

Supplementary Information for
Biosynthesis of pyomelanin from methanol with engineered
***Komagataella phaffii* and its characterizations**

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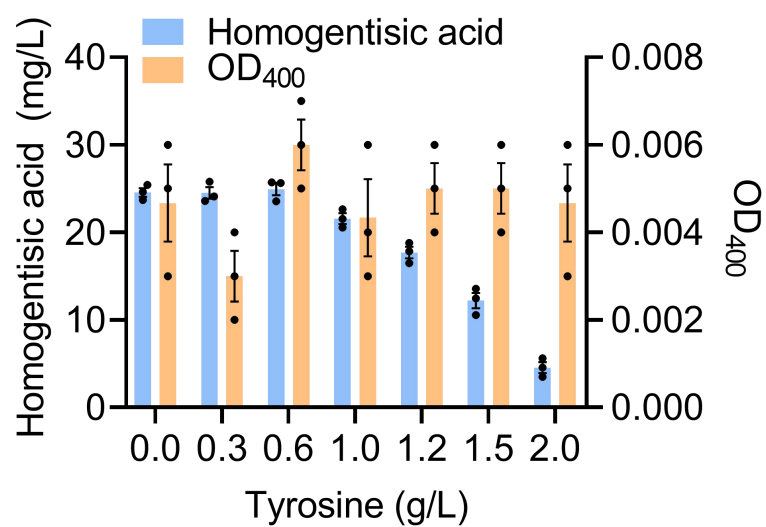
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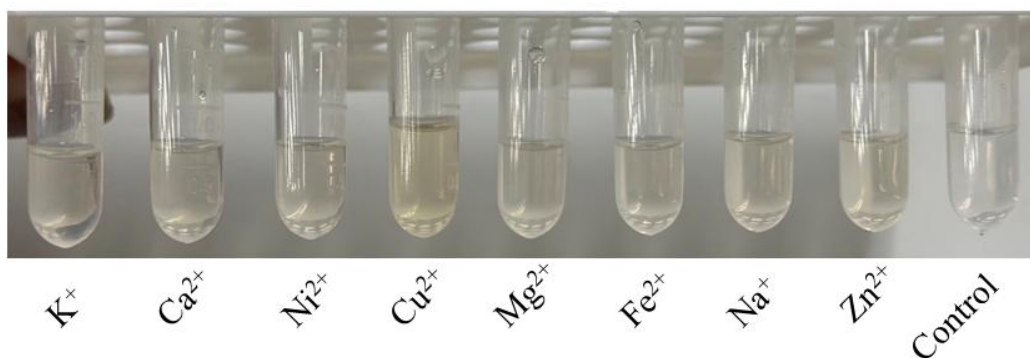
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24 **Supplementary Fig. 1.** Effect of exogenous addition of L-tyrosine on HGA
 25 production and OD₄₀₀ of wild-type *Komagataella phaffii*. Data are the means of 3
 26 biological replicates with error bars representing standard deviations. Source data are
 27 provided as a Source Data file.



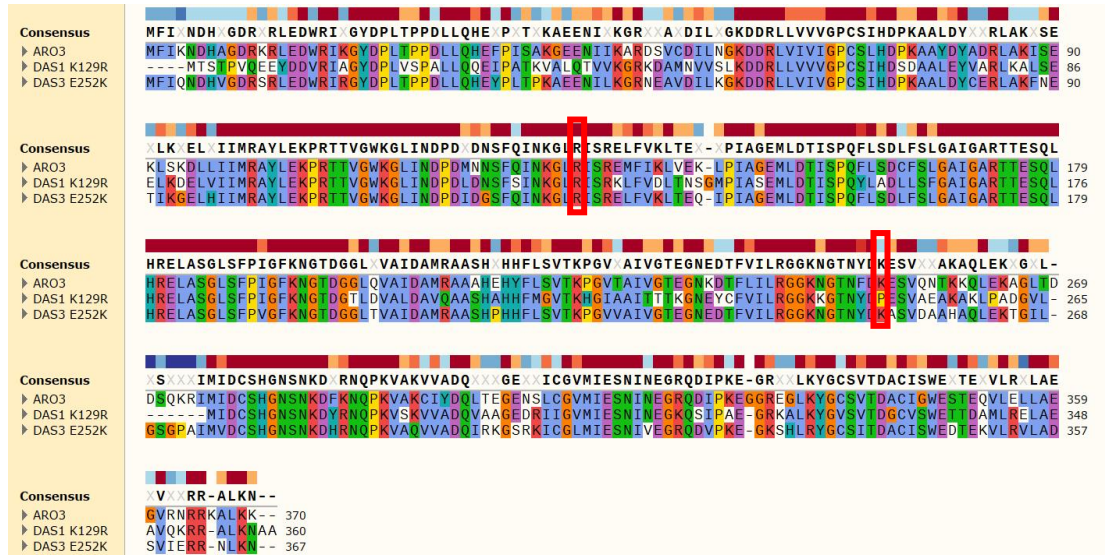
28 **Supplementary Fig. 2.** Effect of adding different metal ions on *Af*HPPD enzyme
29 activity. The generation of HGA is used as the evaluation reference for relative
30 enzyme activity. The enzyme reaction system containing Cu^{2+} exhibits the darkest
31 color after the reaction is completed.

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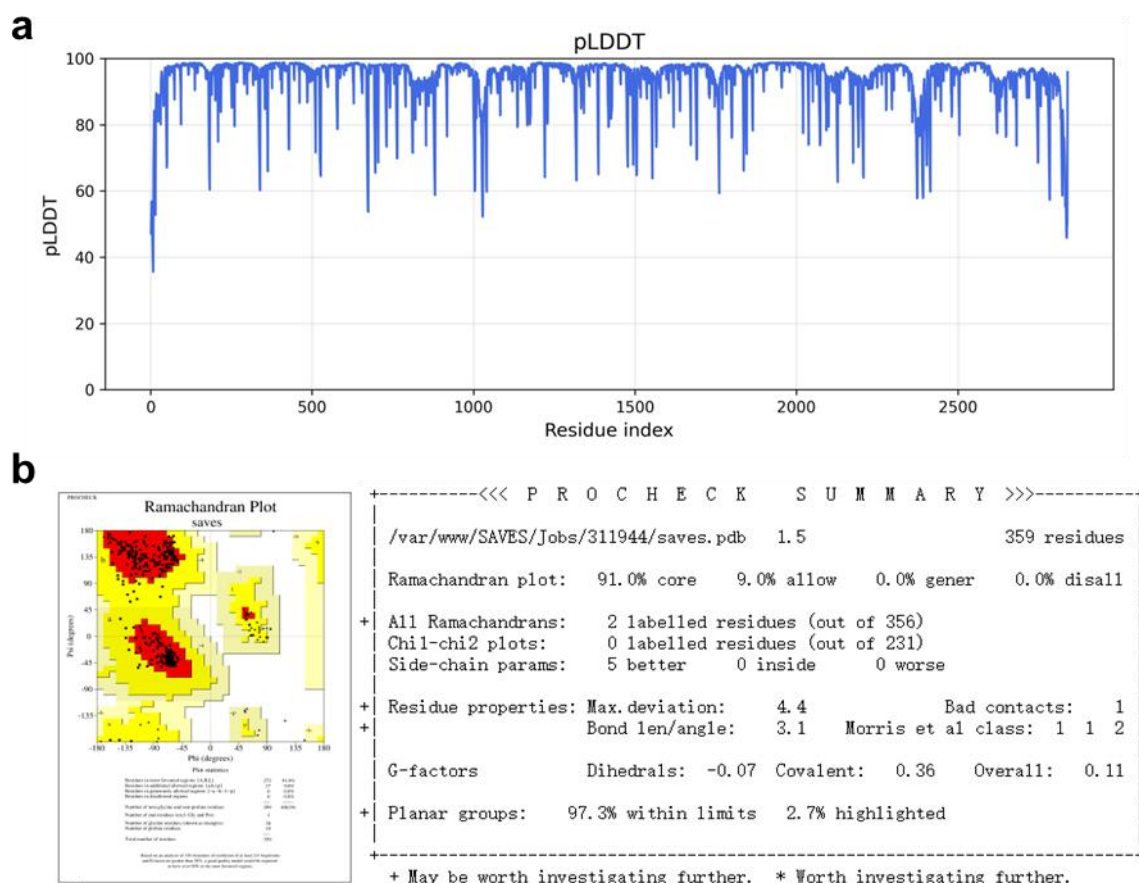


	Control	Cu ²⁺
OD ₄₀₀	0.051	0.123

33 **Supplementary Fig. 3.** Effect of Cu²⁺ of promote non-enzymatic oxidation of HGA.

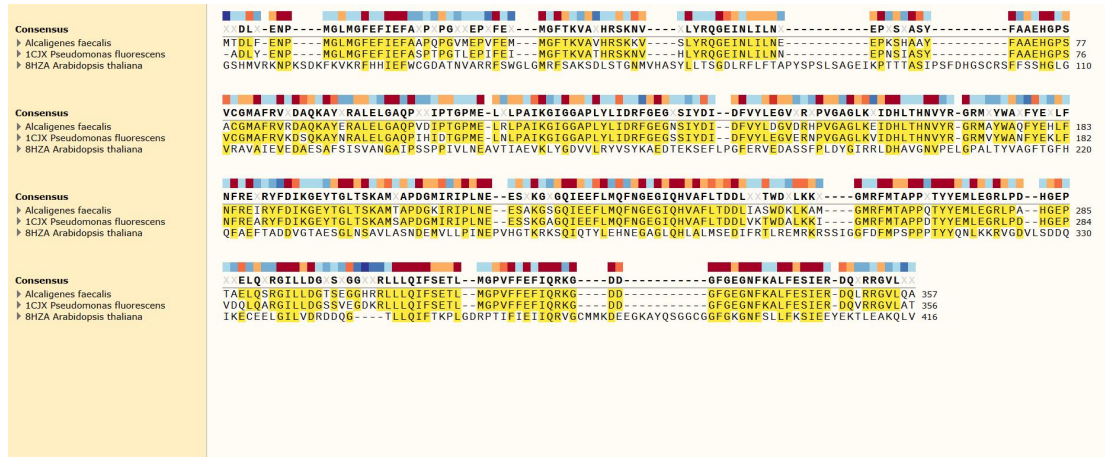


34 **Supplementary Fig. 4.** Alignment of ARO3 with DAHPS1^{K129R} and DAHPS3^{E252K}
35 sequences of *Komagataella phaffii*, showing that the position 133 of ARO3 is aligned
36 with the position 129 of DAHPS1^{K129R}, both are arginine, and the position 252 of
37 ARO3 is aligned with the position 252 of DAHPS3^{E252K}, both are lysine.

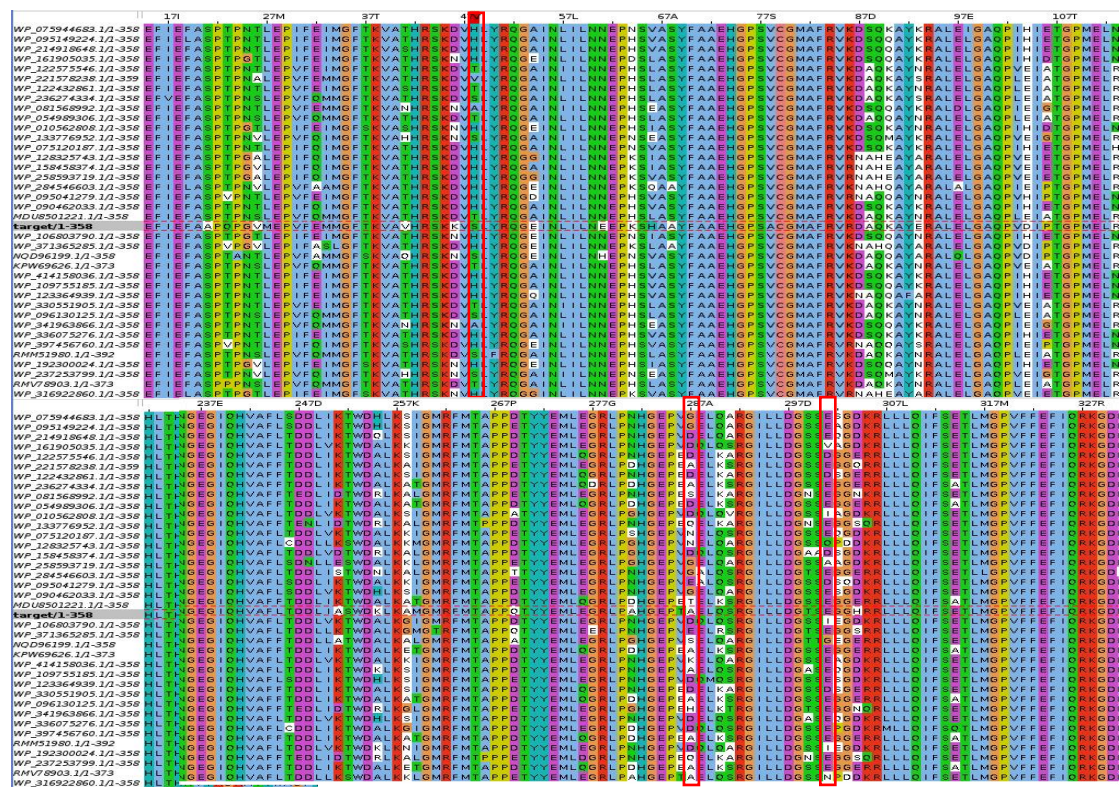


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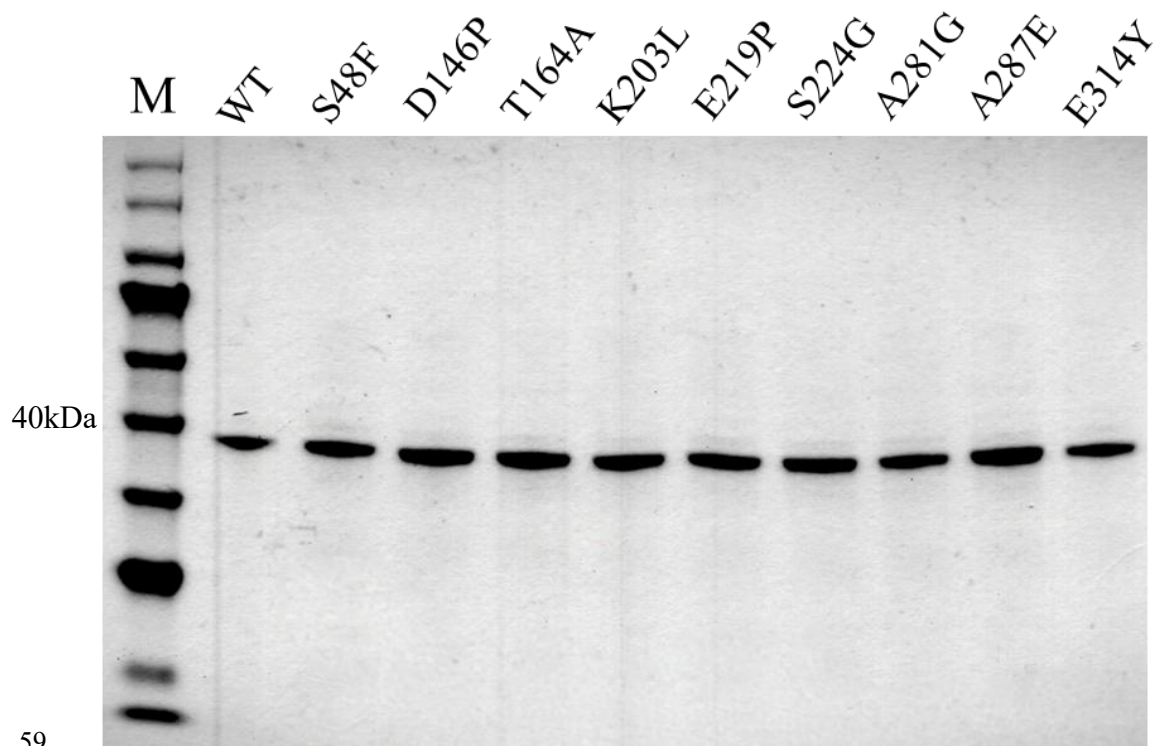
39 **Supplementary Fig. 5.** Reliability analysis of AlphaFold2 modelling. a Standard
 40 quality metrics of AlphaFold2 modelling based on atom-plddts. pTM = 0.93. RMSD =
 41 0.325. b Ramachandran plot of the structure of *A/HPPD* based on AlphaFold2,
 42 showing that in 3-dimensional/1-dimensional analysis, at least 80% of the amino acid
 43 scores are equal to or higher than 0.1, and more than 90% of the amino acids are
 44 detected in appropriate positions.



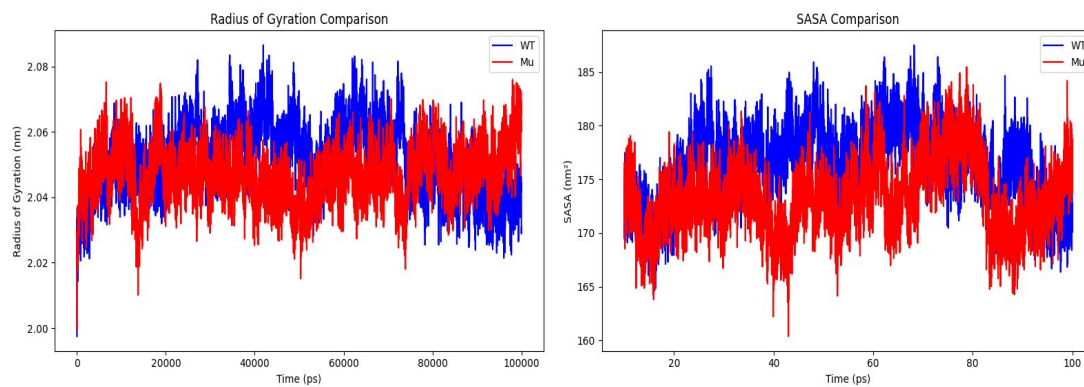
Supplementary Fig. 6. Sequence alignment of *Pf*HPPD, *At*HPPD, and *Af*HPPD.



Supplementary Fig. 7. Multiple sequence alignment between *A/HPPD* and 973 homologous sequences. The sources of the three selected sites, i.e., S48F, E314Y, and A287E, are highlighted in red boxes.

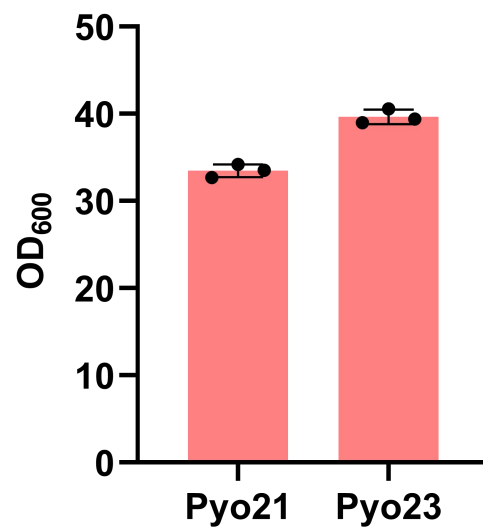


Supplementary Fig. 8. SDS-PAGE analysis of *AfHPPD* proteins of wild-type (WT) enzyme and nine mutated enzymes of *Alcaligenes faecalis*. This data was represented with three biological replicates.



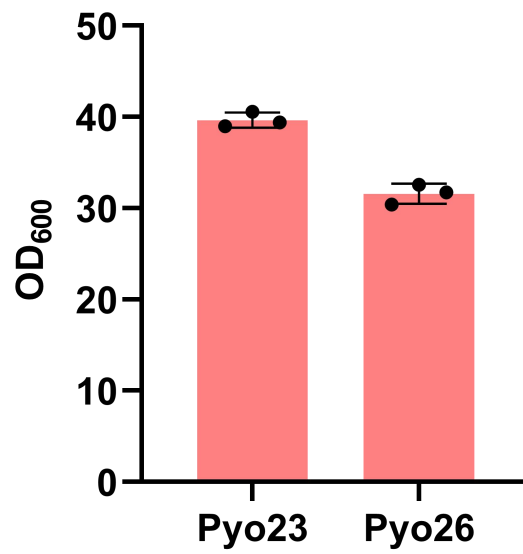
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65 **Supplementary Fig. 9.** Molecular dynamics simulations for *Af*HPPD and
 66 *Af*HPPD^{E314Y/S224G}, showing the radius of gyration (Rg) (left) and the soluble surface
 67 area (SASA) (right). Source data are provided as a Source Data file.



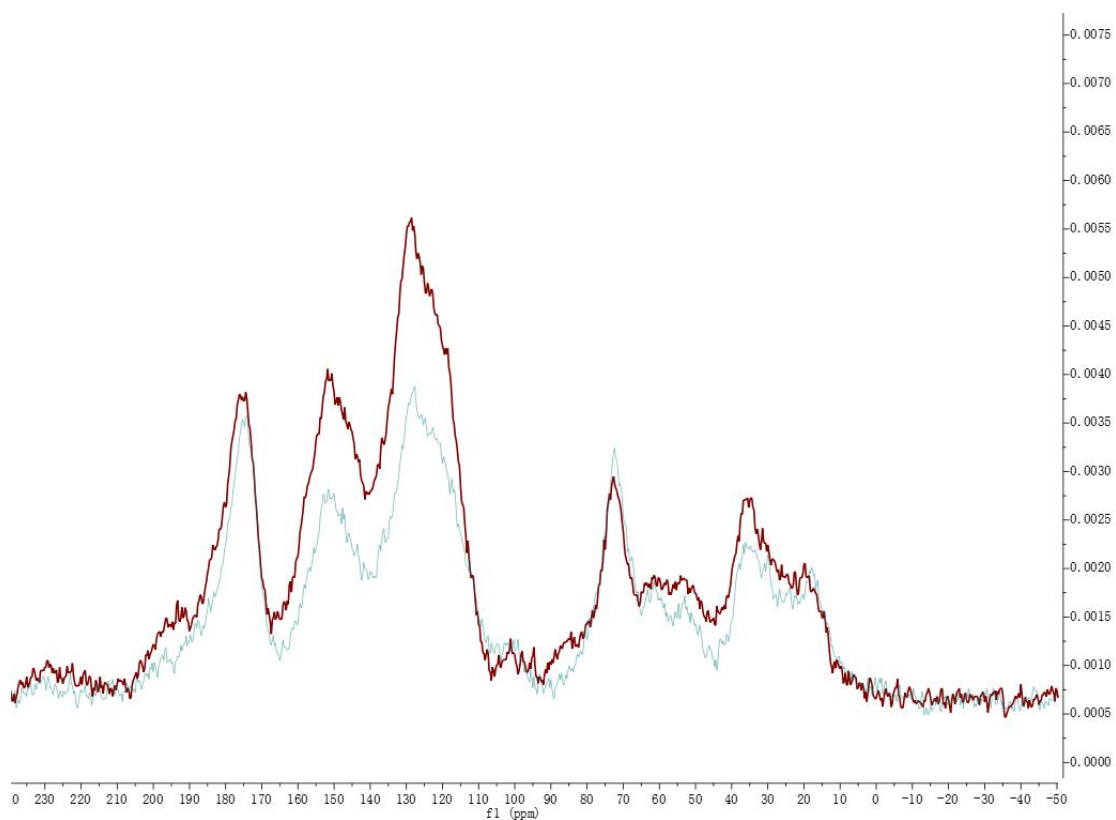
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69 **Supplementary Fig. 10.** Overexpression of gene *DAS2* improves the growth of strain
70 Pyo23 derived from strain Pyo21 of *Komagataella phaffii*. Data are the means of 3
71 biological replicates with error bars representing standard deviations. Source data are
72 provided as a Source Data file.



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74 **Supplementary Fig. 11.** Knockout of gene *PheA* decelerates the growth of strain
75 Pyo26 derived from strain Pyo23 of *Komagataella phaffii*. Data are the means of 3
76 biological replicates with error bars representing standard deviations. Source data are
77 provided as a Source Data file.



Supplementary Fig. 12. ^{13}C Cross-Polarization Magic Angle Spinning (CP-MAS) solid-state NMR spectra of the Pyo-NaOH and Pyo-Lac. The red line represents Pyo-Lac, and the blue line represents Pyo-NaOH.

82 **Supplementary Table 1.** Compared to *At*HPPD, *Pf*HPPD has a higher identify and
83 similarity with *Af*HPPD. For the active center, the important metal binding domains
84 are analyzed. *Pf*HPPD and *At*HPPD bind Fe²⁺, while *Af*HPPD bind Cu²⁺. They are all
85 composed of H-H-E motifs.

Comparison with <i>Af</i> HPPD		Coverage (%)	Identify (%)	Similarity (%)
<i>Pf</i> HPPD		99	84.27	91.29
<i>At</i> HPPD		97	28.10	45.10

HPPD structure	Binding site with metal ion
<i>Af</i> HPPD	H162-H241-E323
<i>Pf</i> HPPD	H161-H240-E322
<i>At</i> HPPD	H226-H308-E394

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