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Discovery of a Fungal Copper Radical Oxidase with High Catalytic Efficiency Towards 5- hydroxymethylfurfural and Benzyl Alcohols for Bioprocessing.

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Supporting Tables

Table S1: Initial activity screens* of *CgrAAO*-WT and its variants

	Substrate	Specific activity ($\mu\text{mole} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)		
		<i>CgrAAO</i> -WT	<i>CgrAAO</i> -Y334F	<i>CgrAAO</i> -Y334W
Carbohydrates	D-Galactose (300 mM)	1.80 \pm 0.03	4.1 \pm 0.1	34.9 \pm 0.3
	D-Lactose (300 mM)	1.93 \pm 0.05	4.4 \pm 0.1	12.6 \pm 0.3
	Melibiose (300 mM)	9.9 \pm 0.7	20.6 \pm 1.2	46.9 \pm 0.5
	Raffinose (300 mM)	8.5 \pm 0.1	38.3 \pm 1.1	51.4 \pm 0.3
	D-Glucose (300 mM)	0.050 \pm 0.002	n.m.§	n.m.§§
	D-Xylose (300 mM)	0.950 \pm 0.005	3.80 \pm 0.08	0.88 \pm 0.01
	L-Arabinose (300 mM)	0.74 \pm 0.01	n.m.§	n.m.§
	D-Ribose (300 mM)	0.37 \pm 0.01	0.67 \pm 0.01	0.38 \pm 0.03
	D-Fructose (300 mM)	0.117 \pm 0.006	n.m.§	n.m.§
	D-Mannose	0.066 \pm 0.004	n.m.§	0.070 \pm 0.006
	Sucrose (300 mM)	0.045 \pm 0.001	n.m.§	n.m.§
	Maltose (300 mM)	0.051 \pm 0.002	n.m.§	n.m.§
	Cellobiose (300 mM)	0.115 \pm 0.002	n.m.§	0.14 \pm 0.01
	Carob Galactomannan (2.5 mg.mL ⁻¹)	0.43 \pm 0.02	0.90 \pm 0.02	0.063 \pm 0.004
	Xyloglucan (2.5 mg.mL ⁻¹)	0.060 \pm 0.002	n.m.§	n.m.§
Polyols	Glycerol (300 mM)	7.2 \pm 0.4	18.4 \pm 0.3	12.3 \pm 0.4
	Sorbitol (300 mM)	0.760 \pm 0.007	0.60 \pm 0.03	0.32 \pm 0.02
Diols	1,2-Propanediol (300 mM)	4.02 \pm 0.04	6.3 \pm 0.2	0.35 \pm 0.02
	1,3-Propanediol (300 mM)	10.9 \pm 0.1	42.7 \pm 0.9	1.87 \pm 0.03
	1,4-Butanediol (300 mM)	2.6 \pm 0.1	n.m.§	2.2 \pm 0.1
Aldehyde	Methyl glyoxal (5 mM)	n.m.§	1.3 \pm 0.1	n.m.§
Primary Alcohols	Methanol (300 mM)	0.81 \pm 0.02	1.50 \pm 0.07	0.19 \pm 0.01
	Ethanol (300 mM)	0.42 \pm 0.04	0.70 \pm 0.01	0.050 \pm 0.002
	1-Butanol (300 mM)	0.85 \pm 0.02	n.m.§	n.m.§
	1-Propanol (300 mM)	0.50 \pm 0.01	n.m.§	n.m.§
Secondary Alcohols	2-Propanol (10 mM)	0.036 \pm 0.002	n.m.§	n.m.§
	1-Phenyl Ethanol (10 mM)	n.m.§	n.m.§	n.m.§
	2-Phenyl Ethanol (10 mM)	n.m.§	n.m.§	n.m.§
Benzyl Alcohols	Benzyl alcohol (5 mM)	3.4 \pm 0.1	10.6 \pm 0.1	1.24 \pm 0.06
	m-Anisyl alcohol (5 mM)	3.1 \pm 0.2	8.2 \pm 0.2	1.63 \pm 0.05
	p-Anisyl alcohol (5 mM)	2.9 \pm 0.1	6.6 \pm 0.2	1.03 \pm 0.02
	Veratryl alcohol (5 mM)	3.71 \pm 0.05	10.8 \pm 0.3	1.48 \pm 0.08
	Cinnamyl alcohol (5 mM)	2.8 \pm 0.2	6.4 \pm 0.3	0.64 \pm 0.08
	4-Hydroxy benzyl alcohol (5 mM)	3.3 \pm 0.1	7.8 \pm 0.4	1.3 \pm 0.1
	Coniferyl alcohol (5 mM)	n.m.§	n.m.§	n.m.§
Furans	HMF (5 mM)	26.4 \pm 1.1	16.4 \pm 0.9	1.39 \pm 0.06
	HMFA (5 mM)	2.8 \pm 0.3	2.1 \pm 0.1	0.66 \pm 0.04
	DFF (5 mM)	0.0010 \pm 0.0001	0.051 \pm 0.001	0.100 \pm 0.005
	FFCA (5 mM)	0.0020 \pm 0.0001	0.003 \pm 0.001	0.0030 \pm 0.0001

* Measurements were performed in triplicates at 25 °C in 100 mM sodium phosphate buffer pH 7 using the HRP/ABTS assay. Activities were monitored using concentrations indicated within parentheses for each substrate.

§No activity detected with a specific activity limit of detection of $9 \times 10^{-4} \mu\text{mole} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ using 65 μmole of protein, which is 5-fold

Table S2: EPR spin Hamiltonian parameters from simulations of cw X band spectra for *CgrAAO*-WT, -Y334F and -Y334W^a

		<i>CgrAAO</i> -WT	<i>CgrAAO</i> -Y334F	<i>CgrAAO</i> -Y334W
<i>g</i> values	g_1	2.059	2.059	2.049
	g_2	2.072	2.072	2.061
	g_3	2.278	2.278	2.275
A_{Cu} (MHz)	$ A_1 $	40	40	50
	$ A_2 $	45	40	50
	$ A_3 $	530	530	515
SHF principal values (MHz) *	A_N	43, 43	43, 43	45, 45
		± 3	± 3	± 3
A_{Cu} strains (MHz)		55, 65, 130	35, 75, 130	50, 65, 130
Line widths (mT)		0.7, 0.7	0.7, 0.7	0.8, 0.8
Frequency (GHz)		9.2986	9.2995	9.2982

* error estimated from quality of simulated fits

^a. Spectra were recorded in the presence of 10% glycerol in 100 mM Na phosphate buffer pH 7.0. For coupled nitrogen nuclei, only the principal coupling value could be determined from the simulations of the superhyperfine (SHF); the two values refer to the two different N nuclei.

Table S3: Comparison of catalytic parameters of *Cgr*AAO with other enzymes acting on HMF and its derivatives*

	HMF			DFF			HMFCA			FFCA		
	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (M ⁻¹ .s ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (M ⁻¹ .s ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (M ⁻¹ .s ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (M ⁻¹ .s ⁻¹)
Bacterial HMFO^a	1.4	9.9	7.1 x 10 ³	1.7	1.6	940	73	8.5	120	NM	NM	<10
PerAAO^b	1.6 ± 0.2	0.33 ± 0.01	220 ± 43	3.3 ± 0.2	0.52 ± 0.01	158.0 ± 9.2	NM	NM	NM	NM	NM	NM
MtGLOx^c	20.2 ± 9.0	15.9	982	NM	NM	NM	NA	NA	NA	NA	NA	NA
Pciglox1^d	15.66 ± 2.35	1.59 ± 0.12	101.66 ± 0.01	4.3 ± 0.1	0.54 ± 0.24	124.39 ± 0.01	NA	NA	NA	0.85 ± 0.14	0.03 ± 0.01	38.55 ± 0.01
Pciglox2^d	5.87 ± 2.04	0.56 ± 0.09	96.04 ± 0.01	0.0 ± 0.1	4.80 ± 0.24	2.34 ± 0.01 x 10 ⁴	NA	NA	NA	1.40 ± 0.39	2.02 ± 0.03	1.40 ± 0.01 x 10 ³
Pciglox3^d	6.35 ± 1.32	0.75 ± 0.07	118.35 ± 0.01	0.0 ± 0.1	1.28 ± 0.09	7.30 ± 0.01 x 10 ³	NA	NA	NA	0.61 ± 0.58	0.04 ± 0.01	72.03 ± 0.01
CgrAAO^e	6.5 ± 0.3	126.0 ± 1.5	0.09 x 10 ⁴	NM	NM	NM	26.9 ± 3.0	28.3 ± 1.3	1.1 ± 0.1 x 10 ³	NM	NM	NM

* NM not measurable; NA non assessed

^a Kinetic data from ¹; ^b Kinetic data from ²; ^c Kinetic data from ³; ^d Kinetic data from ⁴; ^e Kinetic data derive from Table 1

Table S4 : PCR primers^a

	Primers name	Primers sequence 5' - 3'
Mutagenesis	<i>Cgr</i> AAO-Y334W-f	GGTGGGCTTggTCAGGTGAGC
	<i>Cgr</i> AAO-Y334W-r	AATAGTGAAGACCTTACCATTAC
	<i>Cgr</i> AAO-Y334F-f	GGTGGGGCTTtTTCAGGTGAG
	<i>Cgr</i> AAO-Y334F-r	AATAGTGAAGACCTTACCATTAC

^a. Primer sequences used for site directed mutagenesis. Mutated bases are in lowercase.

A

FgrGalOx Q01745	G L G R W G P T D L P I V P A A A A I E P - - - T S G R N L L V W S S Y R N D A F G G S P G G I - - T L T S S W D P S T G	56
CgrAlcOx EFQ30446	N V G K W G P M V K F P V V P V A V A L V P - - - E T G N L L V W S S G W P N R W T T A G N G K - T Y T S L Y N V N T G	56
CglAlcOx ELA25906	Q L G Q W S P L I K F P V V P V S V A L L P - - - E S G N L L V W S S G W P N R W T T A G N G K - T Y T S L Y N V Q T G	56
CgrRafOx EFQ36699	N G N G W S P I Q T L K L P N P V A V L V P A Y P V V Q D F L S F S S F S P T F G G G A P Y N T A F M R Y I K S S	60
PorAlcOx XP_003719369	S A G Q W G P I V V K F P V V P V S V A L L P - - - E S G D L I V W S S G W P D R F T T N G N G K - T Y T S I Y N V Q T G	56
ChiAlcOx OBRO5259	N V G Q W G P M V K F P V V P V A V A L L P - - - E T G N M L V W S S G W P N R W T T A G N G K - T Y T S I Y D V K T G	56
PruAA5_2A CAP96757	N G G V W G P T I D L P V V A V S G A V I P - - - E T N E V L V W S S W A K D D Y L H S - R G Y - T L T A V W N M N D N	55
CgrAAO EFQ27661	V K G K W G D L I R L P V I P V A A Y I V P S Y P E P S R L L F F S S W S N D A F S G A - S G M - T Q F G D Y D F A T G	58
	* : : : : * : : : : * : : : : * : : : : *	
FgrGalOx Q01745	I V S D R T V T V T K H D M F C P G I S M D G N G Q I V V T G G N D A K K T S L Y D S S - - - S D S W I P G P D M Q V A	113
CgrAlcOx EFQ30446	N I S D A I V Q N T Q H D M F C P G T S L D A D E G R I I V T G G S S A A K T S V L D F K K G E S S P W T P L S N M Q I S	116
CglAlcOx ELA25906	N V S D A I V Q N T Q H D M F C P G T S L D A E G R I I V T G G S S A A K T S V L D F K N G E S S W T A L S N M Q I S	116
CgrRafOx EFQ36699	A A S Q F N V A E T K H D M F C P G M N H L A D G R L V I N G G N T D A A V T I Y D P F - - - A N T W T R A A N M N M G	117
PorAlcOx XP_003719369	N V S E A I I Q N T S H D M F C P G T S M D E F A G R I V V T G G S G A A K T S V D F Q N G Q R S P W M P A S D L T N P	116
ChiAlcOx OBRO5259	K V S D A L I Q N T Q H D M F C P G T S M D E N G R I I V T G G S S A A K T S V L D F K K G E F S S W T P L S N M Q I S	117
PruAA5_2A CAP96757	S V T Q R K V Q E T H H D M F C S G M S Y D G K G E L L V T G G N N D K S T I F D P A - - - S G K W T E G N T M I I T	115
CgrAAO EFQ27661	A I S Q R T V T N T H D M F C P G I S Q L E D G R I L I Q G G S D A D T V S I Y D P A - - - T N E F T R G P N M T L A	115
	* : : : : * : : : : * : : : : * : : : : *	
FgrGalOx Q01745	R G Y Q S S A T M S D G R V F T I G G S W S G G V - - - F E K N G E V Y S P S S K T W T S L P N A K V N P M L T A	167
CgrAlcOx EFQ30446	R G Y Q S S C T T S E G K I F V I G G S F S G A G - - - - - T R N G E V Y D P K A N T W T K L A G C P V K P L V M Q	169
CglAlcOx ELA25906	R G Y Q S S C T T S E G K I F V I G G S F S G A G - - - - - T R N G E I Y D T A T N K W T K L A G C P V K P L V M Q	169
CgrRafOx EFQ36699	R G Y Q S S V T L S D G R G F T I G G S Y T G G I G G Q N G T P M K N G E V Y D P K L N K W T L P G A L V A P M L T T	177
PorAlcOx XP_003719369	R G Y Q S S V T S E G K I F T I G G T F S G N G - - - - - K R D G E V Y D V N A N K W T K L P G C P A T I M R V A	169
ChiAlcOx OBRO5259	R G Y Q S S C T T S E G K I F V I G G S F S G A G - - - - - R R D G E V Y D P K A N T W T K L A G C P V K P L V M Q	169
PruAA5_2A CAP96757	R G Y Q A S A T A D G R V F I G G S W N G G T N - - - - - Y D K N G E I Y D P D T E K Y S F L K N A L V R P M W T D	167
CgrAAO EFQ27661	R G Y Q T S C T L S N G K V F T I G G A Y S G E R - - - - - V G K N G E V Y D P V A N A W T Y L P G A D F R P M L T N	169
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FgrGalOx Q01745	- D K Q G L R S D N H A W L F G W K K G S V F Q A G P S T A M N W Y Y T S G S D V K S A G K R Q S N R G V A P D A M	226
CgrAlcOx EFQ30446	- - - - - L G M F P D S H A W L S W K N G S V L Q A G P S K M N W Y D T K G T S N T A G L R G - - - - - T D E D S M	221
CglAlcOx ELA25906	- - - - - L G M F P D S H A W L S W K N G S V L Q A G P A K M M W Y D T K G T G A N T A G L R G - - - - - A D Q D S M	221
CgrRafOx EFQ36699	Y D N A G A W R T D N H A W L Y A W S N G S V F Q A G P S K M N W Y S T S G G S V K G A G Q R N - - - - - T Q N D Q M	233
PorAlcOx XP_003719369	- - - - - G G L Y P D S H T L W L W G K D G F V L Q A G P S K M N W F D T K G T G N K P A G T R G - - - - - A D Q D S M	221
ChiAlcOx OBRO5259	- - - - - R G L F P D S H A W L S W K N G T V L H A G P A K M M W Y Y T K G T A N T A G L R G - - - - - A D D S M	221
PruAA5_2A CAP96757	- D Q D S G Y R R D S H G W L F G W K N D T V F Q G G P S K M N W Y Y T H G D G D Q K P A G T R A - - - - - D A N D S M	222
CgrAAO EFQ27661	- D H E G I W R E D N H A W L F G W K N G S I F Q A G P S K D Q H W Y Y I Q G N G T V A K A A T R - - - - - D D D D A M	223
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FgrGalOx Q01745	C G N A V M Y D A V K G K I L T F G G S P D Y Q D S D A T T N A H I I T L G - E P G T S P N T V F A - - - - - S N G L Y F	281
CgrAlcOx EFQ30446	C G V S V M Y D A V A G K I F T Y G G G K G Y T G Y D S T S N A H I L T L G - E P G Q A V Q V K - - - - - L A N G K Y	275
CglAlcOx ELA25906	C G V S V M Y D A V A G K I F T Y G G G K G Y T G Y D S T S N A H I L T L G - E P G Q A V Q V K - - - - - L Q N G Q Y	275
CgrRafOx EFQ36699	C G V T V M Y D S - G K I F A A G A Q S Y S D K A L Y A A H R I T L N - G V N Q S P T V Q Q - - - - - L P N A K Y	285
PorAlcOx XP_003719369	C G V T A M Y D A A A G K V A F T Y G G K L R Y T G E S G S N A A H V L T L P D T P G D L V A E R - - - - - V S D G Q F	276
ChiAlcOx OBRO5259	C G V S V M Y D A V A G K I F T Y G G G K A Y T G V A S S N A H I L T L G - E P G Q A V Q V K - - - - - L Q N G K F	275
PruAA5_2A CAP96757	S G N A V M F D A V N G K I F T Y G G S Q Y S E N S Y A T T D A Y L I E I D - E P G S Q P K V T A A K N P N G E M A Y	281
CgrAAO EFQ27661	C G V W V M Y D A V A G K I F S A G G S P D Y T D S P A T Q R A H I T T I G - E P N T P A E V E R - - - - - V A D M G F	277
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FgrGalOx Q01745	A R T F H T S V L P D G S T F I T G G Q R R G I P F E D S T P V F T P E I Y V P E Q D T F Y K Q N P N S I V R V Y H S	341
CgrAlcOx EFQ30446	N R G F A N A V M P D G K I W V V G G M K M W L F S D T P Q L T P E L F D P A T G S F T P T T P H T V P R N Y H S	335
CglAlcOx ELA25906	N R G F A N A V M P D G K I W V V G G M K M A L F S D A T P Q L T P E L F D P A T G K F T P T A A H T V P R N Y H S	335
CgrRafOx EFQ36699	A R I F A Q A I V L P N G Q V F V T G G Q A Y A A G F T D T L S V L Q A E I W D P V A N T F T P V A L A V P R N Y H S	345
PorAlcOx XP_003719369	G R G Y H N A V V L P D G K V F V V G G M S R M A L F S D G S P L V L F P E I W D P A T G G F T T M R P H T I P R N Y H S	346
ChiAlcOx OBRO5259	N R G F A N A V M P D G K I W V V G G M R Q M Q L F S D S T P Q L T P E L F D P A T G V F T P T T P H T V P R N Y H S	335
PruAA5_2A CAP96757	A R T F H T S V V L P D G G V F T A G G Q S Y G V F T N D S N A H L T P E L Y D P K T N F N E Q Q P N S I V R V Y H S	347
CgrAAO EFQ27661	P R G F A N A V L P D G G Q V L V T G G Q R M S L V F F T N T D G I L V A E L F N P E T R E W K Q M A P A V P R N Y H S	341
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FgrGalOx Q01745	I S L L L P D G R V F N G G G G L C G - - - - - - - D C K T N H F D A Q I F T P N Y L Y N S G N - L A T R P K	389
CgrAlcOx EFQ30446	T A L L M A D A T I W S G G G G L C G - - - - - - - A N C K E N H F D G Q F W S P P Y L F E A D G V T P A K R P V	387
CglAlcOx ELA25906	T A L L M A D G T I W S G G G G L C G - - - - - - - A G C A A N H F D G Q F W S P P Y L F E A D G K T P A K R P V	387
CgrRafOx EFQ36699	T G L L L P D G R V M N G G G G L C Y V G G - - - - - - G - C N S G N H P L D Q F W T P P Y M F D A R G N - P A T R P Q	395
PorAlcOx XP_003719369	T A M L M A D G T V F S G G G G L C G - - - - - - - A G C S A N H F D G Q F W S P P Y L F Q A D G K T P A K R P V	386
ChiAlcOx OBRO5259	T A L L M A D A T I W S G G G G L C G - - - - - - - A N C K E N H F D G Q F W S P P Y L F E A D G K T P A K R P V	385
PruAA5_2A CAP96757	I S L L L P D G R V F N G G S G L G V - - - - - - - S A P T N H F D A Q I Y S P H Y L F N D G D G S - L A T R P T	389
CgrAAO EFQ27661	V S I L L P D A T V F S G G G G M C W V Q N V G D S T A G C D K T V D H S D A E I F E P P Y L F N E D G S - R A A R P V	396
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FgrGalOx Q01745	I T R T S T Q S - - - - - V K V G G R I T I S T D - - S S I S K A S L I R Y G T A T H T V N T D Q R R I P L T L T N	440
CgrAlcOx EFQ30446	I Q S L S D T - - - - - A V R A G A P I T I M Q D A - G A Y T F S M I R V S A T T H T V N T D Q R R I P L D G Q D	437
CglAlcOx ELA25906	I E S L S D E - - - - - T V K A G A A L T I N M Q D E - G K Y T F S M I R V S A T T H T V N T D Q R R I P L D G Q D	437
CgrRafOx EFQ36699	I S S I S A S Q S G N Q V R V S P G K L T V L L G S S G A N L G H V L V M G S G T H S I D T D Q R R I P L T V Y S	445
PorAlcOx XP_003719369	I R S L G P A S G A N G A V E V R A G D Q V T V M Q D A - G A Y S F S M I R T G S T T H T V N T D Q R R I P L A G Q D	457
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PruAA5_2A CAP96757	I D S V A N K N - - - - - L R A G D K L S I S A S - I D V K N A S L I R Y G T T T H T V N T D Q R R I S L D S W T	440
CgrAAO EFQ27661	I S A I S A D P - - - - - I K A G A T L T F T V E G V E G Q G T A A L I R L G S V T H S V N S D Q R R V P L N V T V	449
	* : : : : * : : : : * : : : : * : : : : *	
FgrGalOx Q01745	N G - G N S Y S F Q V P S D S G V A L P G Y W M L F V M N S A G V P S V A S T I R V T Q	483
CgrAlcOx EFQ30446	G G D G K S F T V N V P N D Y G V A I P G Y Y M L F A M N E A G V P C V A Q F F K V L S	481
CglAlcOx ELA25906	G G D G K S F S V N M P S D Y G V I P G Y Y M M F A M N E A G T P C V A K F F K V L S	481
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PorAlcOx XP_003719369	V G G G - S Y V V T V P S D Y G I A T P G Y Y M L F A S E A G V P A V A K F R V G L G	488
ChiAlcOx OBRO5259	G G D G Q A F T V N V P A D Y G V A I P G Y Y M L F A M N E A G V P C V A K F F K V L S	481
PruAA5_2A CAP96757	A N - E G S Y E T T L P G D S G I L L P G W M L F I L N D D G V P S V S Q T I H I Q V G	483
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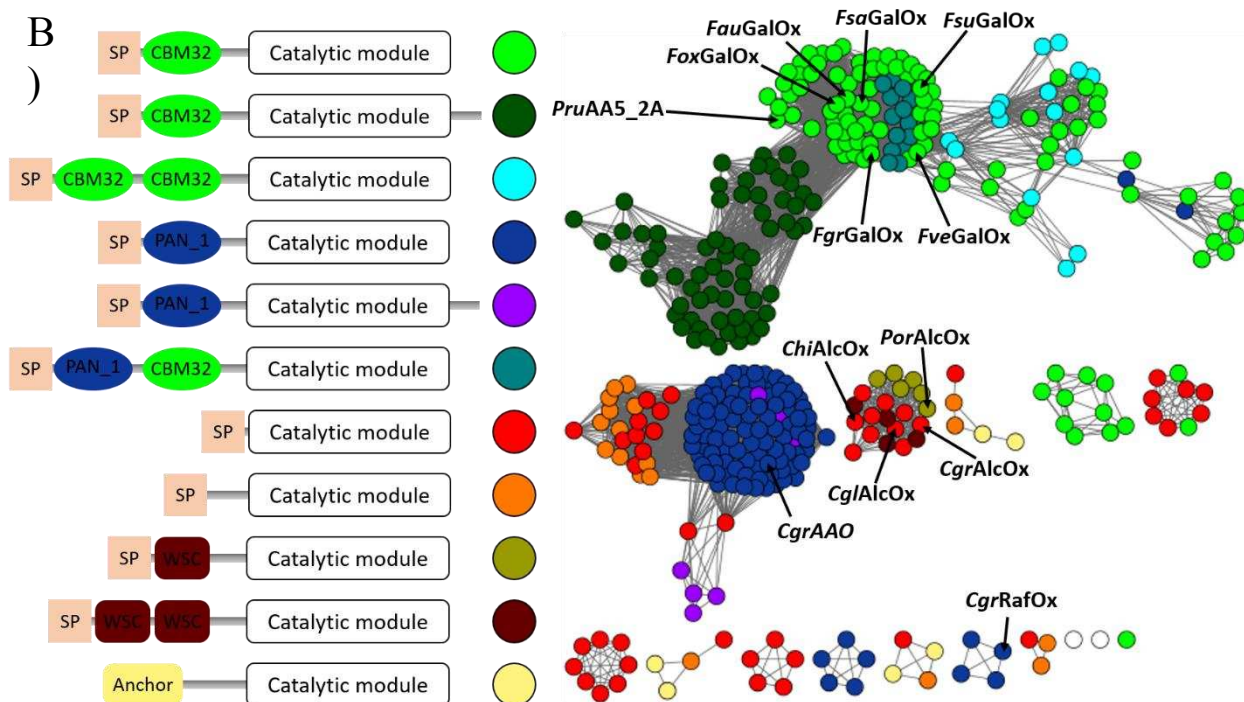


Figure S1. (A) Sequence alignment of *Colletotrichum graminicola* aryl alcohol oxidase (CgrAAO) with characterized AA5_2 members. (B) Sequence similarity network at an alignment score cut-off of 10^{-550} of 392 catalytic modules from the AA5_2 subfamily with their corresponding modularity. For each panel, predicted native signal peptides and additional N-terminal modules have been removed. Conserved active-site catalytic residues and residues involved in substrate recognition are highlighted in yellow and green, respectively (A). Each node is colored according to its modularity. Catalytic modules are shown in white, carbohydrate binding modules are in green⁵, PAN_1 domains are blue⁶, WSC are brown⁷ and GPI anchor are yellow (B). CgrAlcOx = *Colletotrichum graminicola* alcohol oxidase, CglAlcOx = *Colletotrichum gloeosporioides* alcohol oxidase, CgrRafOx = *Colletotrichum graminicola* raffinose oxidase, PruAA5_2A = *Penicillium rubens* Wisconsin 54–1255 AA5_2 oxidase, FgrGalOx = *Fusarium graminearum* galactose oxidase, ChiAlcOx = *Colletotrichum higginsianum* alcohol oxidase and PorAlcOx = *Pyricularia oryzae* alcohol oxidase.

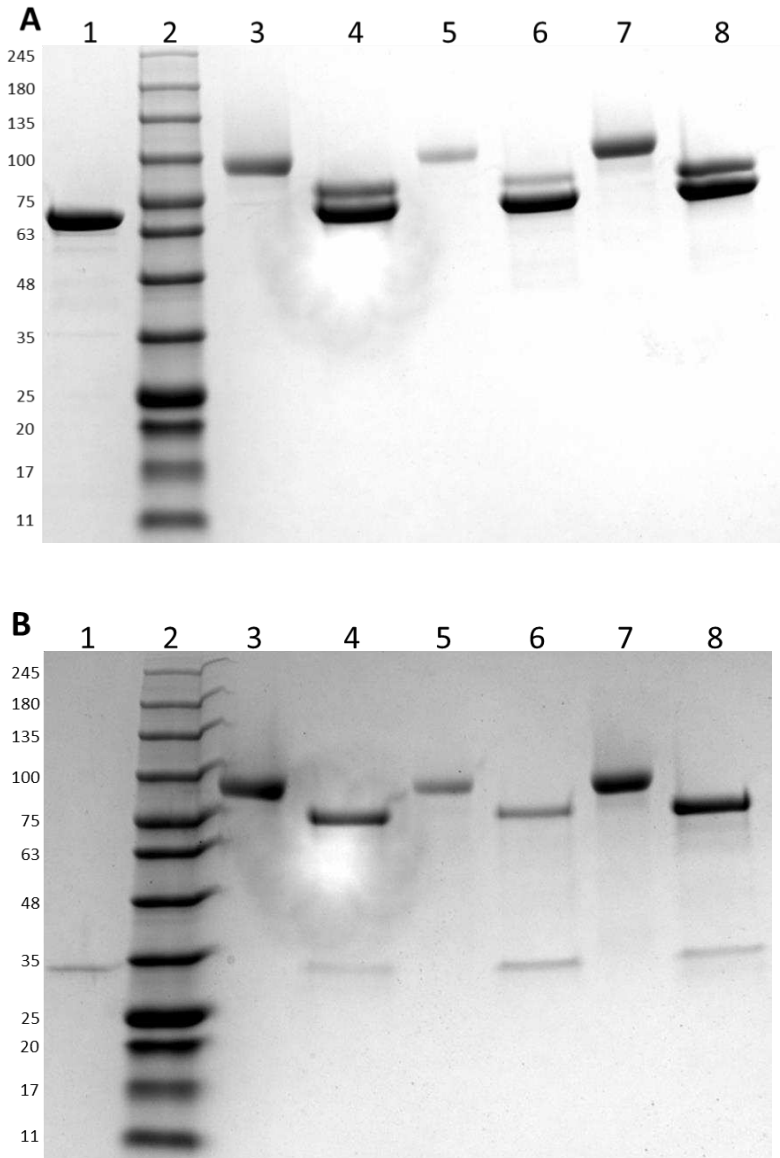


Figure S2. SDS-PAGE of *CgrAAO*-WT, *CgrAAO*-Y334F and *CgrAAO*-Y334W & *N*-deglycosylation studies.

Enzymes were *N*-deglycosylated under denaturing conditions with either PNGaseF (A) or EndoH (B).

(A): 1: EndoH, 2: molecular weight marker, 3: *CgrAAO*-WT (5 μ g), 4: *CgrAAO*-WT (5 μ g) + EndoH, 5: *CgrAAO*-Y334W (5 μ g), 6: *CgrAAO*-Y334W (5 μ g) + EndoH, 7: *CgrAAO*-Y334F (5 μ g), 8: *CgrAAO*-Y334F (5 μ g) + EndoH

(B): 1: PNGaseF, 2: molecular weight marker, 3: *CgrAAO*-WT (5 μ g), 4: *CgrAAO*-WT (5 μ g) + PNGaseF, 5: *CgrAAO*-Y334W (5 μ g), 6: *CgrAAO*-Y334W (5 μ g) + PNGaseF, 7: *CgrAAO*-Y334F (5 μ g), 8: *CgrAAO*-Y334F (5 μ g) + PNGaseF

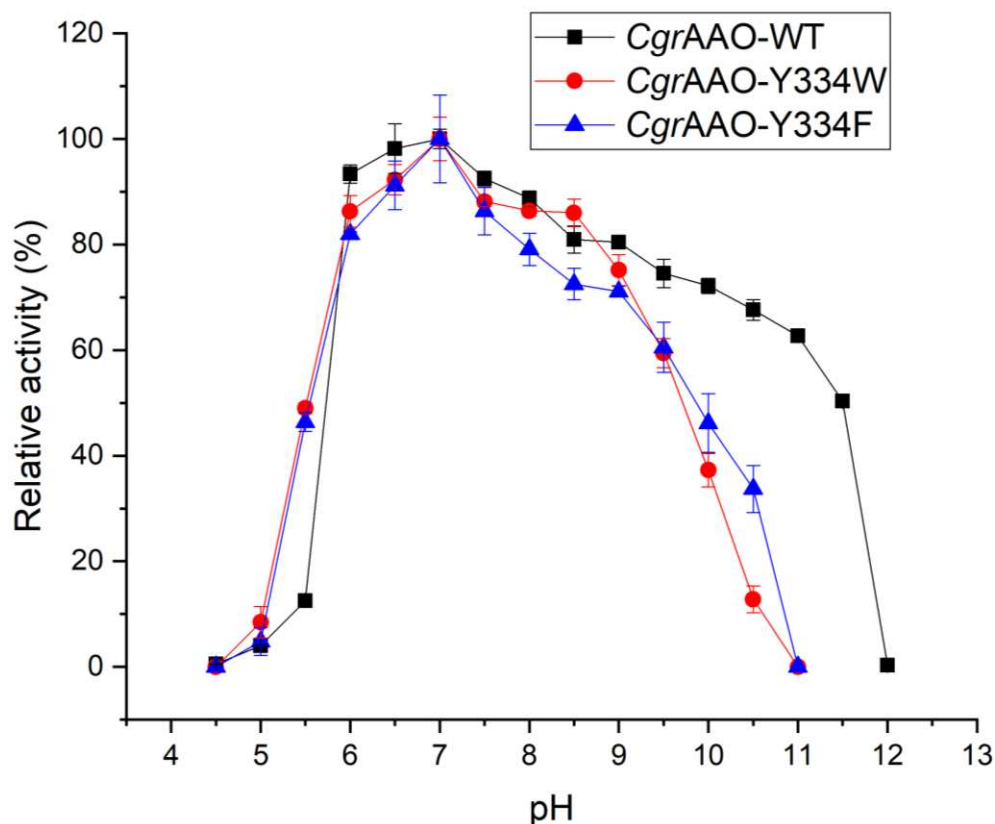


Figure S3. pH-rate profiles of *CgrAAO*-WT and mutants. Data are represented as means \pm standard deviations ($n = 3$). Activities were determined by the HRP/ABTS assay monitoring absorbance at 420 nm using 50 mM HMF for *CgrAAO*-WT and *CgrAAO*-Y334F, and 500 mM melibiose for *CgrAAO*-Y334W. pH rate profiles were determined after 1-min incubations at the desired pH, pH range 4-6 was maintained using 100 mM phosphate-citrate buffers, pH range 6-8 was maintained using 100 mM phosphate buffers and pH range 8- 12 was maintained using glycine-sodium hydroxide buffers.

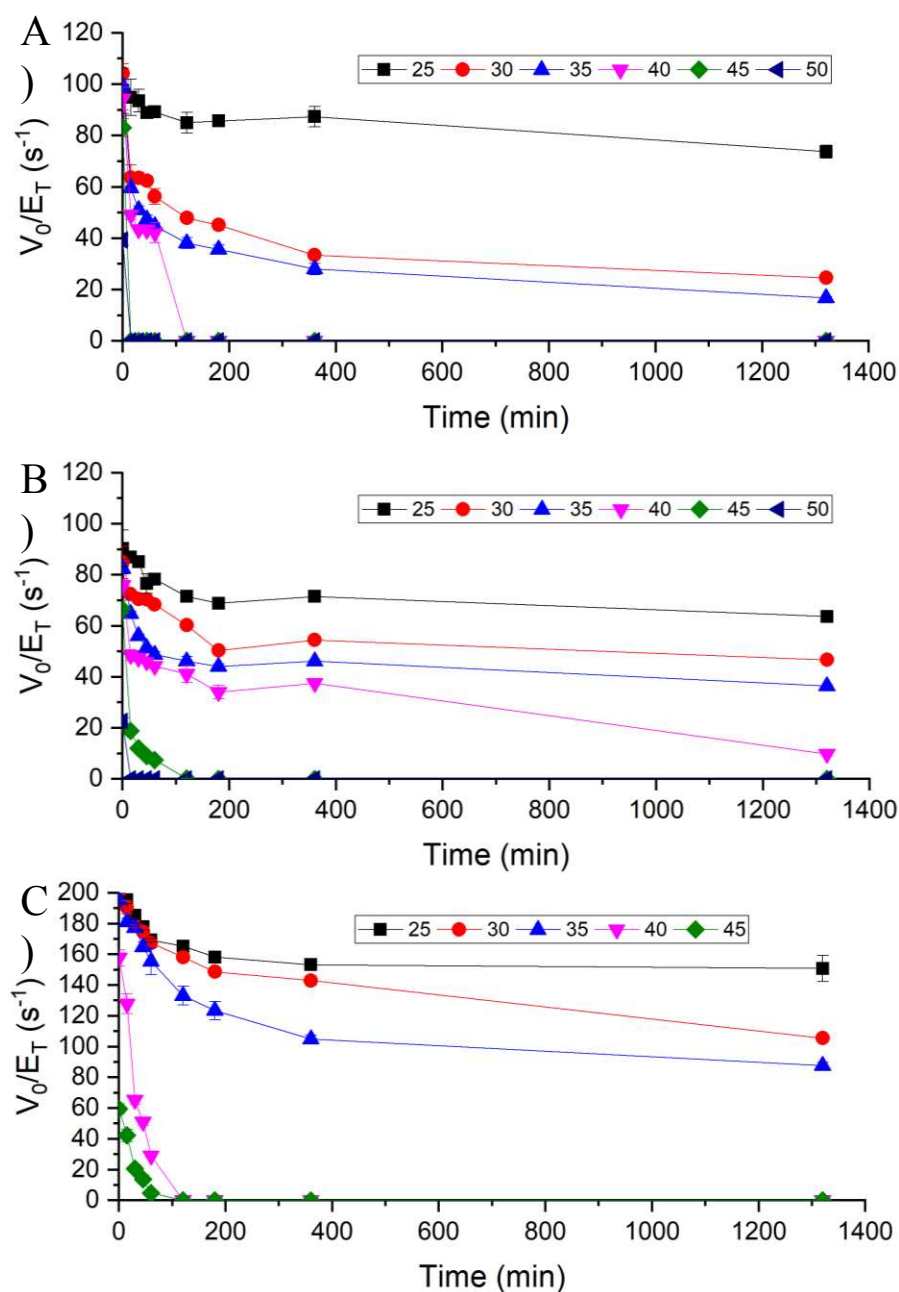
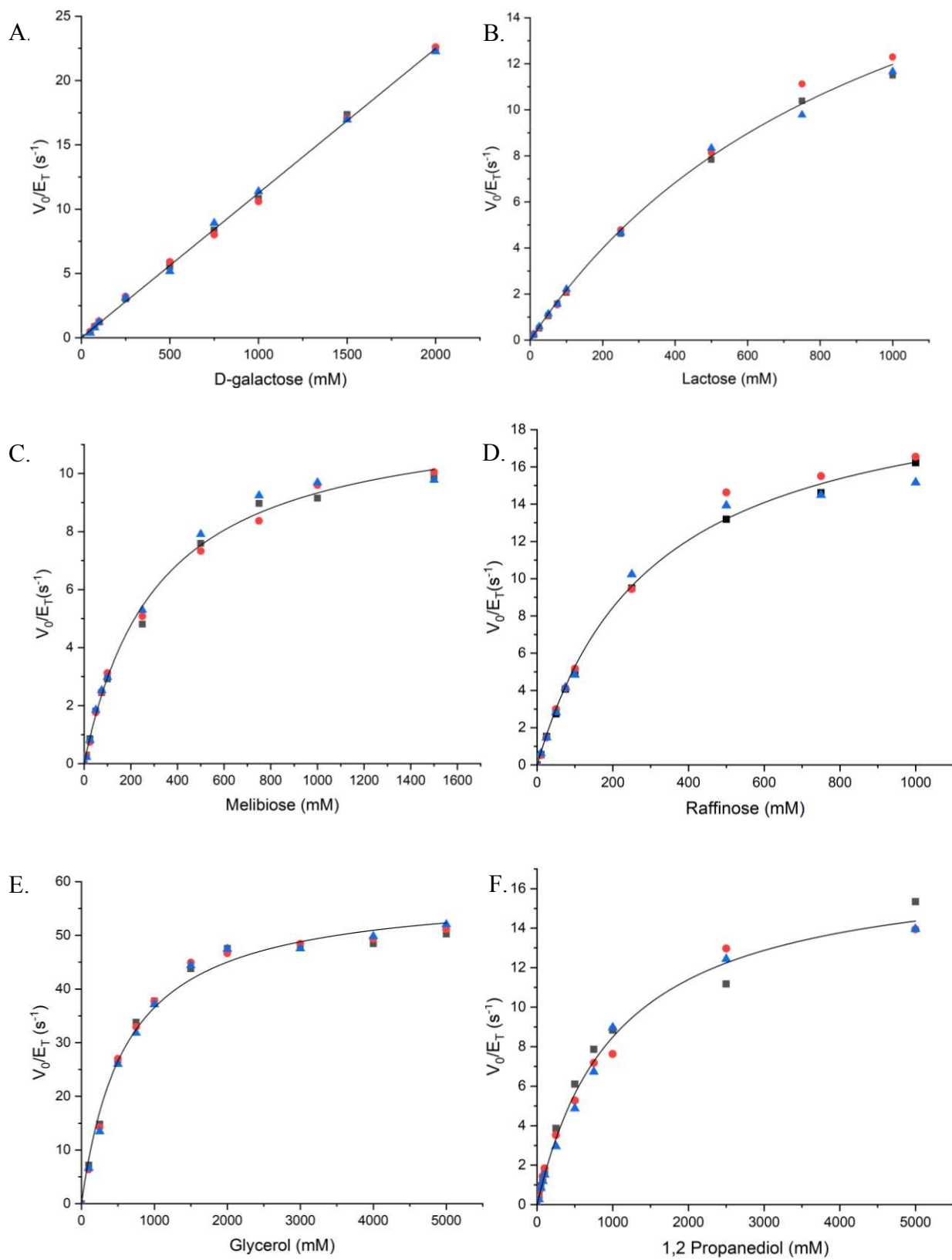
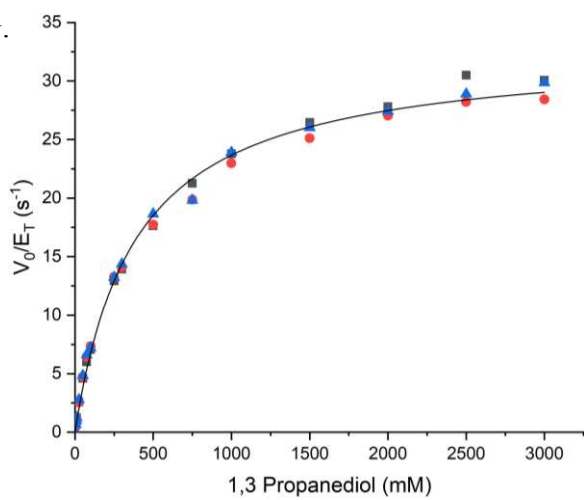


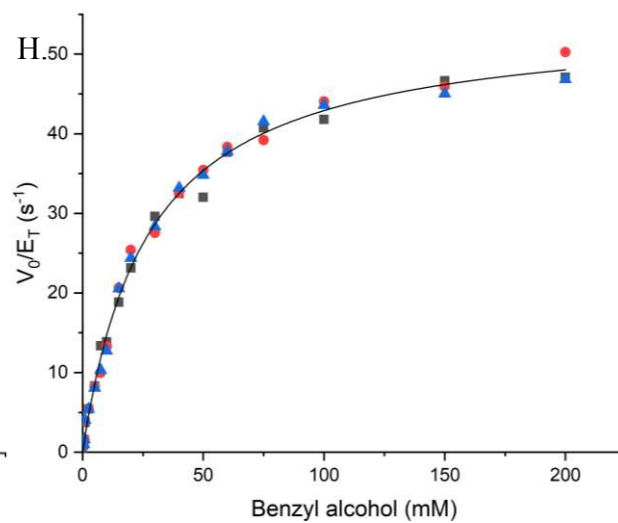
Figure S4. Temperature stability. A) *CgrAAO*-WT; B) *CgrAAO*-Y334W; C) *CgrAAO*-Y334F. Data are represented as means \pm standard deviations ($n = 3$). Activities values were determined by the coupled HRP/ABTS at each temperature, maintained by a gradient thermocycler, using 50 mM HMF for *CgrAAO*-WT and *CgrAAO*-Y334F, and 500 mM melibiose for *CgrAAO*-Y334W.



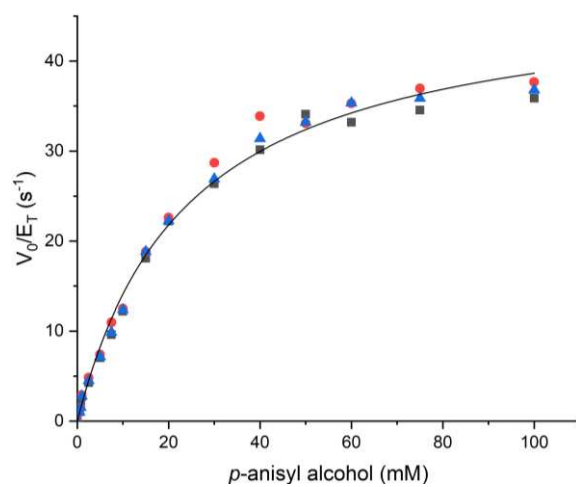
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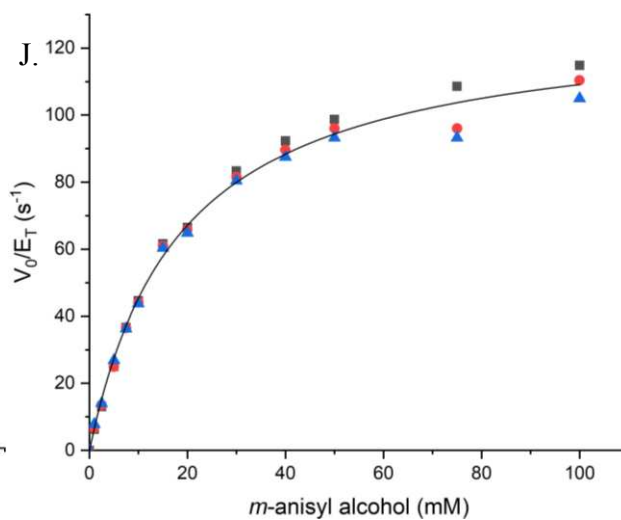
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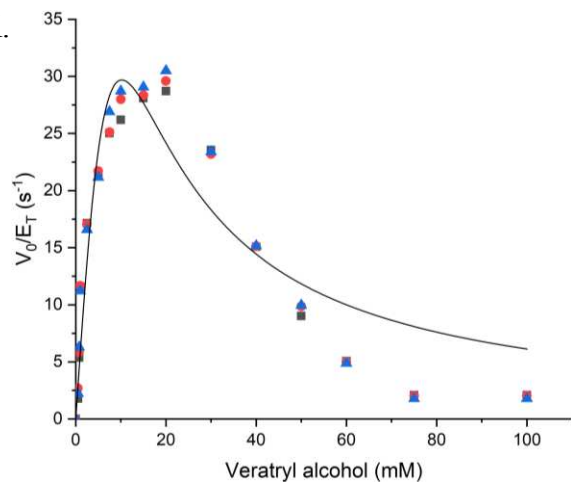
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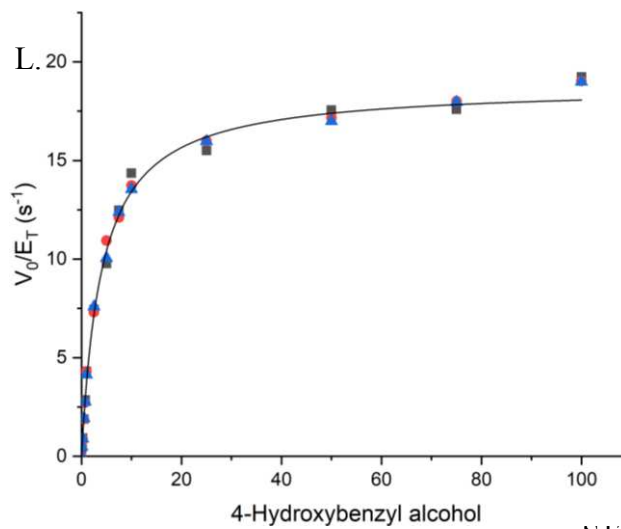
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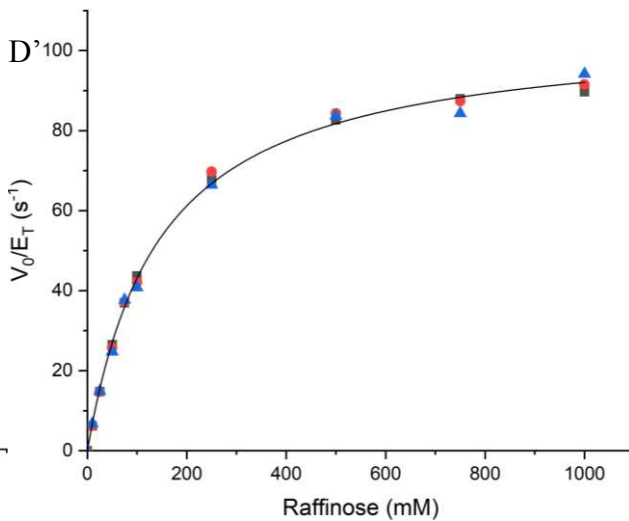
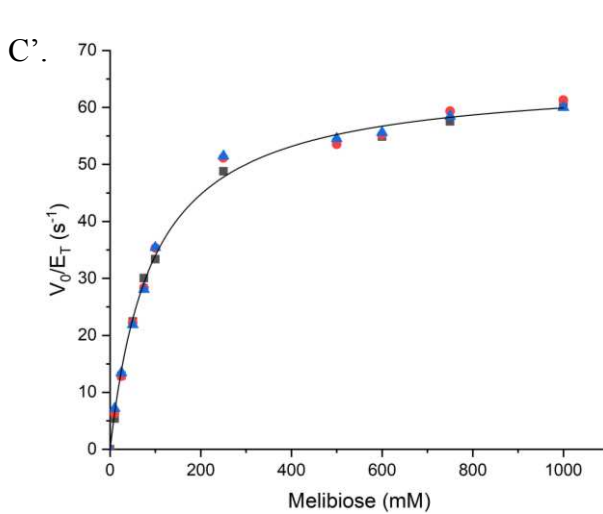
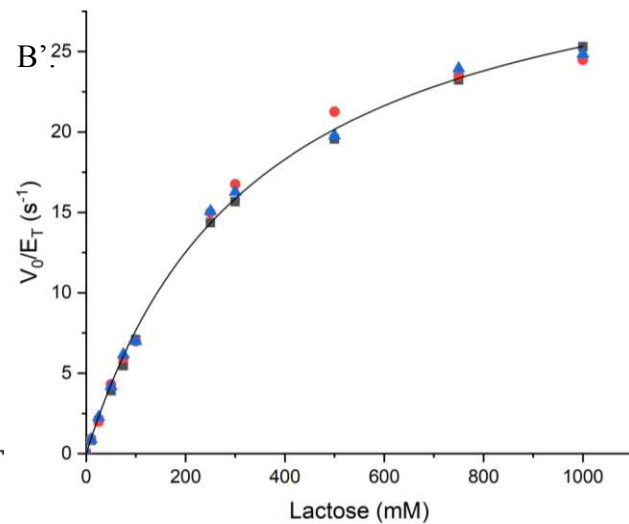
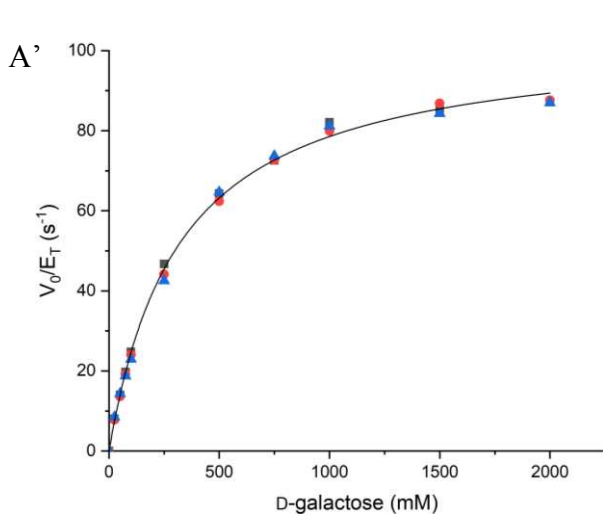
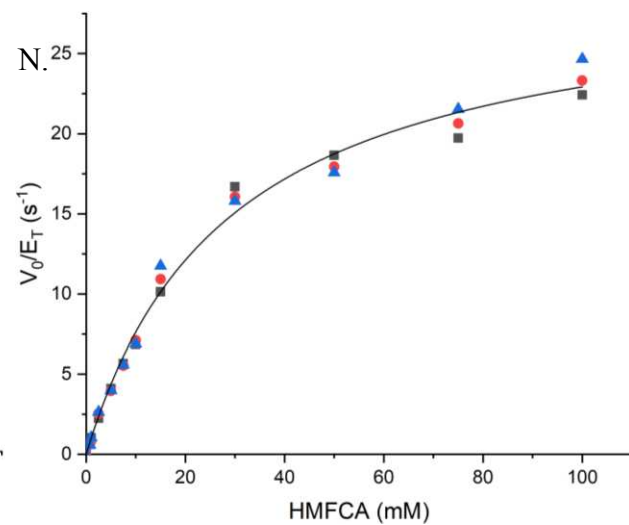
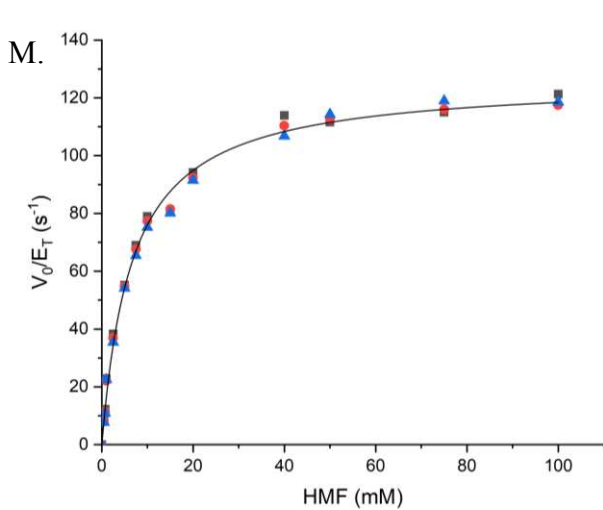


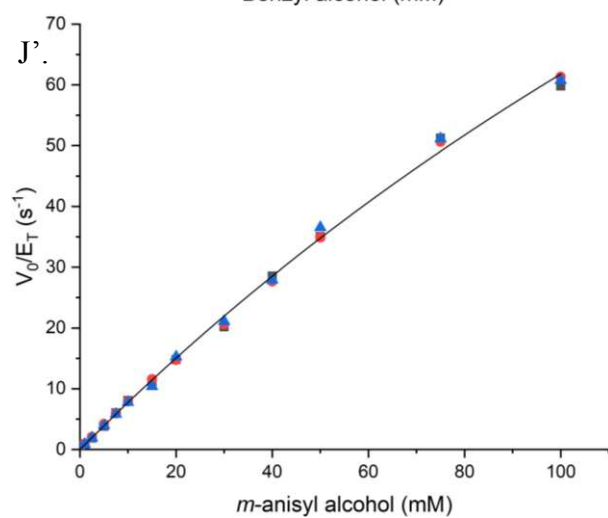
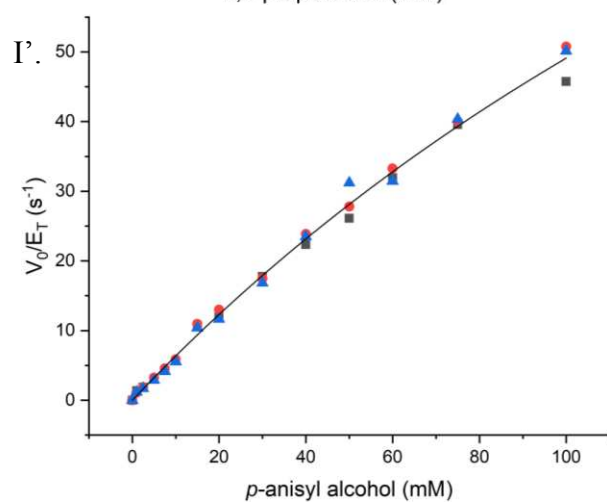
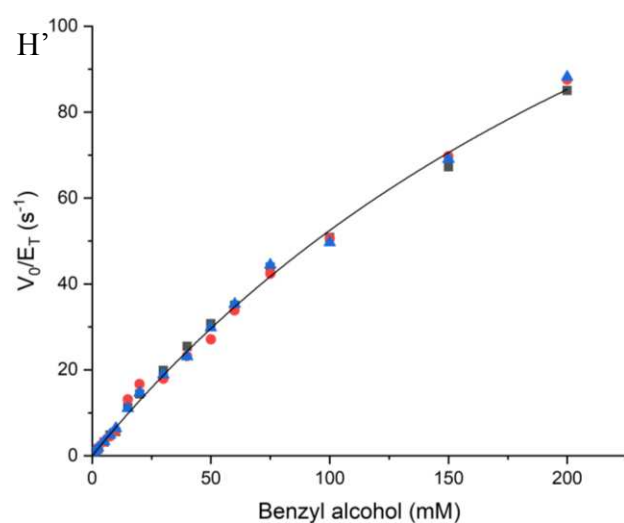
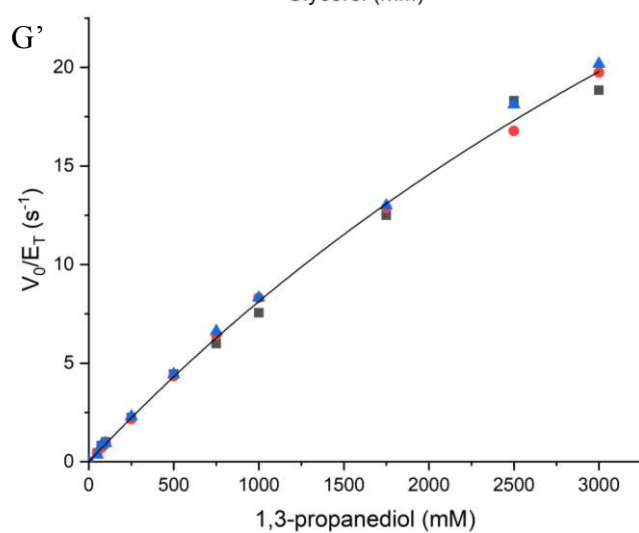
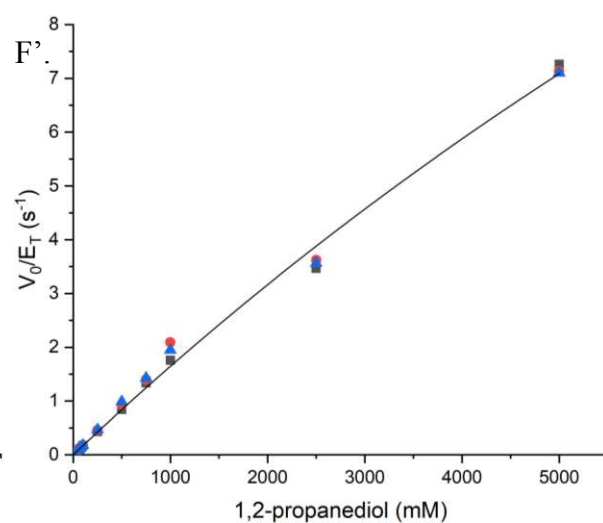
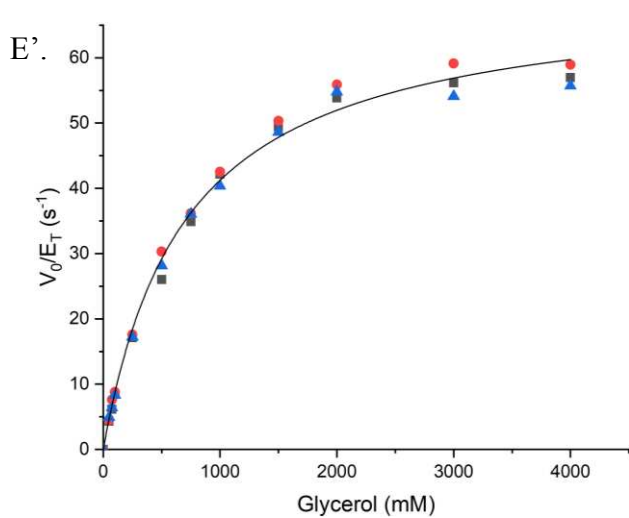
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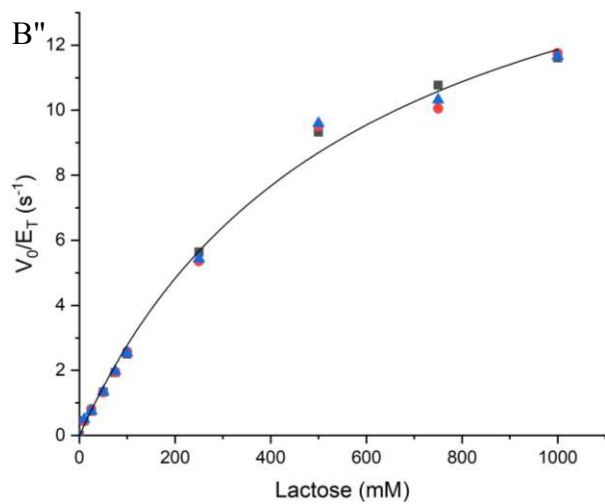
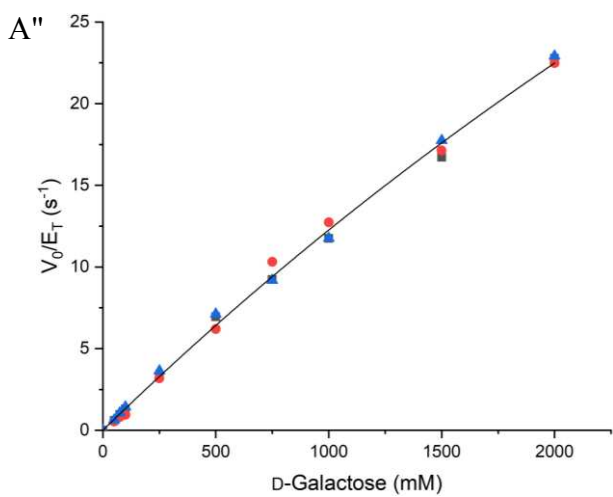
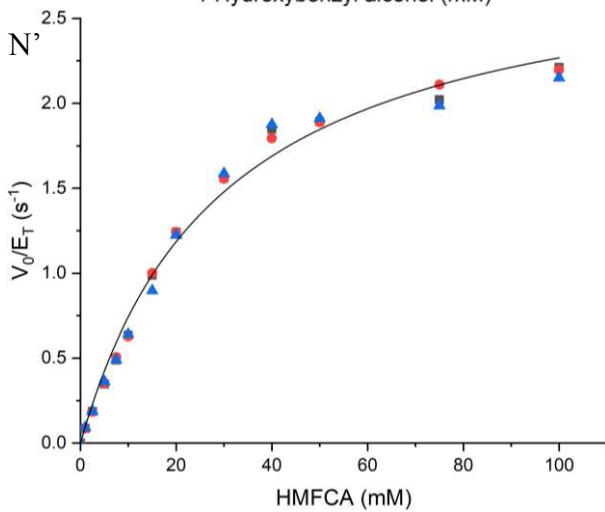
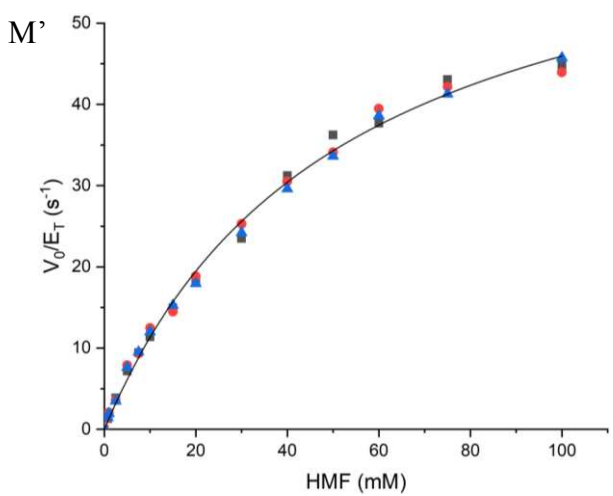
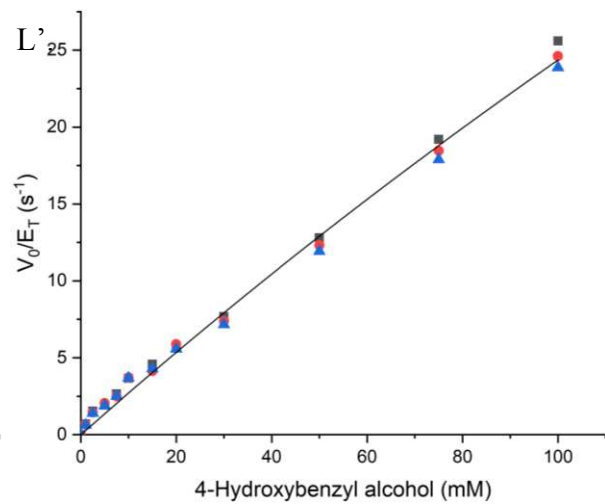
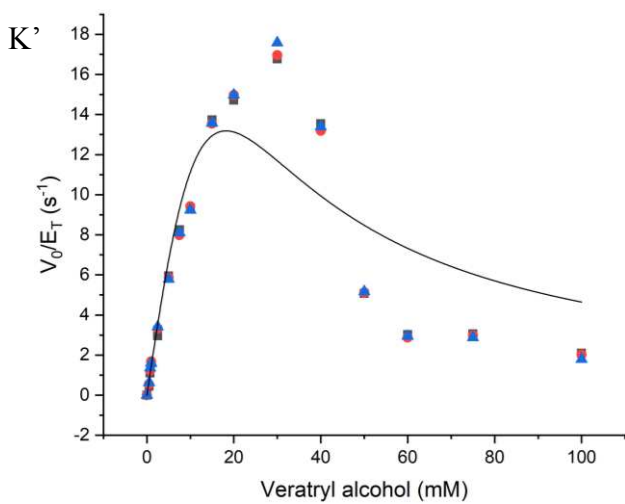


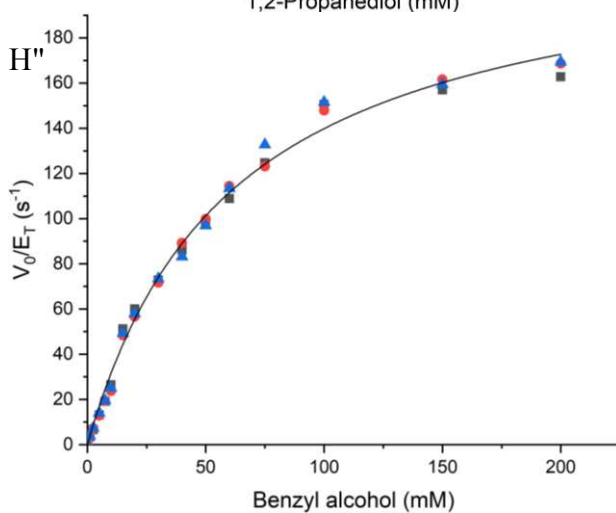
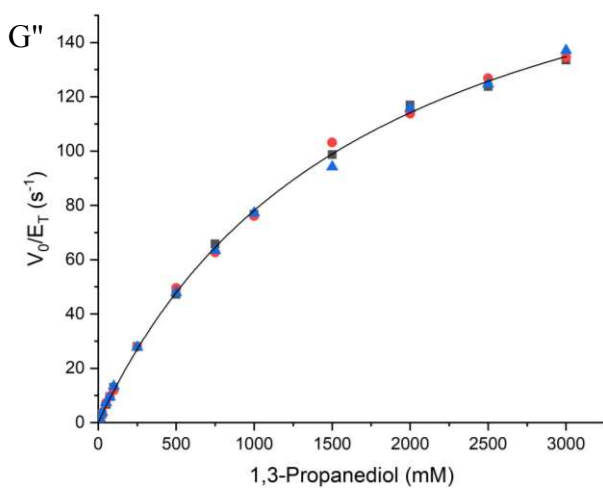
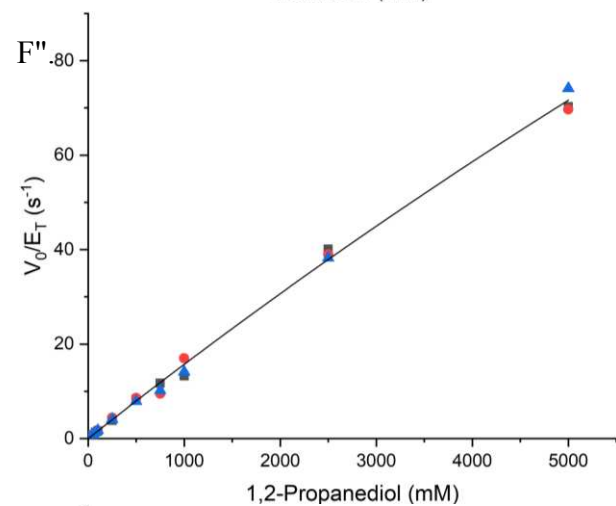
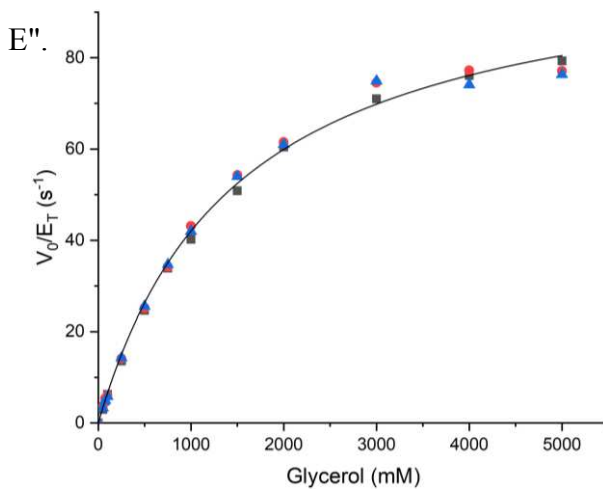
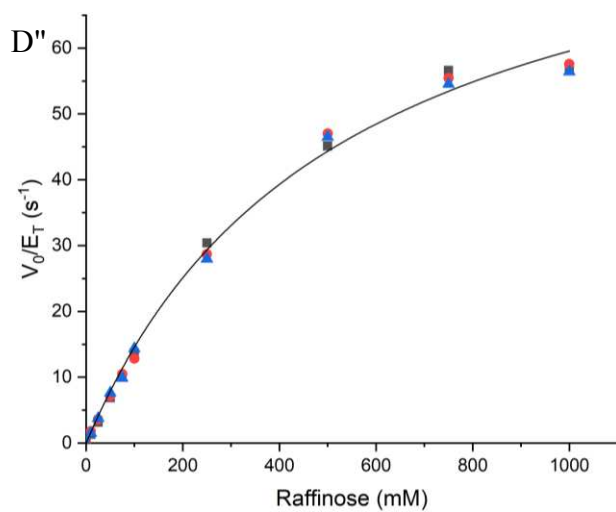
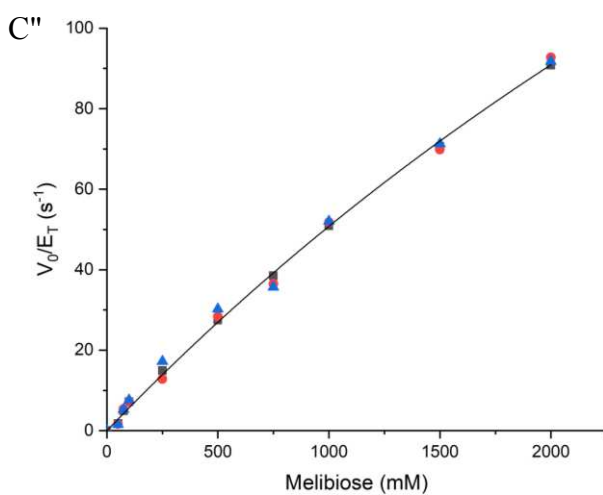
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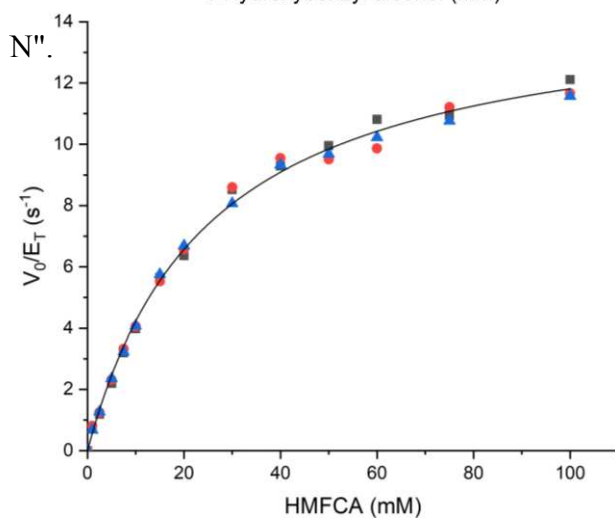
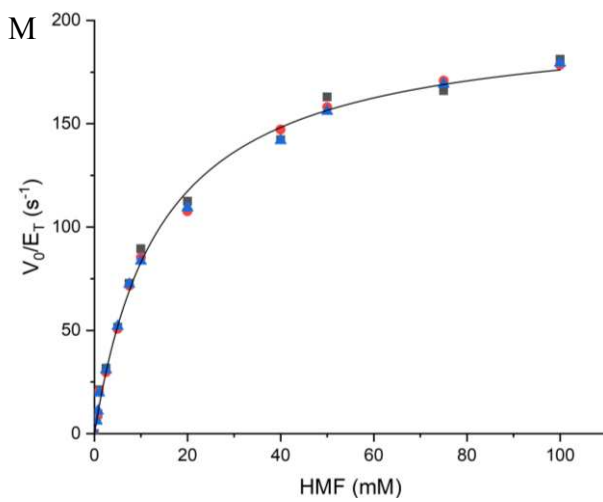
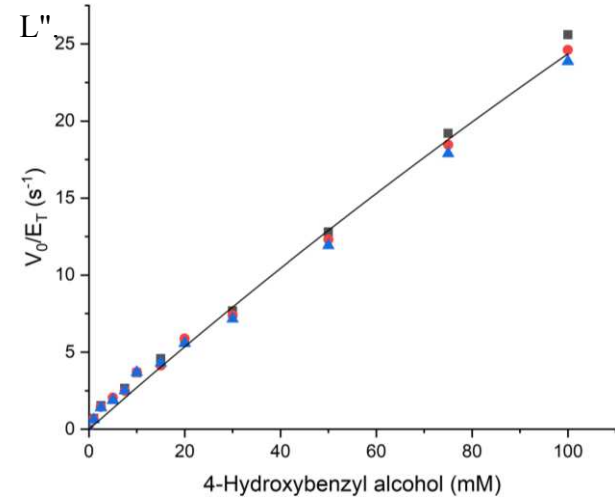
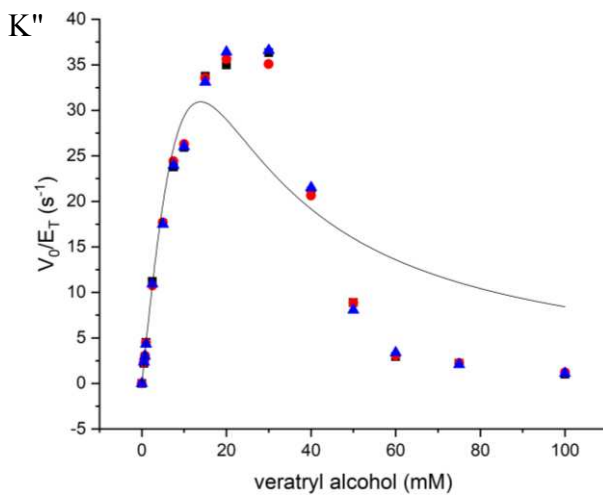
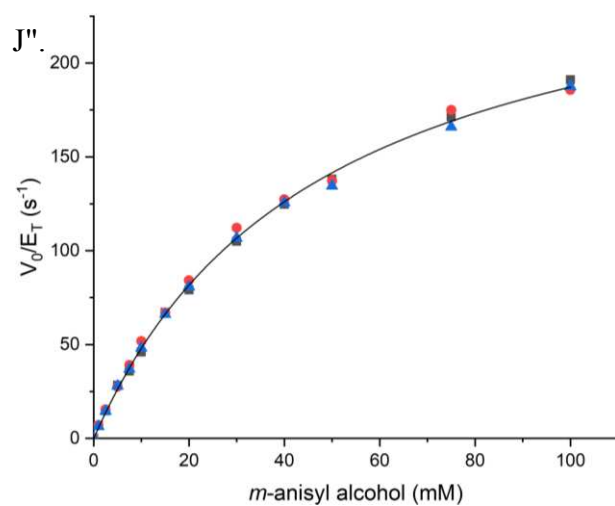
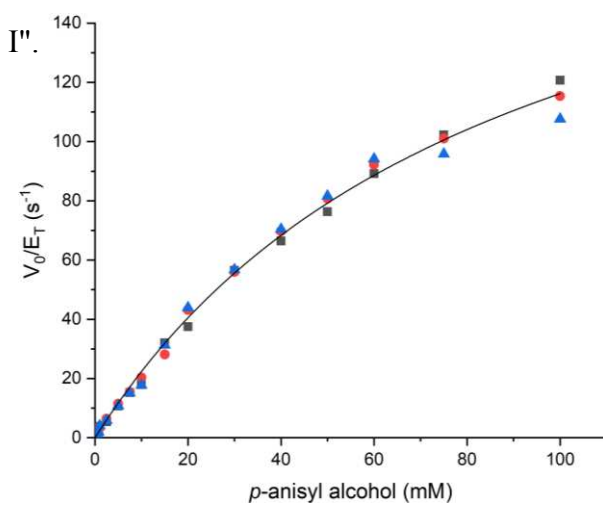
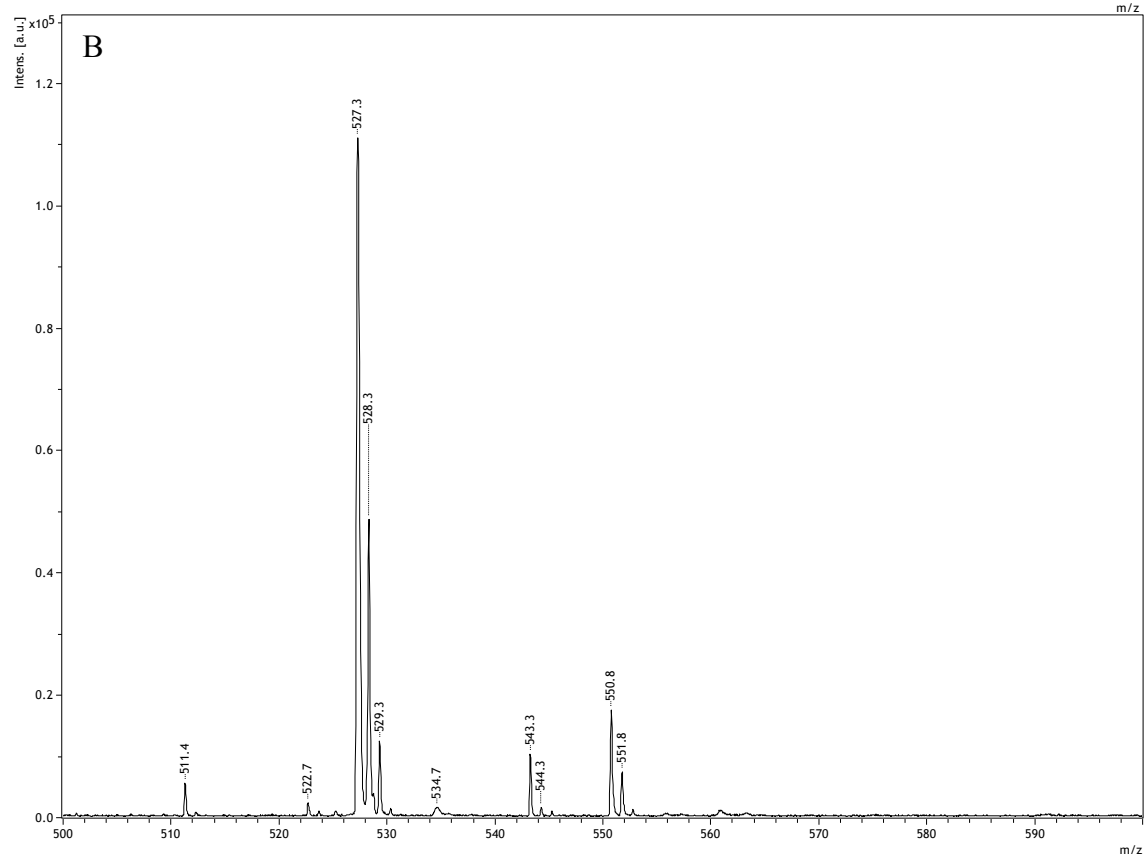
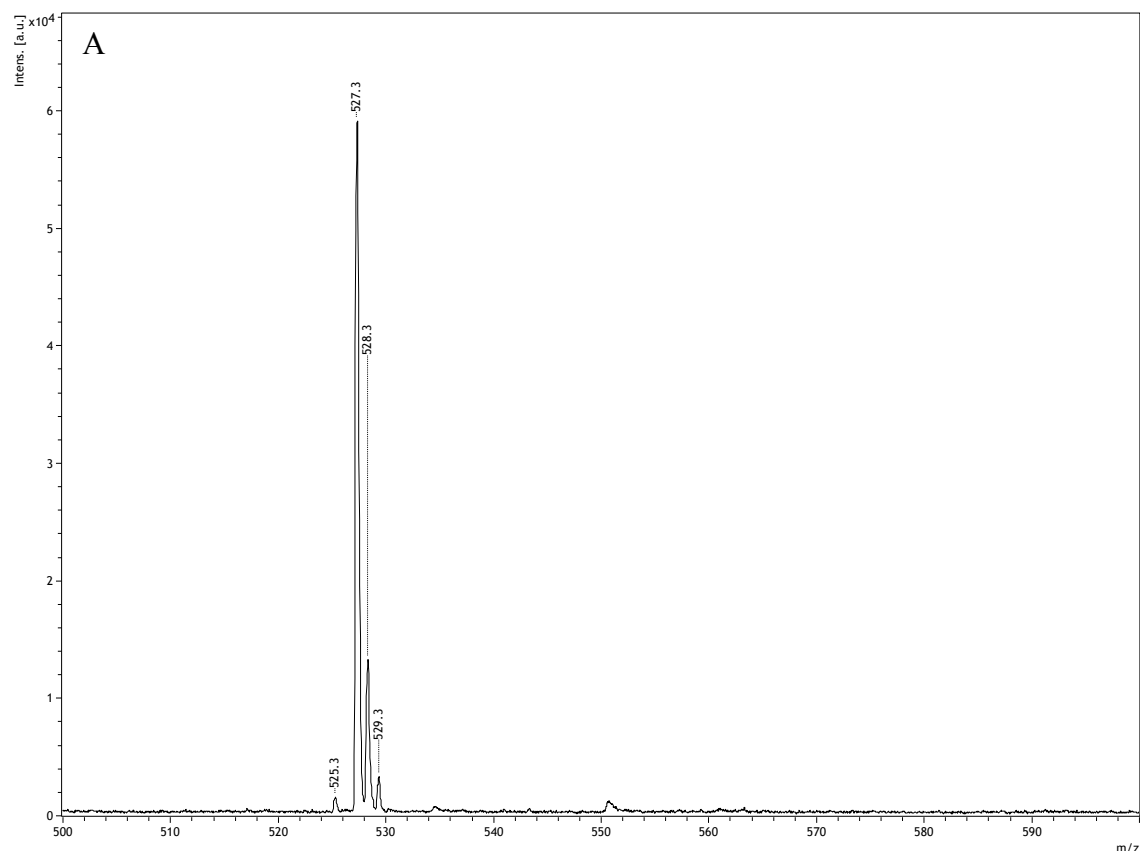
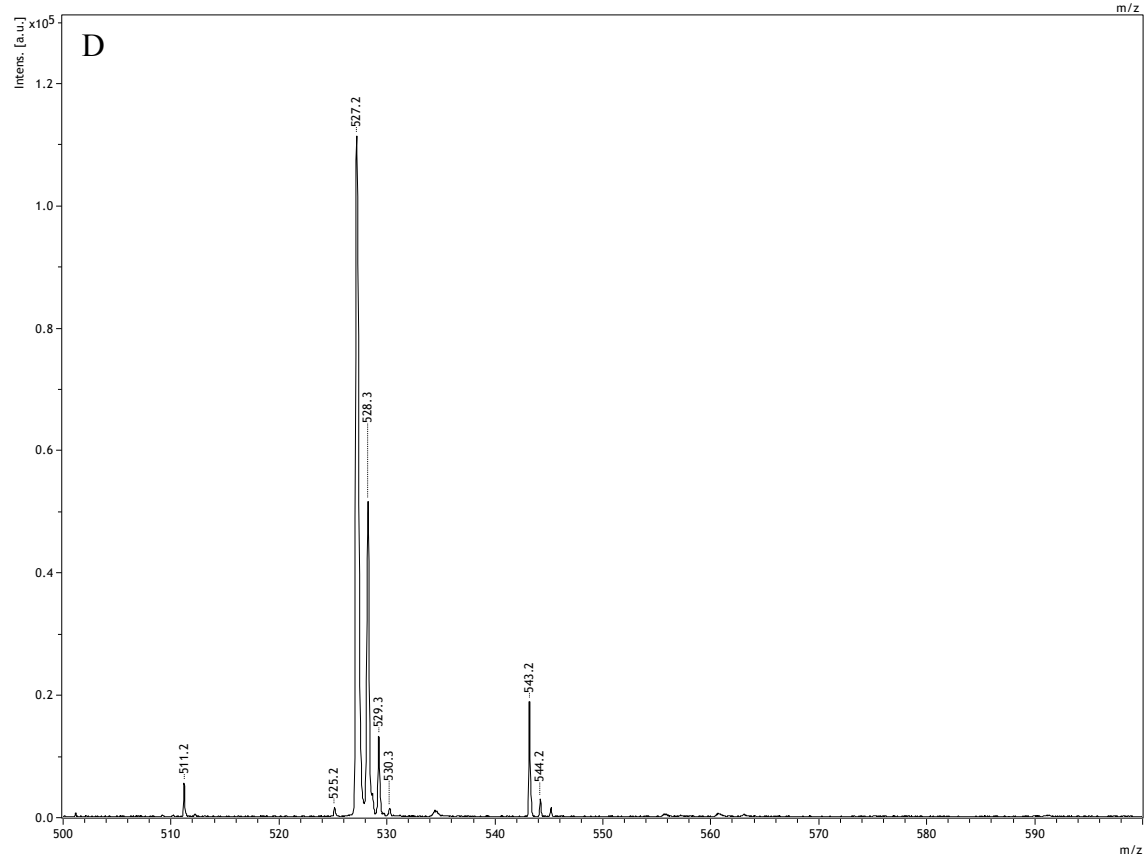
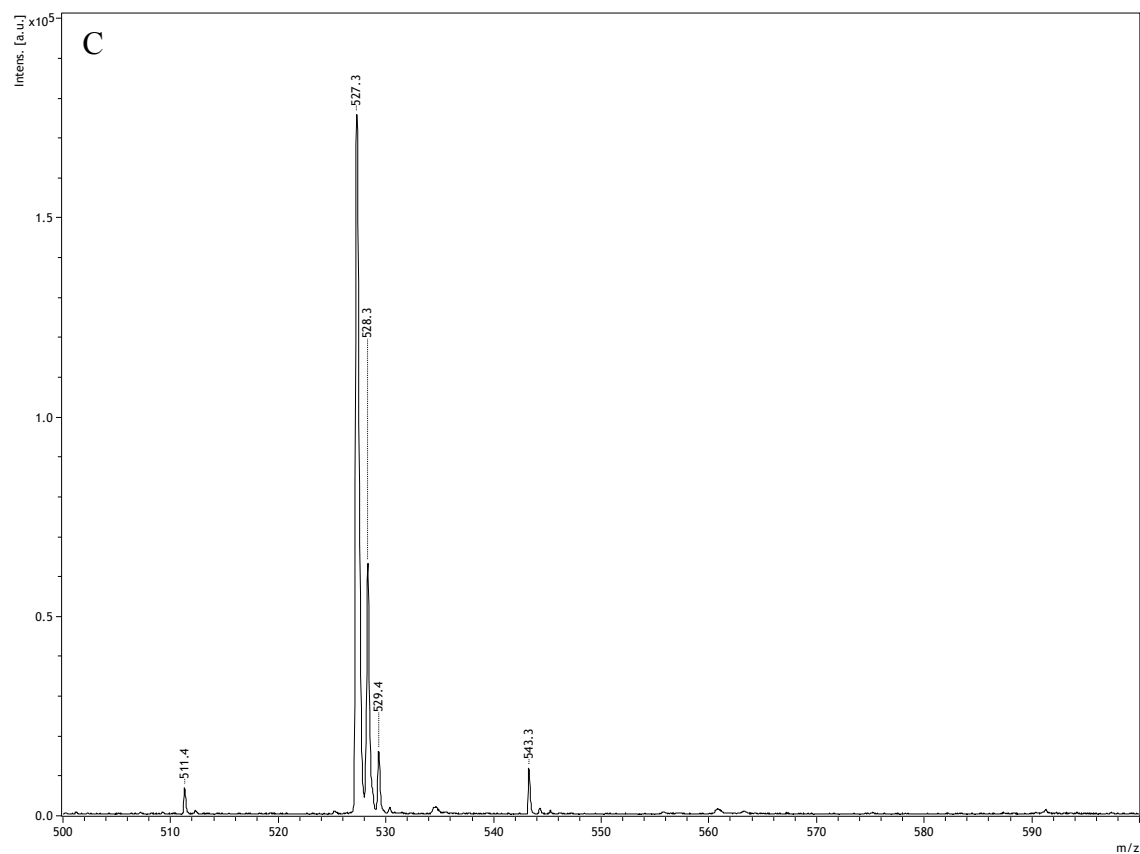
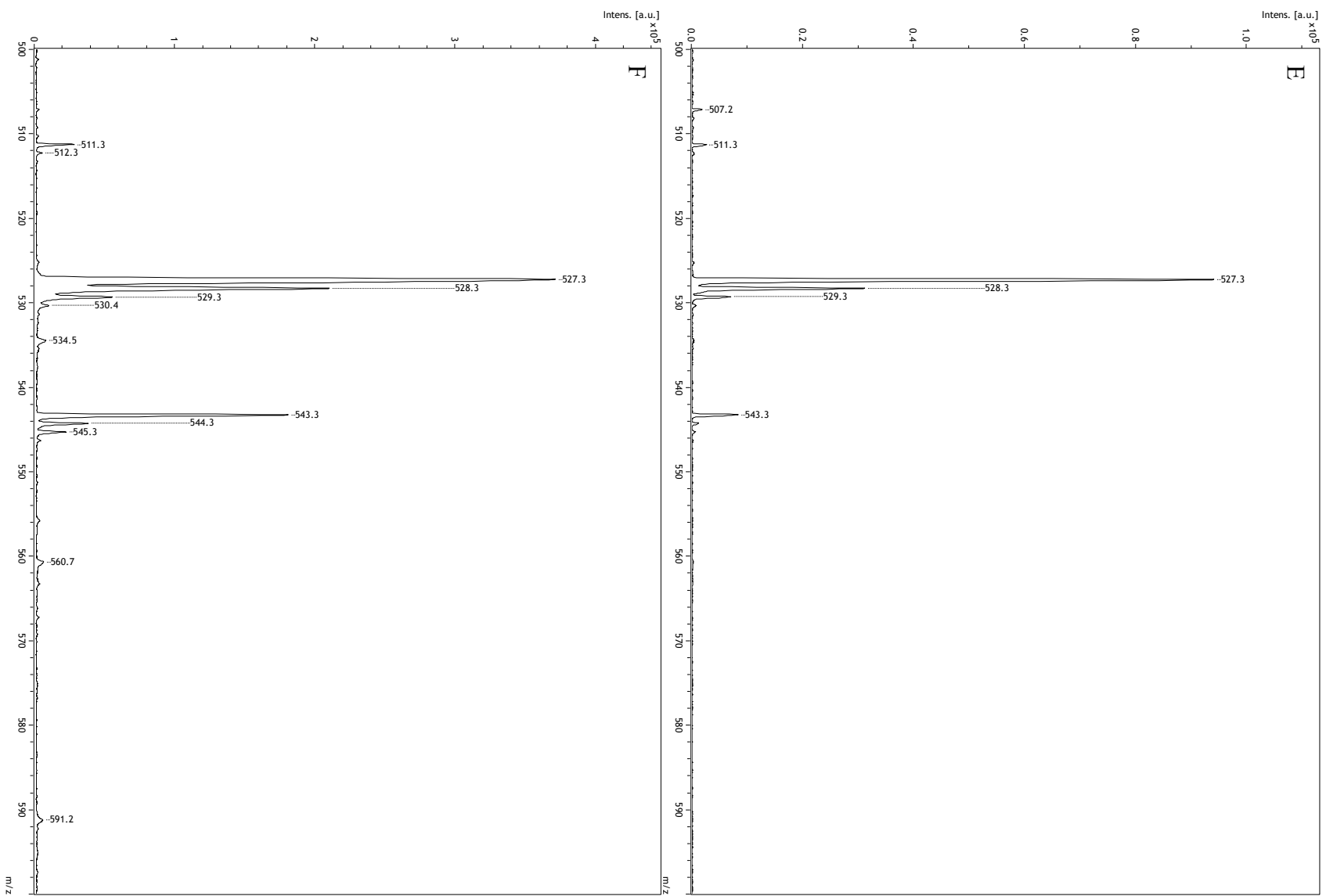
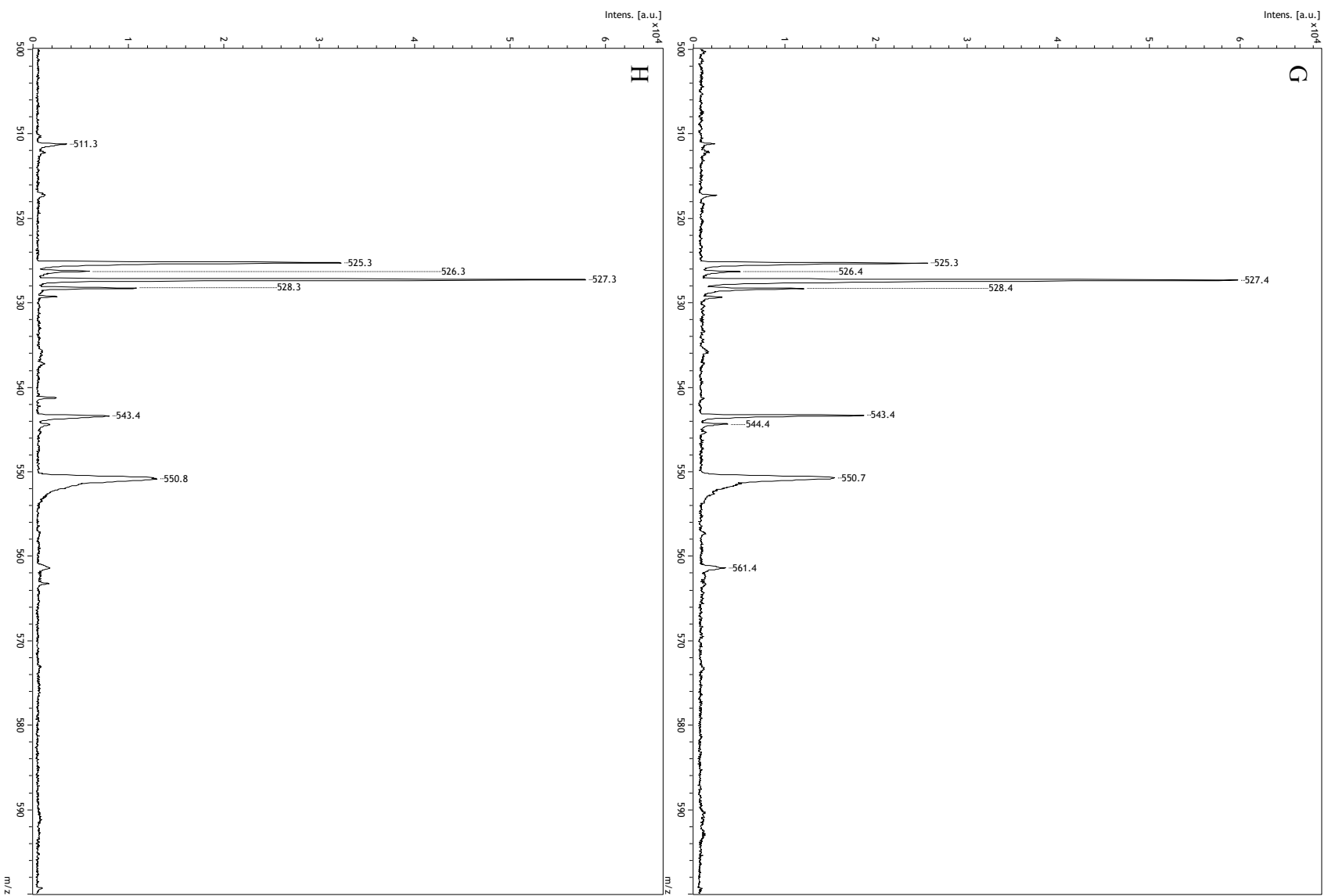


Figure S5. Initial-rate kinetics. Initial-rate values were measured in triplicate at each substrate concentration. Individual k_{cat} and K_m values were derived by non-linear fitting of the standard Michaelis-Menten or substrate-inhibition (veratryl alcohol) equations to the data using OriginLab 9.55. For substrates that did not display saturation kinetics, composite k_{cat}/K_m values were calculated from the slope of linear fits. Individual substrates are indicated in the x-axis labels of Panels A-N for *CgrAAO*-WT; A'-N' for *CgrAAO*-Y334W and A''-N'' for *CgrAAO*-Y334F.









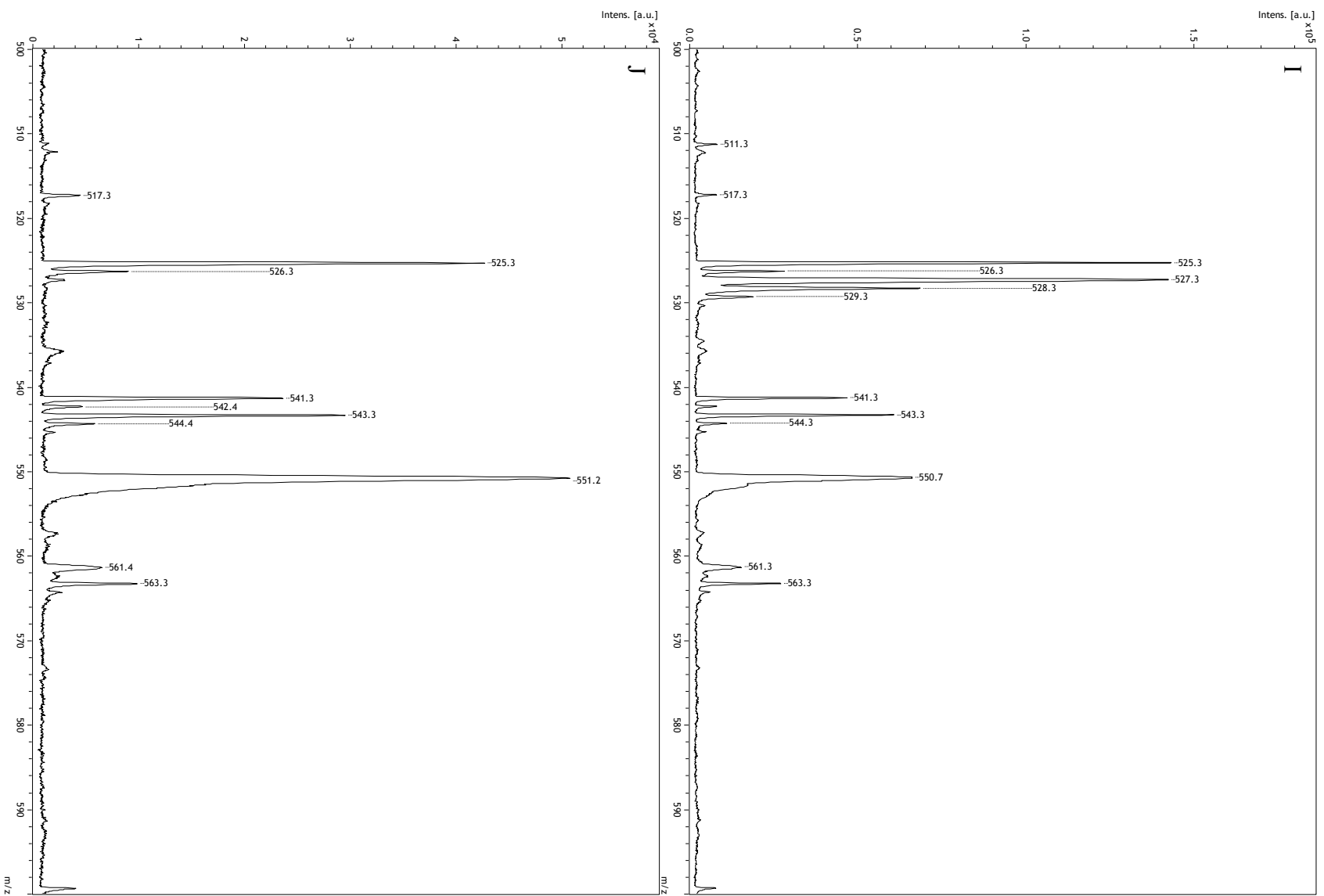


Figure S6. Time course analysis of raffinose oxidation by *Cgr*AAO by MALDI-TOF. (A-E) 10 mM raffinose incubated with 1 U of HRP/mg of substrate and 115 U of catalase/mg substrate at times 0 h (A), 2 h (B), 4 h (C), 8 h (D) and 16 h (E). (F-J) 10 mM raffinose incubated with 1 U of HRP/mg of substrate, 115 U of catalase/mg substrate and 200 μ g of *Cgr*AAO at times 0 h (F), 2 h (G), 4 h (H), 8 h (I) and 16 h (J). m/z 527.3 = raffinose sodium adduct, m/z 525.3 = raffinose aldehyde product sodium adduct, m/z = 543.3 raffinose aldehyde product in hydrate form sodium adduct, m/z = 541.3 uronic acid derivative sodium adduct. The identity of the broad peak at m/z = 550.7 is unknown.

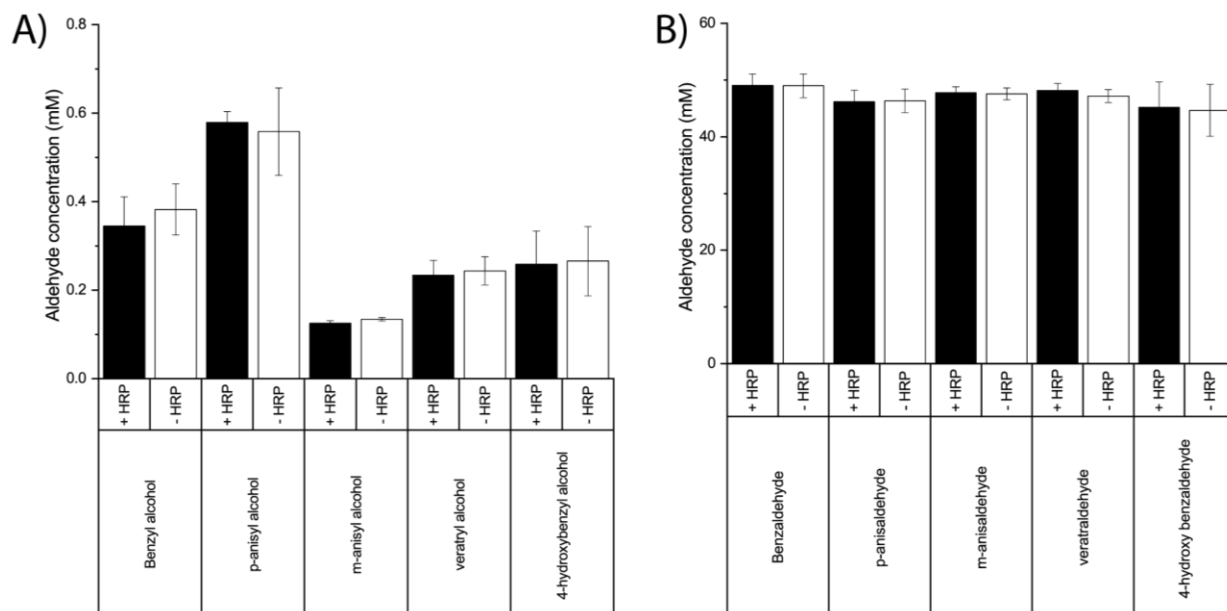


Figure S7. Aldehyde detection by Purpald. (A) 50 mM aryl alcohol incubated with 10 mM H₂O₂ in presence or absence of 2.3 μ M HRP for 15 minutes. (B) 50 mM aryl aldehyde incubated with 10 mM H₂O₂ in presence or absence of 2.3 μ M HRP for 15 minutes. Standard curves for each aromatic aldehyde made between 20 mM and 100 μ M gave a linear response ($r^2 > 0.99$) with a limit of detection of 50 μ M.

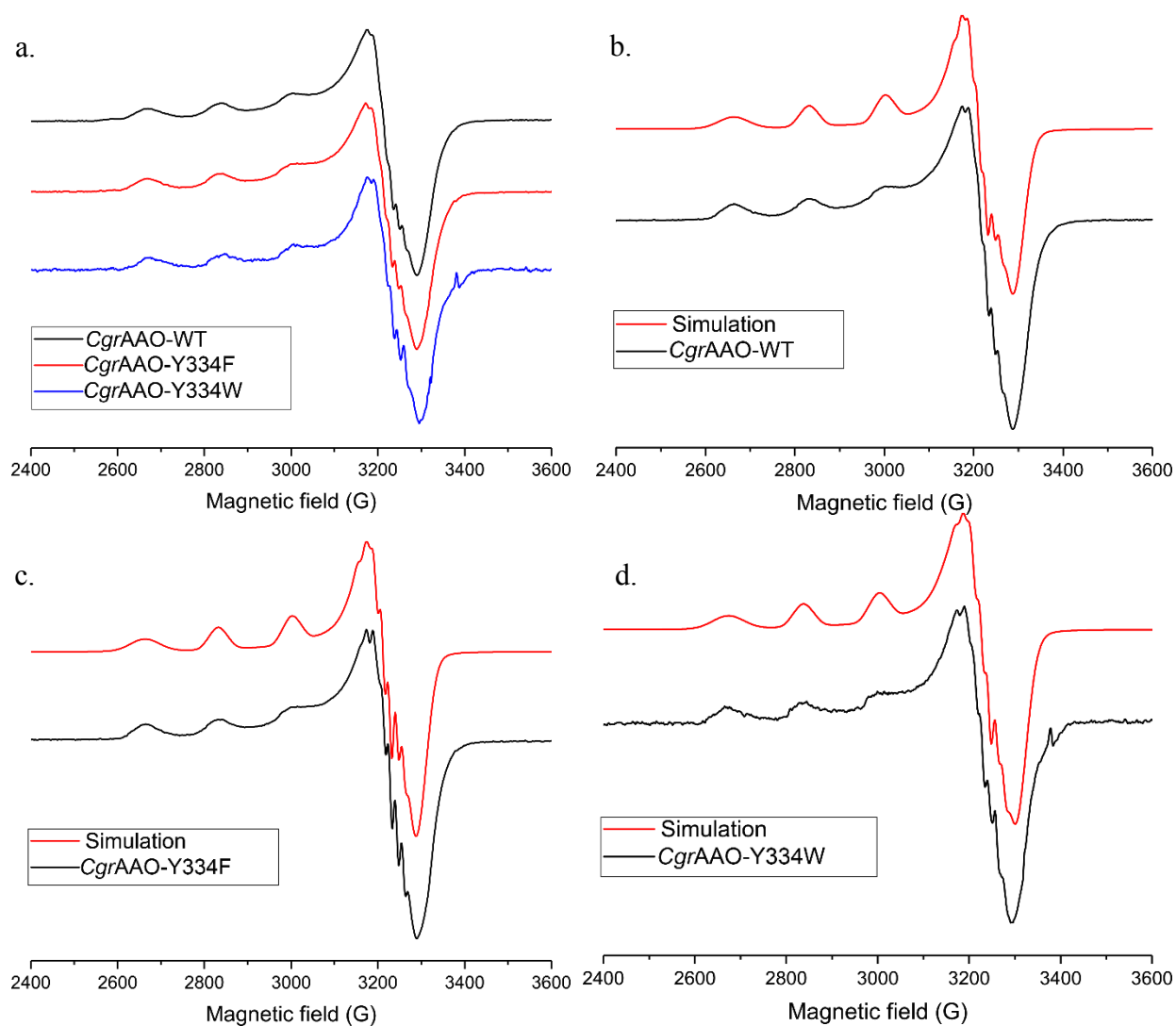


Figure S8: Continuous wave X band frozen solution spectra of *CgrAAO*_(AA5_2)-WT, -Y334F and -Y334W collected in 100 mM Na phosphate buffer pH 7.0 without (a) and with 10% (v/v) glycerol (b, c, d). Simulations of the experimental data for *CgrAAO*-WT (b), *CgrAAO*-Y334F (c) and *CgrAAO*-Y334W (d) are shown in red.

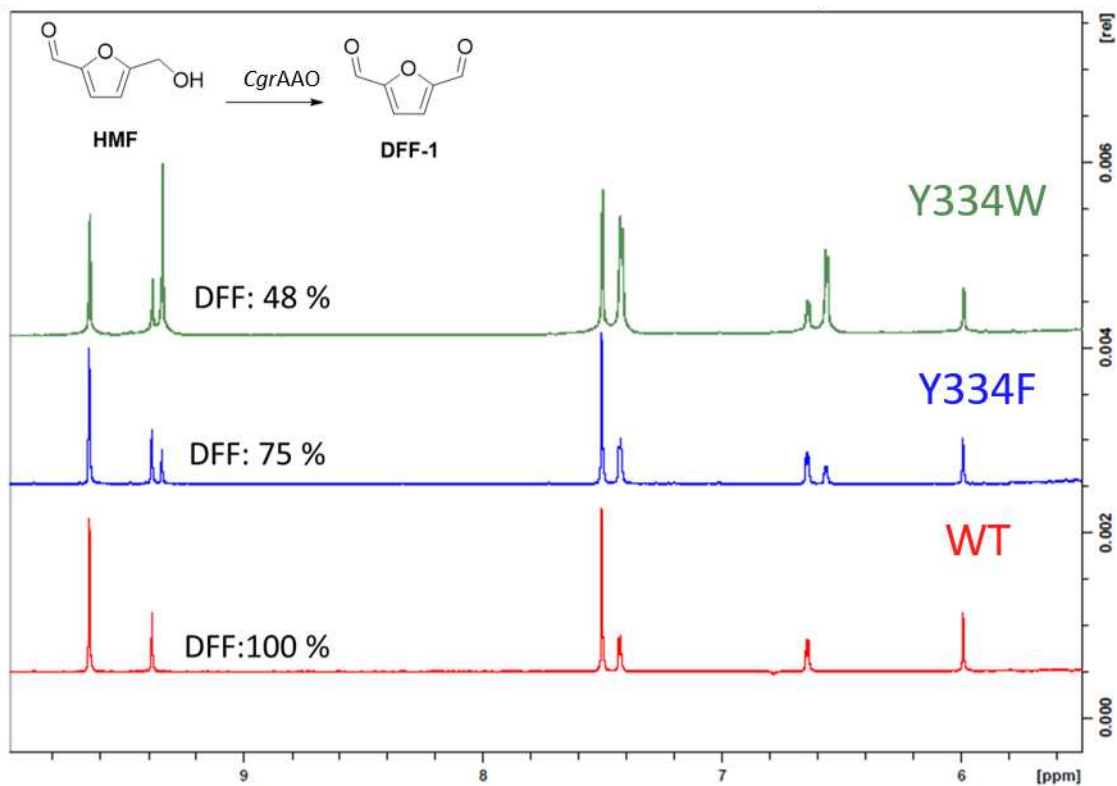


Figure S9. ¹H NMR spectra (400 MHz, 1:9 D₂O:phosphate buffer, 20 mM, pH 7) showing product profiles for the oxidation of 20 mM HMF by *CgrAAO* wild type and variants, as indicated.

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