0.79324	0.019543	1.71E-05	9.86E-09	2.23E-08	2.13E-06
439	MET	GLN	0.072977	0.8699816	3.575	3.61E-08	1.05E-07	3.39E-07	2.32E-07	1.02E-05	0.869982	0.056997	2.88E-10	2.16E-06	3.35E-07	6.51E-06	2.32E-05	0.072977	3.27E-08	8.51E-10	2.28E-07	5.93E-07	1.11E-08	3.06E-08	9.74E-08
202	VAL	ILE	0.080541	0.917354	3.51	3.50E-06	1.35E-05	0.000376	1.18E-06	0.000209	5.84E-06	1.39E-07	1.00E-07	1.50E-07	0.917354	2.00E-05	1.76E-07	8.92E-06	6.30E-09	4.01E-08	4.88E-06	0.001461	2.29E-08	1.77E-08	0.080541
84	ARG	GLN	0.048705	0.51252	3.395	8.14E-06	0.048705	0.01109	0.001246	0.000646	0.51252	0.041848	3.09E-06	0.014947	0.000241	0.004828	0.257361	0.105695	0.00026	3.35E-06	1.28E-05	1.78E-05	0.000378	0.000168	2.21E-05
277	PHE	TRP	0.085784	0.8797484	3.358	1.15E-08	1.16E-05	5.92E-08	6.83E-09	5.58E-08	2.05E-06	1.08E-07	1.94E-09	0.000111	2.26E-06	1.64E-07	1.50E-06	3.62E-05	0.085784	1.59E-09	3.03E-09	1.83E-08	0.879748	0.034302	9.37E-07
457	ALA	SER	0.089971	0.9023634	3.326	0.089971	2.37E-05	0.000143	3.73E-05	0.004705	0.000347	1.26E-05	5.33E-06	9.41E-06	1.08E-07	3.67E-07	4.32E-06	1.57E-05	1.53E-07	3.91E-06	0.902363	0.002343	4.78E-08	9.63E-08	1.50E-05
402	LYS	GLN	0.059417	0.514146	3.113	4.92E-06	0.025128	0.015865	0.001898	0.000376	0.514146	0.09614	1.34E-06	0.263326	8.72E-05	0.001802	0.059417	0.011086	0.004147	1.98E-06	2.11E-05	0.001308	0.002049	0.00287	0.000326
170	LYS	ASN	0.062605	0.4963086	2.987	0.000115	0.005383	0.496309	0.323363	0.003067	0.002801	0.066845	1.11E-05	0.005649	0.006378	0.000953	0.062605	0.000381	0.000206	5.87E-05	0.002393	0.008934	1.65E-05	0.000201	0.014334
573	ASN	LYS	0.065478	0.4683028	2.838	0.036487	0.054246	0.065478	0.020267	0.00467	0.055886	0.062065	0.000124	0.022718	0.00457	0.022515	0.468303	0.010458	0.002186	0.000316	0.036897	0.099571	0.00013	0.000997	0.032115
437	TRP	MET	0.123367	0.8613015	2.804	6.26E-05	0.000508	3.45E-05	4.03E-05	5.06E-05	0.000469	3.03E-05	5.42E-07	0.000576	2.02E-06	0.001051	0.000323	0.861302	0.007608	3.11E-07	1.67E-05	4.29E-07	0.123367	0.004556	8.89E-07
567	LYS	GLU	0.073762	0.4502934	2.61	6.75E-06	0.001998	0.102105	0.010447	9.89E-05	0.163419	0.450293	7.70E-07	0.002149	0.000268	0.190274	0.073762	0.004657	0.000282	4.46E-06	1.38E-05	1.70E-05	6.62E-05	0.000105	3.32E-05
350	ARG	LYS	0.123385	0.7465928	2.597	1.55E-07	0.123385	4.14E-06	2.53E-06	4.01E-06	0.049858	0.032833	1.82E-08	0.00275	7.77E-07	4.03E-05	0.746593	0.043115	0.000746	2.56E-08	1.03E-07	3.39E-07	7.53E-05	0.000593	5.40E-08
77	SER	ALA	0.1469	0.8316153	2.501	0.831615	0.00109	0.000572	0.009306	0.000202	0.001355	0.004956	0.000406	1.14E-05	9.18E-07	9.93E-06	0.002847	8.49E-05	9.44E-07	0.00033	0.1469	0.000306	4.48E-07	1.32E-06	4.68E-06
159	ASN	ASP	0.151453	0.8480359	2.485	6.91E-07	2.22E-07	0.151453	0.848036	0.000301	6.85E-06	0.000133	2.56E-09	4.21E-05	1.27E-07	7.80E-07	2.94E-06	6.38E-07	9.18E-08	9.73E-09	1.56E-05	6.91E-06	1.12E-08	3.93E-08	9.67E-07
31	ASN	GLN	0.084314	0.4405599	2.385	0.000222	4.24E-05	0.084314	0.049783	0.000591	0.44056	0.262984	3.84E-06	0.015177	0.000266	0.123431	0.000539	0.020222	0.000125	3.09E-06	0.000259	0.000758	2.20E-05	9.67E-05	0.000602
88	ALA	SER	0.123142	0.6250075	2.344	0.123142	9.56E-08	3.12E-05	0.00026	0.001737	4.51E-06	3.38E-06	0.244073	7.12E-07	7.65E-08	1.42E-07	5.37E-08	1.86E-07	4.64E-08	0.000371	0.625007	0.005369	1.26E-08	5.12E-08	1.33E-06
340	LYS	ARG	0.147817	0.7499333	2.343	0.004768	0.749933	0.004787	0.001524	6.51E-05	0.021775	0.059929	6.08E-05	0.000417	6.86E-05	0.000799	0.147817	0.004298	4.16E-05	9.83E-06	0.002685	0.000751	7.91E-05	6.07E-05	0.00013
580	THR	SER	0.054472	0.2713338	2.316	0.088995	9.04E-06	0.25088	0.075679	0.257296	0.000206	0.000248	4.53E-06	4.84E-05	1.89E-06	4.89E-07	7.79E-06	5.30E-06	2.31E-07	4.93E-06	0.271334	0.054472	4.83E-08	3.41E-07	0.000806
107	PHE	TYR	0.172036	0.8279406	2.267	2.18E-12	1.65E-09	1.67E-09	5.12E-11	1.44E-10	1.54E-07	5.52E-09	3.81E-13	1.43E-05	1.47E-11	2.06E-09	1.77E-08	4.95E-07	0.172036	4.20E-13	1.58E-11	8.50E-13	8.74E-06	0.827941	7.68E-12
337	ASP	GLU	0.169755	0.7427953	2.13	0.000177	0.001565	0.011659	0.169755	0.000323	0.015268	0.742795	3.08E-06	0.00108	0.001954	0.012823	0.006864	0.00692	9.02E-05	8.57E-06	0.00187	0.007952	1.96E-05	0.000116	0.018759
65	ASN	GLU	0.098707	0.4279543	2.116	0.002103	0.019932	0.098707	0.261347	0.000429	0.098522	0.427954	0.0147	0.003693	3.44E-05	0.003957	0.055595	0.004528	0.001081	2.55E-05	0.004918	0.000672	0.000584	0.001192	2.78E-05
454	PHE	TYR	0.197714	0.7990873	2.015	5.16E-11	2.08E-07	2.53E-06	1.63E-07	1.03E-08	6.36E-07	5.35E-08	1.47E-11	0.003167	7.73E-10	1.28E-06	2.15E-06	7.35E-06	0.197714	2.13E-11	7.43E-10	5.38E-09	1.78E-05	0.799087	2.60E-09
460	THR	VAL	0.068138	0.2585358	1.924	4.94E-05	9.44E-05	0.136226	0.040786	0.119084	0.045734	0.074423	2.45E-07	9.57E-05	0.039176	0.208217	0.001243	0.007225	4.30E-06	1.49E-06	0.000961	0.068138	1.44E-06	2.25E-06	0.258536
43	GLN	SER	0.13289	0.5040752	1.923	0.005339	4.91E-05	0.046581	0.014268	0.007928	0.13289	0.255981	7.99E-06	0.000108	0.000911	0.000382	0.005349	0.002437	1.38E-06	1.74E-06	0.504075	0.022627	2.72E-07	2.72E-06	0.001062
212	GLU	ALA	0.149405	0.5549331	1.893	0.554933	0.040757	0.004851	0.016434	0.001369	0.035494	0.149405	0.029184	0.002233	0.000125	0.000862	0.083955	0.001469	0.000804	5.58E-05	0.071421	0.004823	9.19E-05	0.000749	0.000984
558	VAL	ILE	0.212576	0.7873192	1.889	2.76E-07	1.07E-08	6.85E-07	8.99E-08	6.63E-06	9.45E-08	1.74E-07	5.38E-09	2.88E-08	0.787319	7.92E-05	1.11E-07	1.60E-06	5.79E-08	2.59E-08	3.40E-08	1.62E-05	3.02E-09	1.23E-08	0.212576

512	PHE	TYR	0.216883	0.780973	1.848	2.55E-11	4.64E-05	2.87E-07	1.83E-08	3.98E-09	2.15E-06	1.04E-06	1.60E-11	0.000883	3.76E-09	4.55E-08	0.000289	5.38E-06	0.216883	1.49E-11	4.22E-11	4.33E-10	0.000916	0.780973	1.30E-10
390	ASP	ALA	0.087479	0.3080705	1.816	0.308071	0.001368	0.010154	0.087479	0.002975	0.000552	0.010597	0.274643	4.02E-05	0.000196	0.000201	0.001221	0.000325	2.60E-05	0.09503	0.196477	0.009556	6.05E-06	2.08E-05	0.001061
93	LYS	TYR	0.141802	0.4894597	1.787	5.71E-06	0.007018	0.000206	0.000109	4.33E-05	0.00124	0.00018	2.21E-07	0.093309	3.24E-06	0.000653	0.141802	0.000658	0.253205	2.20E-07	1.32E-05	1.31E-06	0.012086	0.48946	6.45E-06
87	GLN	GLU	0.157694	0.5272988	1.741	7.90E-06	0.000522	8.34E-05	1.10E-05	0.000241	0.157694	0.527299	3.97E-07	0.000294	7.51E-06	9.39E-05	0.007634	0.279143	1.37E-05	0.001317	9.93E-06	0.025548	1.32E-06	7.22E-05	7.12E-06
425	LYS	GLN	0.179202	0.5720701	1.675	1.99E-08	0.011204	0.002705	1.00E-05	2.95E-05	0.57207	0.211494	4.06E-09	4.08E-05	0.000579	0.021194	0.179202	0.001444	7.35E-08	2.51E-07	5.62E-08	1.73E-05	4.40E-07	6.16E-08	9.72E-06
409	ALA	SER	0.175606	0.53697	1.612	0.175606	1.20E-06	0.000241	0.000294	0.002572	1.20E-05	6.84E-05	7.45E-06	1.88E-06	3.75E-07	3.59E-08	3.86E-07	8.15E-07	4.57E-08	1.68E-06	0.53697	0.284184	2.12E-08	7.99E-08	3.91E-05
327	VAL	ALA	0.110006	0.3359572	1.611	0.335957	5.51E-05	0.003823	0.222464	0.142178	0.00149	0.005149	0.000181	0.000524	0.000112	4.77E-06	2.50E-05	0.00014	1.12E-05	0.000392	0.052585	0.124881	2.51E-06	2.02E-05	0.110006
540	TRP	PHE	0.206349	0.6084724	1.56	9.33E-09	4.27E-05	1.00E-06	6.76E-08	1.39E-07	4.00E-06	2.89E-07	2.46E-09	0.000322	3.74E-08	9.83E-06	9.13E-07	0.000139	0.608472	8.23E-10	1.90E-08	2.96E-08	0.206349	0.184659	1.34E-08
39	LYS	GLU	0.09979	0.2910535	1.544	1.35E-05	0.026409	0.08934	0.246289	0.0008	0.138916	0.291053	1.68E-06	0.002432	0.008675	0.022693	0.09979	0.037046	0.000257	7.66E-06	0.001299	0.032659	0.001492	0.000134	0.000693
549	SER	ALA	0.25824	0.7409792	1.521	0.740979	2.35E-07	5.76E-06	2.83E-06	6.50E-05	3.56E-07	4.21E-07	0.000421	8.76E-07	1.80E-09	9.19E-09	2.71E-07	3.62E-08	1.57E-08	1.54E-07	0.25824	0.000284	1.84E-09	2.22E-08	1.87E-07
51	VAL	PRO	0.187151	0.5212157	1.478	0.066422	5.68E-06	0.000572	2.88E-05	0.037049	0.000485	0.000645	6.76E-06	5.27E-06	5.79E-05	1.80E-06	3.35E-06	1.76E-05	2.04E-07	0.521216	0.050647	0.135685	6.07E-08	3.92E-07	0.187151
389	ASN	ASP	0.274987	0.7137021	1.376	1.68E-05	1.31E-05	0.274987	0.713702	0.000406	0.000223	0.001709	3.48E-07	7.36E-05	4.24E-05	0.000131	7.45E-05	1.64E-05	7.54E-06	2.23E-06	0.000249	0.002897	3.55E-06	6.62E-06	0.005439
576	GLY	ALA	0.214619	0.5219673	1.282	0.521967	3.76E-06	0.014591	0.001698	0.001548	0.000118	0.000102	0.214619	7.60E-05	1.40E-07	1.96E-06	5.37E-06	5.32E-06	1.28E-06	1.17E-05	0.245173	7.41E-05	1.34E-07	8.84E-07	2.67E-06
459	PHE	TYR	0.257495	0.6044338	1.231	0.000169	0.004287	0.000723	0.000178	0.000205	0.001922	0.000657	1.03E-05	0.038292	6.05E-06	0.000435	0.000908	0.003499	0.257495	4.56E-06	0.000207	2.84E-05	0.08653	0.604434	1.12E-05
248	LYS	LEU	0.095949	0.2207681	1.202	0.007438	0.047717	0.05655	0.025968	0.027256	0.068581	0.059991	0.000163	0.020981	0.039105	0.220768	0.095949	0.045828	0.006171	0.000293	0.010773	0.11709	0.001552	0.004339	0.143485
359	ARG	LYS	0.306669	0.693142	1.176	1.38E-07	0.306669	2.14E-07	1.53E-08	5.90E-07	0.000105	2.08E-05	8.46E-09	5.58E-06	3.46E-06	7.66E-06	0.693142	3.76E-05	1.68E-07	7.58E-09	2.01E-06	5.59E-07	3.32E-07	1.63E-06	2.95E-06
224	GLU	GLN	0.306134	0.6908181	1.174	0.000708	3.04E-06	1.77E-05	1.61E-05	4.81E-05	0.690818	0.306134	8.57E-05	9.70E-07	8.04E-06	1.58E-05	4.80E-06	0.000924	1.30E-08	2.13E-07	0.000967	0.000232	1.93E-08	3.00E-08	1.73E-05
452	ALA	GLU	0.113442	0.2457205	1.115	0.113442	0.023607	0.010925	0.045719	0.007798	0.210687	0.24572	4.86E-05	0.001757	0.000155	0.004178	0.111579	0.01345	0.000163	2.13E-05	0.197723	0.010568	9.16E-05	0.000457	0.00191
242	SER	THR	0.193455	0.4170451	1.108	0.386601	6.04E-07	8.98E-06	7.05E-07	0.002787	1.24E-06	1.25E-06	5.75E-06	3.35E-08	1.25E-07	1.14E-07	6.55E-08	7.42E-07	7.53E-09	8.30E-06	0.193455	0.417045	1.97E-09	4.65E-09	8.43E-05
392	SER	ALA	0.317942	0.6818087	1.101	0.681809	3.36E-08	3.95E-06	1.92E-05	7.84E-05	1.38E-07	1.61E-07	6.21E-05	2.40E-08	1.85E-09	9.35E-10	1.49E-08	2.81E-08	1.78E-09	7.56E-08	0.317942	8.48E-05	2.55E-10	1.78E-09	9.40E-08
117	LEU	ILE	0.319845	0.6733751	1.074	5.04E-07	1.70E-06	0.000708	0.000378	6.43E-05	0.001646	0.000737	6.21E-08	0.000116	0.673375	0.319845	7.38E-05	0.001537	1.20E-05	3.05E-07	2.12E-06	9.43E-06	1.28E-06	1.90E-06	0.001491
123	ARG	LYS	0.160293	0.3341106	1.06	0.004106	0.160293	0.004006	0.004506	0.000305	0.138541	0.076898	0.000434	0.003254	0.052213	0.0091	0.334111	0.023423	0.002089	0.146441	0.003554	0.009786	0.001886	0.003451	0.021604
361	ASN	ASP	0.324281	0.6699073	1.047	4.34E-06	7.94E-07	0.324281	0.669907	0.005133	2.06E-06	6.04E-06	4.57E-08	3.82E-07	8.35E-07	1.16E-07	1.64E-06	2.09E-07	4.51E-09	2.68E-08	0.000506	0.000147	2.65E-09	6.06E-09	8.65E-06

Supplementary Table 4: The top 20 mutants ranked by the fold change in probability between the predicted amino acid and the wild-type amino acid.

Ranking	position	wtAA	prAA	wt_prob	pred_prob	avg_log_ratio
1	528	GLN	ASP	0.000593	0.687982	10.181
2	207	GLN	ASN	0.001223	0.688738	9.137
3	341	GLU	SER	0.001235	0.347408	8.136
4	135	ILE	VAL	0.003481	0.905840	8.023
5	199	GLU	ASP	0.008748	0.763433	6.447
6	586	GLN	GLU	0.012542	0.940884	6.229
7	316	SER	THR	0.020298	0.970487	5.579
8	254	LEU	MET	0.020965	0.894239	5.415
9	75	ALA	SER	0.022796	0.920131	5.335
10	554	TRP	PHE	0.016817	0.531547	4.982
11	81	GLN	ASN	0.022707	0.641597	4.82
12	144	ASN	ASP	0.034432	0.962491	4.805
13	42	GLN	ASN	0.021044	0.557211	4.727
14	136	ASN	HIS	0.036520	0.895015	4.615
15	300	ARG	GLN	0.023288	0.530322	4.509
16	30	SER	THR	0.044466	0.955477	4.425
17	323	ASP	ALA	0.036167	0.650021	4.168
18	429	LEU	ASN	0.043901	0.750578	4.096
19	560	LEU	ASP	0.023406	0.394006	4.073
20	486	HIS	TYR	0.048336	0.751457	3.959

Supplementary Table 5: Hydrogen bond occurrence analysis of 8M/HMF and 8M/FFA systems.

	8M/HMF	8M/FFA
Substrate_O - N557	65.7%	0%

Supplementary Table 6: Binding free energies of HMF and FFA calculated for 4M and 8M using MM/GBSA.

	4M/HMF	8M/HMF	4M/FFA	8M/FFA
Binding affinity (kcal/mol)	-14.77 ± 0.32	-16.26 ± 0.25	-14.46 ± 0.39	-16.59 ± 0.35