

Catalysts

Enhanced Thermostability of *Pseudomonas nitroreducens* Isoeugenol Monooxygenase by the Combinatorial Strategy of Surface Residue Replacement and Consensus Mutagenesis

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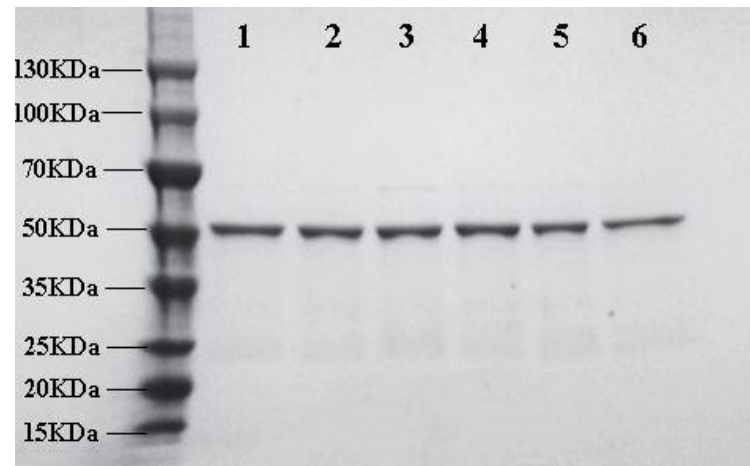


Figure S1. SDS-PAGE assay of purified IEM and its mutants. Lane 1: Wild-type IEM; Lane 2: mutant K83R; Lane 3: mutant K95R; Lane 4: mutant L273F; Lane 5: K83R/K95R; Lane 6: K83R/K95R/L273F.

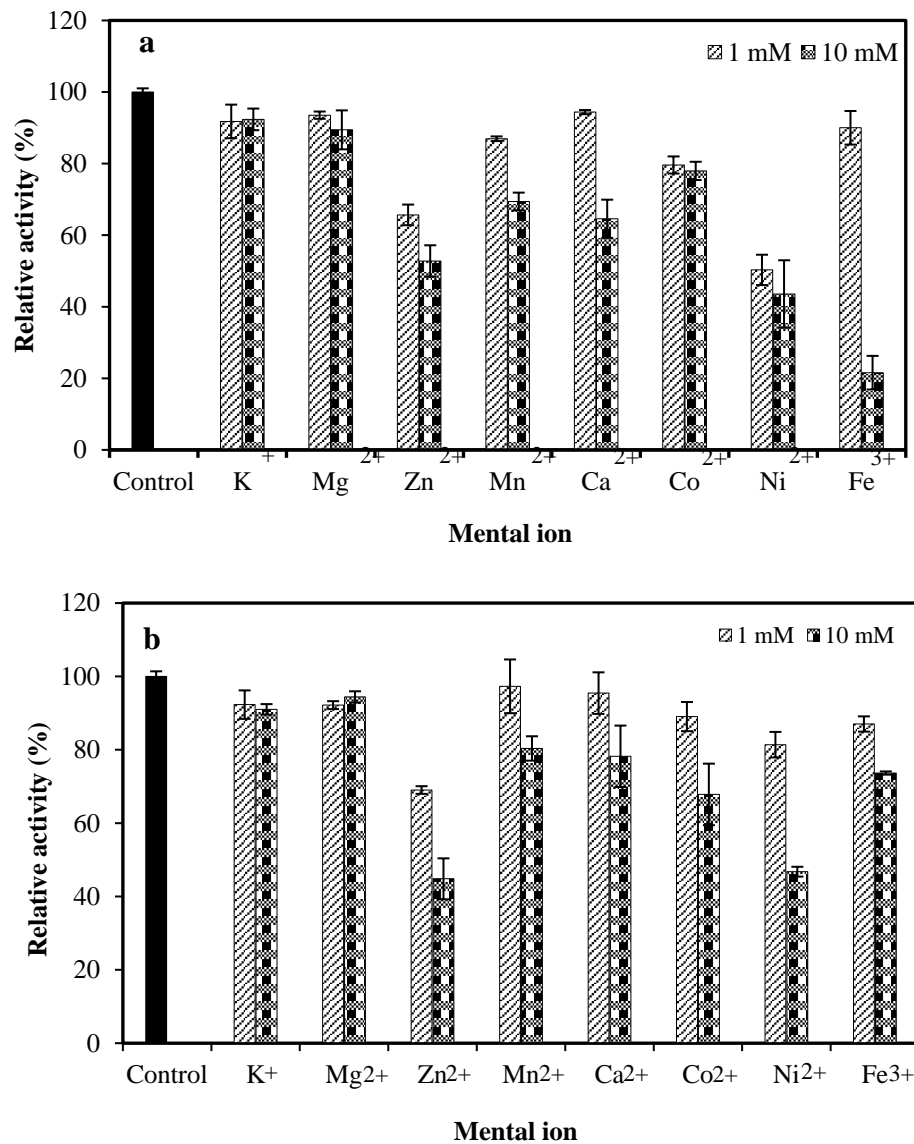


Figure S2. The effects of metal ions on IEM and K83R/K95R/L273F activities. (a) Wild-type; (b) mutant K83R/K95R/L273F. The enzyme activity was measured in the presence of 1 mM and 10 mM metal ions. The control represented the reaction without any metal ions.

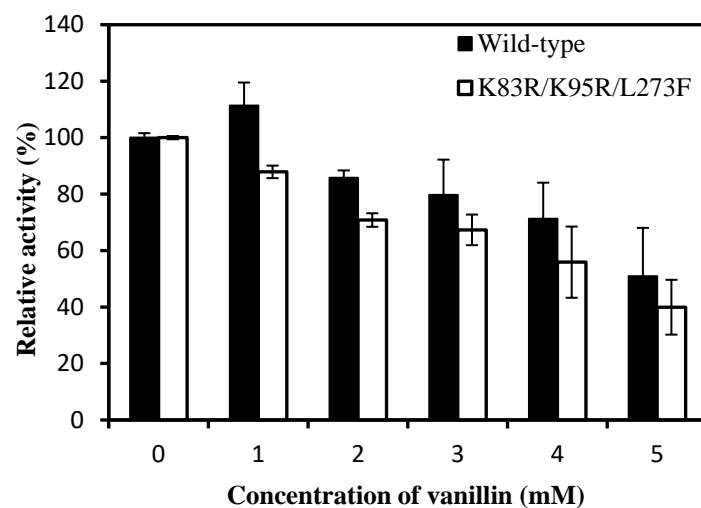


Figure S3. The effects of product-inhibition on IEM and K83R/K95R/L273F activities. The enzyme activity was measured in the presence of 1 mM to 5 mM vanillin. The control represented the reaction without vanillin.

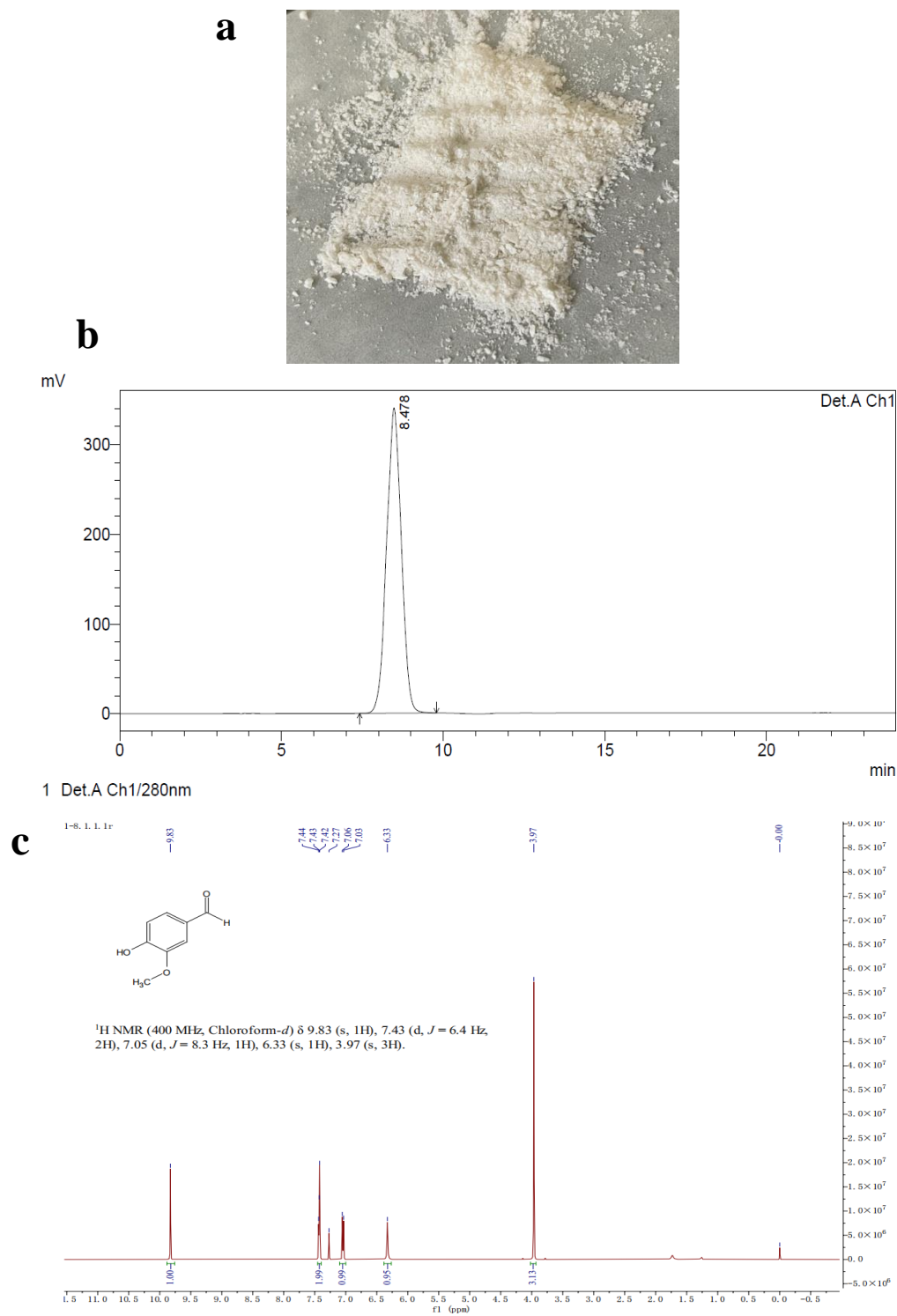


Figure S4. Preparation of natural vanillin. **(a)** The morphology of prepared vanillin; **(b)** HPLC spectrum; **(c)** ^1H NMR spectrum.

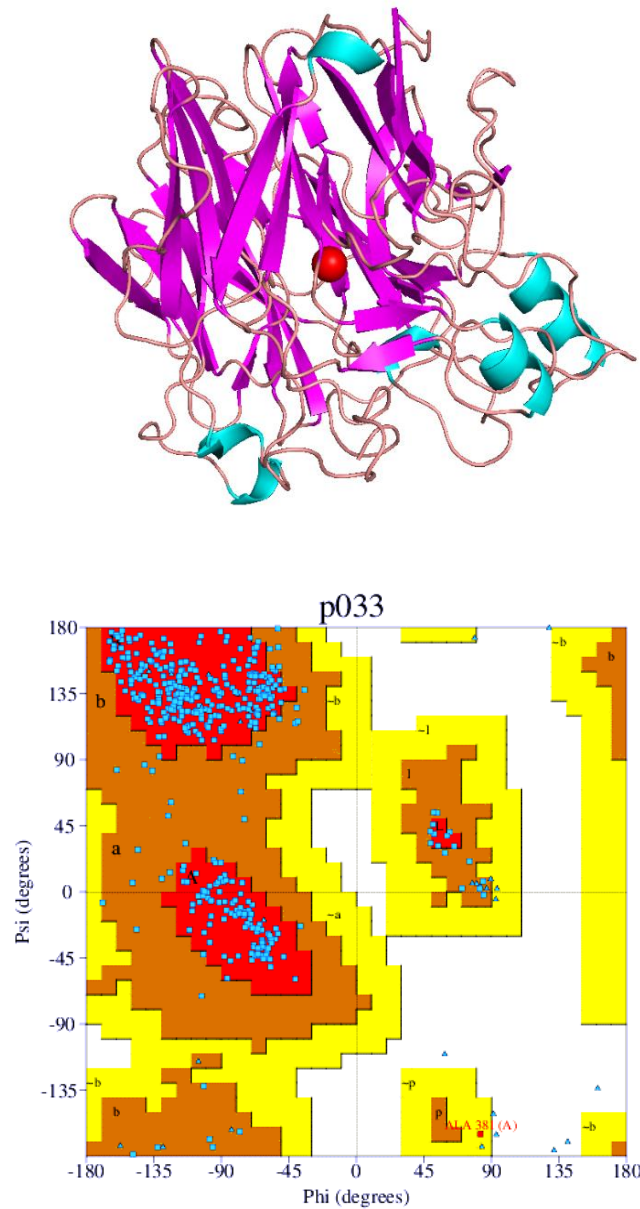


Figure S5. The 3D structure of IEM based on Homology Modeling and qualified by Ramachandran plot. The results showed that 88.6% residues were located in the most favoured regions, 11.4% residues in allowed regions and no residue in disallowed regions. Besides, the model was also qualified as “Pass” by VERIFY 3D for that at least 88.25% of the residues had average 3D-1D score ≥ 0.2 .

Table S1. The comparison of the enzyme activity and residual activity after 35 °C heat-treatment between IEM and mutants.

Strategy	Mutants	Relative activity (%)	Residual activity (%)
-	IEM	100.0±8.0	53.2±2.4
Surface residue replacement	G31A	10.7±0.1	50.8±1.2
	G113A	19.4±1.1	13.2±5.4
	G128A	65.3±3.0	7.7±0.1
	G157A	61.9±0.2	8.6±0.3
	G290A	80.0±1.6	30.9±1.1
	G398A	130.4±1.5	55.6±1.5
	K35R	76.7±0.9	46.0±0.2
	K54R	78.5±0.5	34.5±1.6
	K83R	103.9±3.8	84.2±2.7
	K95R	106.6±3.7	68.0±0.4
	K112R	113.5±0.7	53.8±1.1
	K185R	70.4±1.1	23.7±0.1
	K199R	115.2±0.4	48.1±0.4
	K202R	71.2±1.9	40.7±0.4
	K242R	94.5±0.8	30.3±0.7
	K244R	104.6±2.9	55.8±0.7
	K274R	94.5±0.3	56.2±1.0
	K319R	90.0±2.3	37.7±1.4
	K339R	82.1±0.4	39.2±2.0
	K383R	99.5±0.3	35.1±0.8
Consensus mutagenesis	V130L	71.5±1.4	48.8±1.0
	S182Y	ND	ND
	L303N	ND	ND
	I352R	99.7±1.3	70.4±2.4
	D176E	95.8±1.5	43.0±1.2
	N312G	81.3±4.0	53.3±1.7
	F216M	73.9±0.6	49.1±1.4
	L273F	117.2±0.6	50.4±0.6
	E47D	ND	ND

*ND: enzyme activity was not detectable.

Table S2. Primers for site-directed mutagenesis.

Primers	Sequences (5'→3')	Length (bp)	T _m (°C)
G31A-F	GATCTAGAGGTTGAC <u>GCT</u> GAAATCCCAAATC	32	64.09
G31A-R	GATTTTGGGATTTCA <u>GCGT</u> CAACCTCTAGATC	32	64.09
G113A-F	GACACCAGTGTAAGCTCTAGACCGCACCGTTG	34	68.96
G113A-R	CAACGGTGCGGTCTAG <u>AGCT</u> TTTACACTGGTGTC	34	68.96
G128A-F	CATTAGCCATCAC <u>GCTA</u> AGGTGCTGGCGGTG	31	69.35
G128A-R	CACCGCCAGCACCTT <u>AGCGT</u> GATGGCTAATG	31	69.35
G157A-F	CGCTTCGACTACGAC <u>GCT</u> CAAGTTACCAGCC	31	69.35
G157A-R	GGCTGGTAACTTG <u>AGCGT</u> CGTAGTCGAAGCG	31	69.35
G290A-F	GAATGCTTGGAAGTC <u>GCT</u> ACCAAGATTTATATC	34	64.14
G290A-R	GATATAAATCTTGGT <u>AGCGA</u> CTCCCAAGCATTC	34	64.14
G398A-F	GGGACAACCACCGAG <u>GCT</u> ACTACGACCTCTGG	32	71.78
G398A-R	CCAGAGGTCGTAGTC <u>CAGCT</u> CGGTGGTTGTCCC	32	71.78
K35R-F	GACGGCGAAATCCCA <u>AGG</u> TCAATAAATGGAACG	33	66.60
K35R-R	CGTTCCATTTATTGAC <u>CTT</u> GGGATTTTCGCCGTC	33	66.60
K54R-F	CAAGTTACCCCGCAAAGGTTCCACACCTTCATAG	34	67.75
K54R-R	CTATGAAGGTGTGGAACCTTTGCGGGGTAACCTG	34	67.75
K83R-F	CATCAGTCGCTGGGTT <u>AGG</u> ACCGCTCGATTCACG	34	71.37
K83R-R	CGTGAATCGAGCGGT <u>CCTA</u> ACCCAGCGACTGATG	34	71.37
K95R-F	GAACGACTAGCGCGA <u>AGAT</u> CGCTATTTGGCATG	33	67.84
K95R-R	CATGCCAAATAGCGAT <u>CTT</u> TCGCGCTAGTCGTTT	33	67.84
K112R-F	GACGACACCAGTGTAAGAGGACTAGACCGCACC	33	70.32
K112R-R	GGTGCGGTCTAGTCCTCTTACACTGGTGTCGTC	33	70.32
K185R-F	CTTCGGTTCGGCAGCT <u>AGGG</u> GCGAAGCAACTCC	33	72.81
K185R-R	GGAGTTGCTTCGCC <u>CCT</u> AGCTGCCGAACCGAAG	33	72.81
K199R-F	CTATTACATTGTCGAC <u>AGAC</u> ACGGCAAGGTGAC	33	66.60
K199R-R	GTCACCTTGCCGTG <u>TCT</u> GTCGACAATGTAATAG	33	66.60
K202R-F	GTCGACAAGCACGGC <u>AGAG</u> TGACACATGAAAC	32	67.93
K202R-R	GTTTCATGTGTCAC <u>TCT</u> GCCGTGCTTGTCGAC	32	67.93
K242R-F	GCCTGTCCCGCCTC <u>AGAG</u> GCGAAACAGCC	28	71.29
K242R-R	GGCTGTTTCGCT <u>CTG</u> AGGCGGGACAGGC	28	71.29
K244R-F	CCCGCCTCAAGGCG <u>AGAC</u> AGCCAATTTATATG	32	67.93
K244R-R	CATATAAATTGGCTG <u>TCT</u> CGCCTTGAGGCGGG	32	67.93
K274R-F	CAGATTCGCTGGCTC <u>AGGG</u> CACCGGCGCTCTGG	33	75.29
K274R-R	CCAGAGCGCCGGTGCC <u>CTG</u> AGCCAGCGAATCTG	33	75.29
K319R-F	CCCTTCGCCCCCTGAG <u>AGAG</u> CCGTACCACGCCTG	33	75.29
K319R-R	CAGGCGTGGTACGGC <u>TCT</u> CTCAGGGGCGAAGGG	33	75.29
K339R-F	GCAGCGACGAGATC <u>AGGC</u> GAACCCGGCTACAC	32	73.06
K339R-R	GTGTAGCCGGGTTTCG <u>CCT</u> GATCTCGTCGCTGC	32	73.06
K383R-F	CATCAGCAGGCCGAGAGAATATTTGCGTACAAC	33	66.60
K383R-R	GTTGTACGCAAATATTCTCTCGGCCTGCTGATG	33	66.60

V130L-F	GCCATCACGGCAAGCTGCTGGCGGTGAAGGAAG	33	72.81
V130L-R	CTTCCTTCACCGCCAGCAGCTTGCCGTGATGGC	33	72.81
S182Y-F	CTTGTTGTTCTTCGGTTACGCAGCTAAGGGCGAAG	35	68.84
S182Y-R	CTTCGCCCTTAGCTGCGTAACCGAAGAACAACAAG	35	68.84
L303N-F	GGAAAGTGAAATCAATCCGTTCCCCTTCCCC	31	66.71
L303N-R	GGGGAAGGGGAACGGATTGATTTCACTTTCC	31	66.71
I352R-F	CTTTGCGGAAATGCCACGCATGGATTTTCGCTTC	34	67.75
I352R-R	GAAGCGAAAATCCATGCGTGGCATTTCGCAAAG	34	67.75
D176E-F	GACCCGGAAACGGGTGAGTTGTTGTTCTTCGG	32	69.21
D176E-R	CCGAAGAACAACAACCTCACCCGTTTCCGGGTC	32	69.21
N312G-F	CTTCCCCAACTCACAAGGTCAACCCTTCGCCCCTG	35	72.36
N312G-G	CAGGGGCGAAGGGTTGACCTTGTGAGTTGGGGAAG	35	72.36
F216M-F	CAGCCCTATGGCGCAATGATGCACGACTTTGCC	33	70.32
F216M-R	GGCAAAGTCGTGCATCATTGCGCCATAGGGCTG	33	70.32
L273F-F	GTCAGATTCGCTGGTTCAAGGCACCGGCGCTC	32	71.78
L273F-R	GAGCGCCGGTGCCTTGAACCAGCGAATCTGAC	32	71.78
E47D-F	CTACCGTAATACGCCAGACCCTCAAGTTACCCCG	34	70.16
E47D-R	CGGGGTAACCTTGAGGGTCTGGCGTATTACGGTAG	34	70.16

*Mutated bases were underlined.