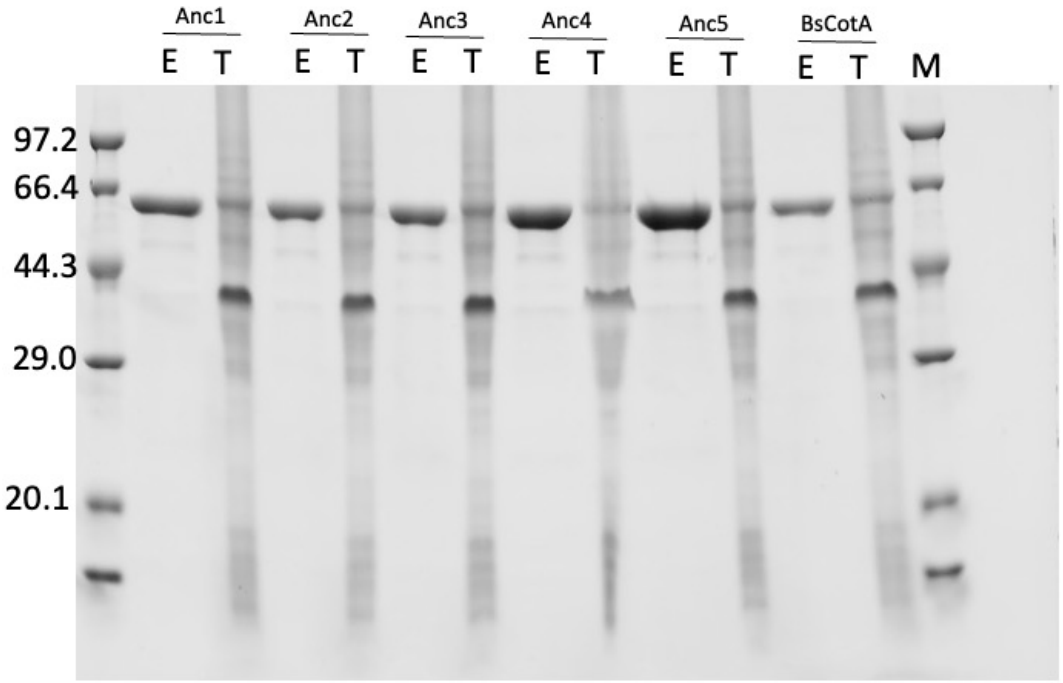
Supplementary Material

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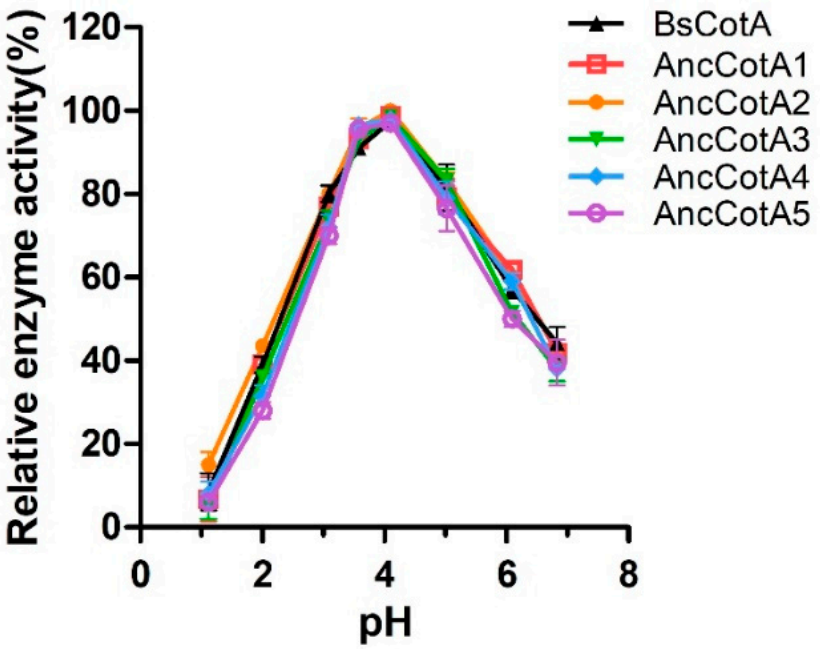
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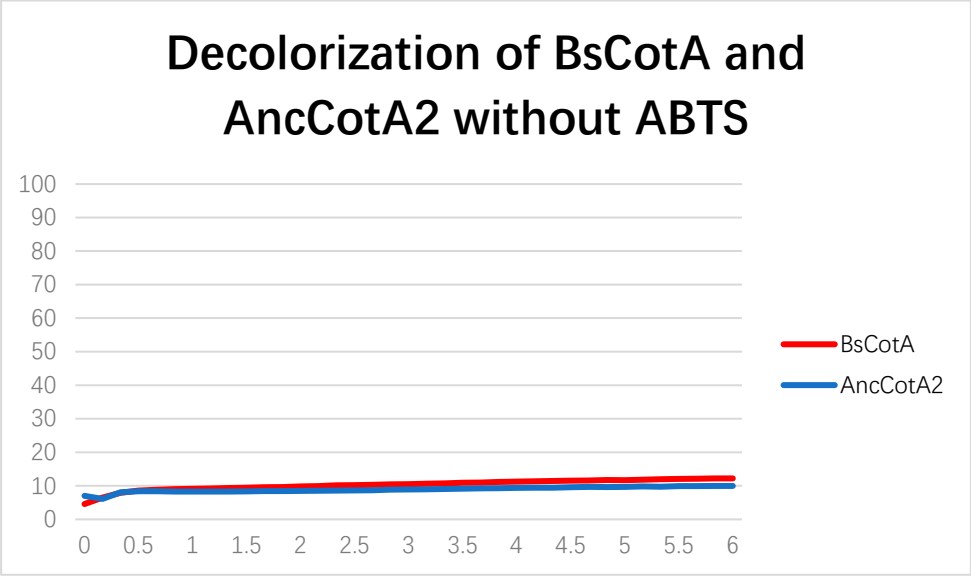
Supplementary Figure S1. A phylogenetic analysis of the BsCotA-like laccases. An unrooted Maximum-likelihood tree was constructed using IQ-Tree showing the phylogenetic relationship of BsCotA-like laccases. The confidence level of each node was estimated by the bootstrap procedure using 10,000 resampling repetitions of the data. Bootstrap values (% re-sampled data set) are indicated for each node. The multiple sequences used for the alignment and to generate the tree are available in Supplemental Dataset S1.



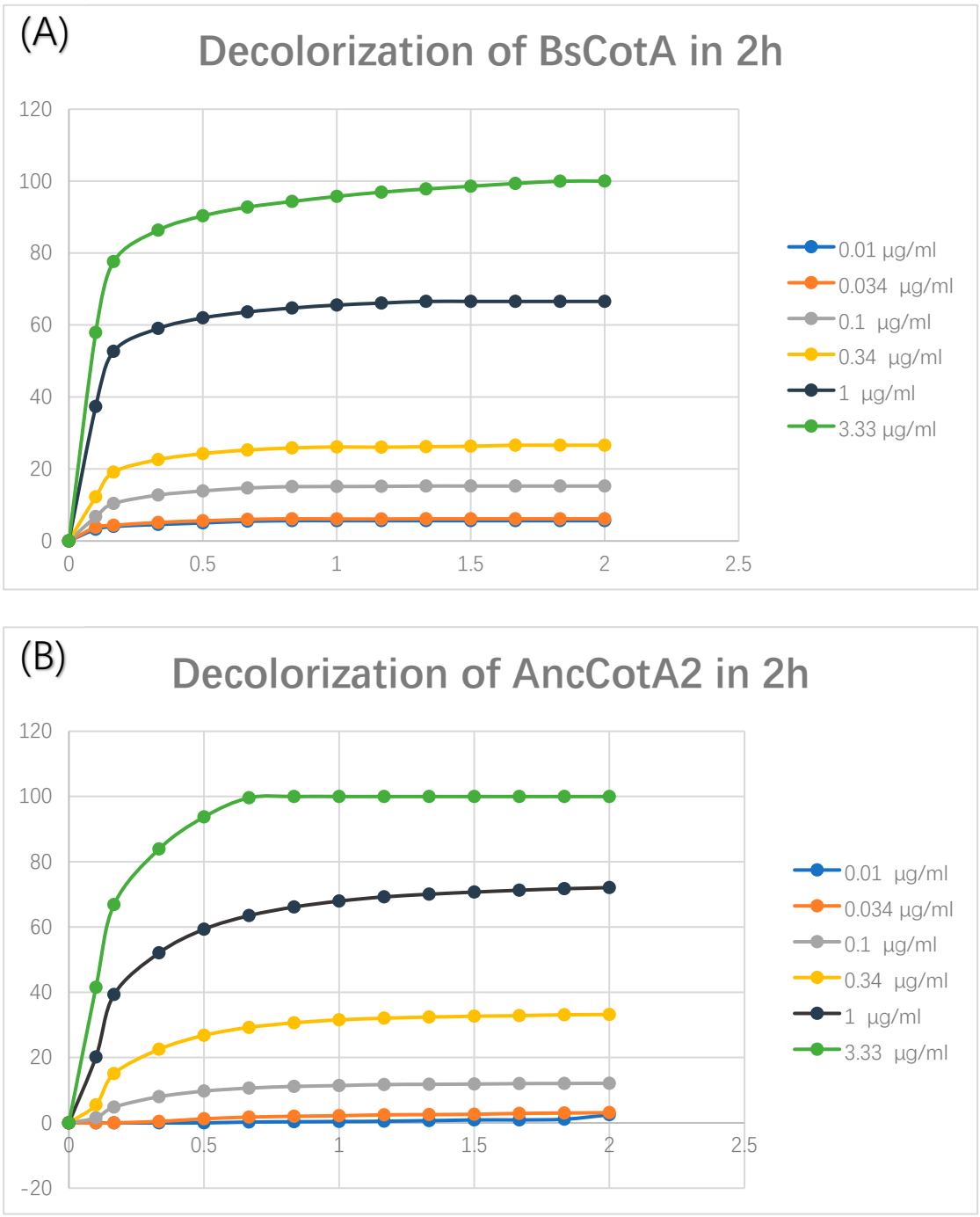
Supplementary Figure S2. SDS-PAGE analysis of laccase BsCotA and CotA Ancestors. The SDS-PAGE shows crude bacterial lysates and purified enzyme samples (T—supernatant, E—eluted fraction).



Supplementary Figure S3. Optimum pH for five designs AncCotA and BsCotA.



Supplementary Figure S4. Decolorization rate of 0.1 mg/mL indigo carmine by BsCotA and AncCotA2 at 3.33 μg/mL without ABTS in 6 h.



Supplementary Figure S5. (**A**) Decolorization rate of 0.1 mg/mL indigo carmine by BsCotA at different concentrations within 2 h. (**B**) Decolorization rate of 0.1 mg/mL indigo carmine by AncCotA2 at different concentrations within 2 h.

Supplementary Table S1. Mutation site analysis of AncCotA2.

Reasons	Positions	Number
Difficult to explain	T2N/K25S/E33K/K63N/Q94H/E96Q/D113P/H175R/D187G/A211G/F292I/E312Q/Q345G/E348T/Q362T/H363S/T400A/S448V/T467V/H512Q/K513Q	21
Conservative replacement	D14E/I84V/D92E/S124T/K125R/V169A/I200L/E213D/S218T/E231D/V239A/L243M/D272E/N290Q/I302V/T307S/Y309F/I334V/Y358L/V403L/Q442N/S444T/L447I/L475I/I508V	25
Core packing	A171M/S186A/L194M/T196M/V254I/I255L/T300F	7
Formed a new hydrogen bond	A317T/A344K/T36Y	3
Improved surface polarity/charge	I438T/A9E/Q20K//Q38K/N75D/S78D//T79K/Q129E/P177K/A356S/A375T/A439E/P511K/	13