Supplementary Material

Identification of a novel ene reductase from *Pichia angusta* with potential application in

(*R*)-levodione production

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Table and figure captions:

Fig. S1 Sequence alignment of PaER with the other nine OYEs. Substrate binding sites and catalytic residue are marked with black triangles.

Fig. S2 HPLC detection of *Pa*ER flavin species.

Fig. S3 (A) Standard curve of size-exclusion chromatography, (B) The size-exclusion chromatography of *Pa*ER.

Fig. S4 SDS-PAGE analysis of engineered *E. coli* cells. M: Molecular weight marker; T: whole cell lysate; S: supernatant; P: precipitate.

Fig. S5 GC-MS spectrum of (*R*)-levodione prepared by *Pa*ER. GC-EI-MS m/z (M+ 154 for $C_9H_{14}O_2$) 139, 111, 95, 83, 69, 56.

Fig. S6 NMR of product (*R*)-levodione prepared by *Pa*ER.

^{1}H NMR (400 MHz, Chloroform-d) δ 3.01 (dp, J = 13.2, 6.5 Hz, 1H), 2.81 – 2.68 (m, 2H), 2.52 (d, J = 15.4 Hz, 1H), 2.34 (dd, J = 17.7, 13.3 Hz, 1H), 1.29 – 1.10 (m, 8H).

Table S1 List of primers sequences.

Table S2 Comparison of *Pa*ER with other OYEs regarding half-lives.

Table S3 Kinetic parameters of *Pa*ER.

Table S4 Comparison of different ene-reductases for asymmetric reduction of ketoisophorone

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                              η1    β1       β2        β3              TT              α1              β4      β5
PaER                      TT   10  222   20        30          TT  40   0000000000  50         60
                            1                                      40                           50          60

PaER    .....MTPSTSLASSNLFKPIKVGKVELKNRLVFAPTTRYRASKD.FVPT.DSMLKYYEQRAENNGGLLTAEATY
OYE1    MSFVKDFKPQALGDTNLFKPIKIGNNELLHRAVIPPLTRMRALHPGNIPNRDWAVEYYTQRAQRPGTMIITEGAF
OYE2    MPFVKDFKPQALGDTNLFKPIKIGNNELLHRAVIPPLTRMRAQHPGNIPNRDWAVEYYAQRAQRPGTLIITEGTF
OYE2.6  ...MSSVKISPLKDSEAFQSIKVGNNTLQTKIVYPPTTRFRALED.HTPS.DLQLQYYGDRSTFPGTLLITEATF
OYE3    MPFVKGFEPISLRDTNLFEPIKIGNTQLAHRAVMPPLTRMRATHPGNIPNKEWAAVYYGQRAQRPGTMIITEGTF
MgER    ....MSVNINPLGETQVFQPIKLGENTLSHRVFFPPTTRTRSLED.HTPS.NLAYKYYDERSKFPGTLIISEGTF
ClER    .....MVAVKPLKDTEIFKPTKVGNHELSNKIVYAPTTRMRAIAD.HTPS.DLAYKYYDDRTKYPGSLVITEATL
NCR     ...............MPSLFDPIRFGAFTAKNRIWMAPLTRGRATR.DHVPT.EIMAEYYAQRAS..AGLIISEATG
PENTR   ..........MSAEKLFTPLKVGAVTAPNRVFMAPLTRLRSIEPGDIPT.PLMGEYYRQRAS..SGLIITEATQ
GYE1    ...........MPTLFDPIDFGPIHAKNRIVMSPLTRGRADK.EAVPT.PIMAEYYAQRAS..AGLIITEATG


                   η2     TT           α2              β6    η3    α3      β7
PaER              222  80           90         100       110  000  120       130          140
                  70                                                        130

PaER    VDFNFGLYPF.TPMIKTPAQVAAWAKIIEAVHKQGSYFSIQLWHLGRAADPKFNKEKGVPFVAPS.AIYLDQDSE
OYE1    ISPQAGGYDN.APGVWSEEQMVEWTKIFNAIHEKKSFVWVQLWVLGWAAFPDNLARDGLRYDSASDNVFMDAEQE
OYE2    PSPQSGGYDN.APGIWSEEQIKEWTKIFKAIHENKSFAWVQLWVLGWAAFPDTLARDGLRYDSASDNVYMNAEQE
OYE2.6  VSPQASGYEGAAPGIWTDKHAKAWKVITDKVHANGSFVSTQLIFLGRVADPAVMKTRGLNPVSAS.ATYESDAAK
OYE3    ISPQAGGYDN.APGIWSDEQVAEWKNIFLAIHDCQSFAWVQLWSLGWASFPDVLARDGLRYDCASDRVYMNATLQ
MgER    PSAQAGLYEG.VPGIWTERQTKTWKHIIDKIHENKSFASIQLWNLGRTGDPALLKKAGKPFLAPS.AIYFDEESK
ClER    MSPKTGLYDR.VPGIYTDEHVAGWKKITDKIHANGSKVSMQLWPLGRVADPVATKKACYPLVAPS.LIYPSEEAK
NCR     ISQEGLGWPY.APGIWSDAQVEAWLPITQAVHDAGGLIFAQLWHMGRMVPSNVS..GMQPVAPSASQAPGLGHTY
PENTR   ISAQAKGYAG.APGLHSPEQIAAWKKITAGVHAEEGRIAVQLWHTGRISHSSIQPGGQAPVSASALNANTRTSLR
GYE1    ISREGLGWPF.APGIWSDAQVEAWKPIVAGVHAKGGKIVCQLWHMGRMVHSSVT..GTQPVSSSATTAPGEVHTY


           α4        β8            α5              β9          α6              TT
PaER     00....00  150            160        170       180  190       200         210

PaER    KA....AREAGNELRELTIPEIEPIVKEFAAAAKRAIHEAKADFIELHSAHGYLLDQFIQPNINKRTDKYGGSIE
OYE1    AK....AKKANNPQHSLTKDEIKQYIKE.YVQAAKNSIAAGADGVEIHSANGYLLNQFLDPHSNTRTDEYGGSIE
OYE2    EK....AKKANNPQHSITKDEIKQYVKE.YVQAAKNSIAAGADGVEIHSANGYLLNQFLDPHSNNRTDEYGGSIE
OYE2.6  EA....AEAVGNPVRALTTQEVKDLVYETYTNAAQKAMDACFDYIELHAAHGYLLDQFLQPCTNQRTDEYGGSIE
OYE3    EK....AKDANNLEHSLTKDDIKQYIKD.YIHAAKNSIAAGADGVEIHSANGYLLNQFLDPHSNKRTDEYGGTIE
MgER    KA....AEKAGNPLRAMTEEEIKDMIYEQYTIAAKNALEAGFDYIELHSAHGYLLHEFLEESSNKRTDKYGGSIE
ClER    KA....AEEAGNPIHVLTTEEVEDLVND.FVHAAKKAVAAGVDYVEVHGAHGYLVDTFFQVSTNKRTDKYGGSIE
NCR     DG....KKPYDVARALRLDEIPRLLDD.YEKAARHALKAGFDGVQIHAANGYLIDEFIRDSTNHRHDEYGGAVE
PENTR   DENGNAIRVDTSTPRALELDEIPGIVND.FRQAVANAREAGFDLVELHSAHGYLLHQFLSPSSNHRTDQYGGNVE
GYE1    EG....KKPFEQARAIDAADISRILND.YENAARNAIRAGFDGVQIHAANGYLIDEFLRNGTNHRTDEYGGVPE
                                                         ▲   ▲ ▲


        α7              η4  β10      TT   η5    η6        α8              β11
PaER  0000000000  220      230       240     250       260       270        280

PaER    NRARLVLEVVDACIEAVGAEHVGIRLSPYAKFQGSEGVDSEINPIALSPFGYILSELEKRARDGNRLAYVSVVEPRV
OYE1    NRARFTLEVVDALVEAIGHEKVGIRLSPYGVFNSMSG.GAETGIVAQYAYVAGELEKRAKAGKRLAFVHLVEPRV
OYE2    NRARFTLEVVDAVVDAIGPEKVGLRLSPYGVFNSMSG.GAETGIVAQYAYVLGELERRAKAGKRLAFVHLVEPRV
OYE2.6  NRARLILELIDHLSTIVGADKIGIRISPWATFQNMKAHKDTVHPLTTFSYLHELQQRADKGQGLAYISVVEPRV
OYE3    NRARFTLEVVDALIETIGPERVGLRLSPYGTFNSMSG.GAEPGIIAQYSYVLGELEKRAKAGKRLAFVHLVEPRV
MgER    NRARFVLELVDHMISIVGAERLGIRISPWATFQGMKSVHGEVHPLTTYSYLVNELEKRAQAGNRLAYISLVEPRV
ClER    NRARFALEILDRLIEEIGAERVAIRISPWAKFQGILAEEGEVNPVAQFGYFLSELENRARAGKRIAYVSIVEPRV
NCR     NRIRLLKDVTERVIATIGKERTAVRLSPNGEIQGTVDSHP..EQVFI.......PAAKMLSDLDIAFLGMREGAV
PENTR   NRARLVLEVVDAVCQEWSPDRIGIRVSPIGTFQN.VDNGPNEEADAL.......YLIEELAKRGIAYLHMSEPDW
GYE1    NRIRFLKEVTERVIAAIGADRTGVRLSPNGDTQGCIDSAP..ETVFV.......PAAKLLQDLGVAWLELREPGP


                   α9       β12             β13   α10      α11
PaER      TT  TT       00000          320        330   340      350
        290      300           310

PaER    SG..NVDSN.DQRKFDTSWIREIWKGILFRAGGYLKENEQ..SLEHDVNQDDRTLIGVSRYYTSNPDLVERLKKG
OYE1    TNPFLTEGEGEYEGGSNDFVYSIWKGPVIRAGNFALHPEV...VREEVK.DKRTLIGYGRFFISNPDLVDRLEKG
OYE2    TNPFLTEGEGEYEGGSNKFAYSIWKGPIIRAGNFALHPEV...VREEVK.DPRTLIGYGRFFISNPDLVDRLEKG
OYE2.6  SGNVDVSEE.DQAG.DNEFVSKIWKGVILKAGNYSYDAPEFKTLKEDIA.DKRTLVGFSRYFTSNPNLVWKLRDG
OYE3    TDPSLVEGEGEYSEGINDFAYSIWKGPIIRAGNYALHPEV...VREQVK.DPRTLIGYGRFFISNPDLVYRLEEG
MgER    DGINSVEKK.DQTG.NNDFVKDLWKGILKAGNYTYDAPKFGQLLDDVS.DGRTLVGFSRYFISNPDLISRLEKG
ClER    SGVIDVAGE.DIQG.DNSFVRSVWKGIVIKAGNYTYDAPEFKTLLQDVS.DGKTLVAFARYFTSNPDLVQRLHDG
NCR     DGTFGKTDQ....PKLSPEIRKVFKPPLVLNQDYTFETAQ.....AALDSGVADAISFGRPFIGNPDLPRRFFEK
PENTR   AG..GQPYS...EAFRQKVRERFHGVIIGAGAYTPEKAE....DLINKGLIDAVAFGRDYIANPDLVARLQQK
GYE1    NG.TFGKTDQ...PKLSPQIRKVFLRPLVLNQDYTFEAAQ....TALAEGKADAIAFGRKFISNPDLPERFARG


              TT     TT          TT        TT    TT   TT
PaER       360     370       380        390       400

PaER    LSLTPYDRSRFYNHSSNDGYLTWPKYGEDE.EKYKAVLDVEPKALA...
OYE1    LPLNKYDRDTFYQMS.AHGYIDYP........TYEEALKLGWDKK....
OYE2    LPLNKYDRDTFYKMS.AEGYIDYP........TYEEALKLGWDKN....
OYE2.6  IDLVPYDRNTFYSDN.NYGYNTFSMDSEE..VDKELEIKRVPSAIEAL.
OYE3    LPLNKYDRSTFYTMS.AEGYTDYP.......TYEEAVDLGWNKN....
MgER    HQLAPYERETFYGRS.DFGYNDYPKYGEKR.EDAEVAKKRVPEELVV..
ClER    ADLTPYKRELFYAPS.NWGYNTFTNAGETKTFSEEEESKRLPAPIDTKA
NCR     APLTKDVIETWYTQI.PKGYTDYP..........LLGD.......
PENTR   AALNPQRPESFYGGG.AEGYTDYP...........TL............
GYE1    IALQPDDMKTWYSQG.PEGYTDYP............SATSGPN......
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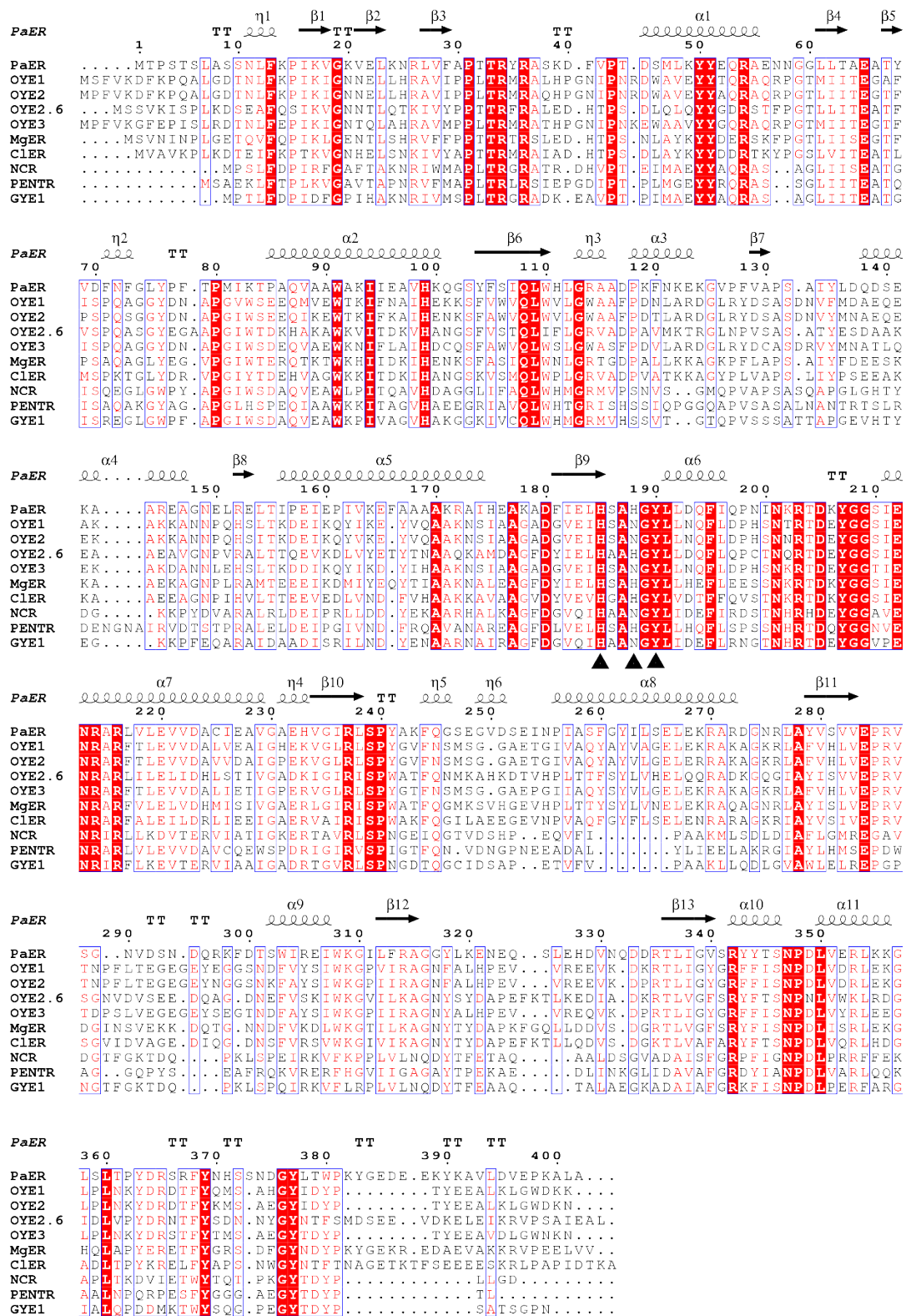
Fig. S1 Sequence alignment of *Pa*ER with the other nine OYEs. Substrate binding sites and catalytic residue are marked with black triangles.
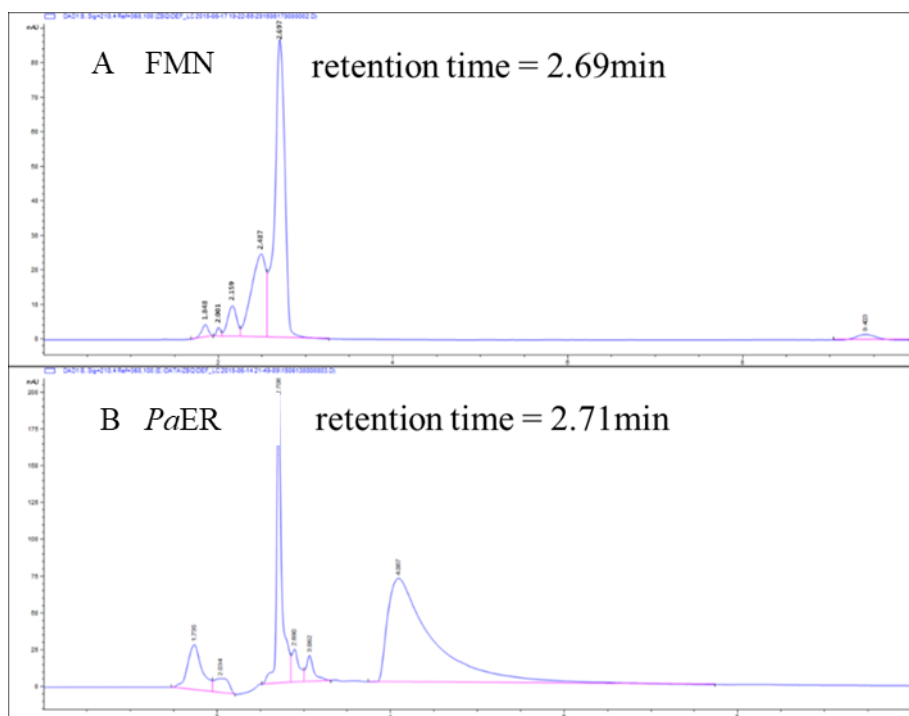
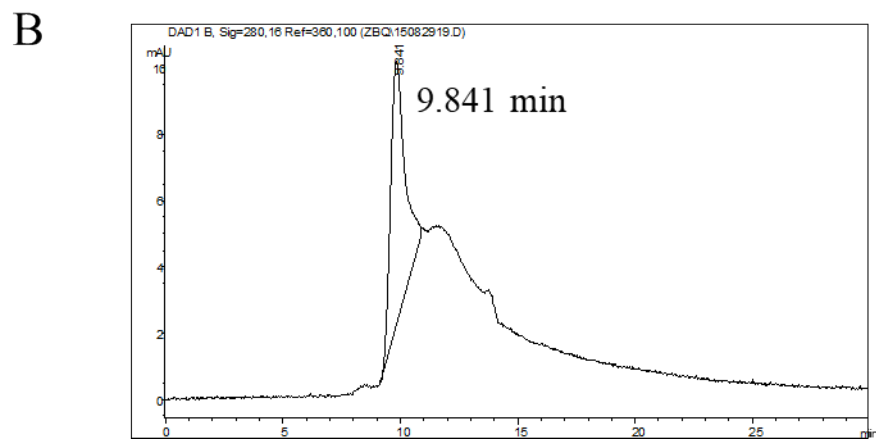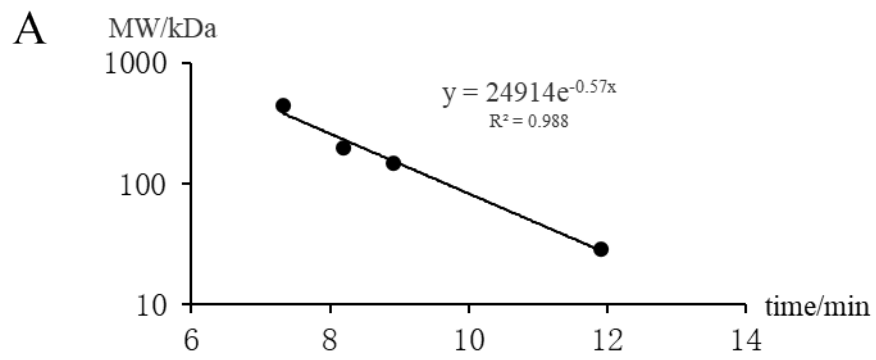**Fig. S2** HPLC detection of *Pa*ER flavin species.

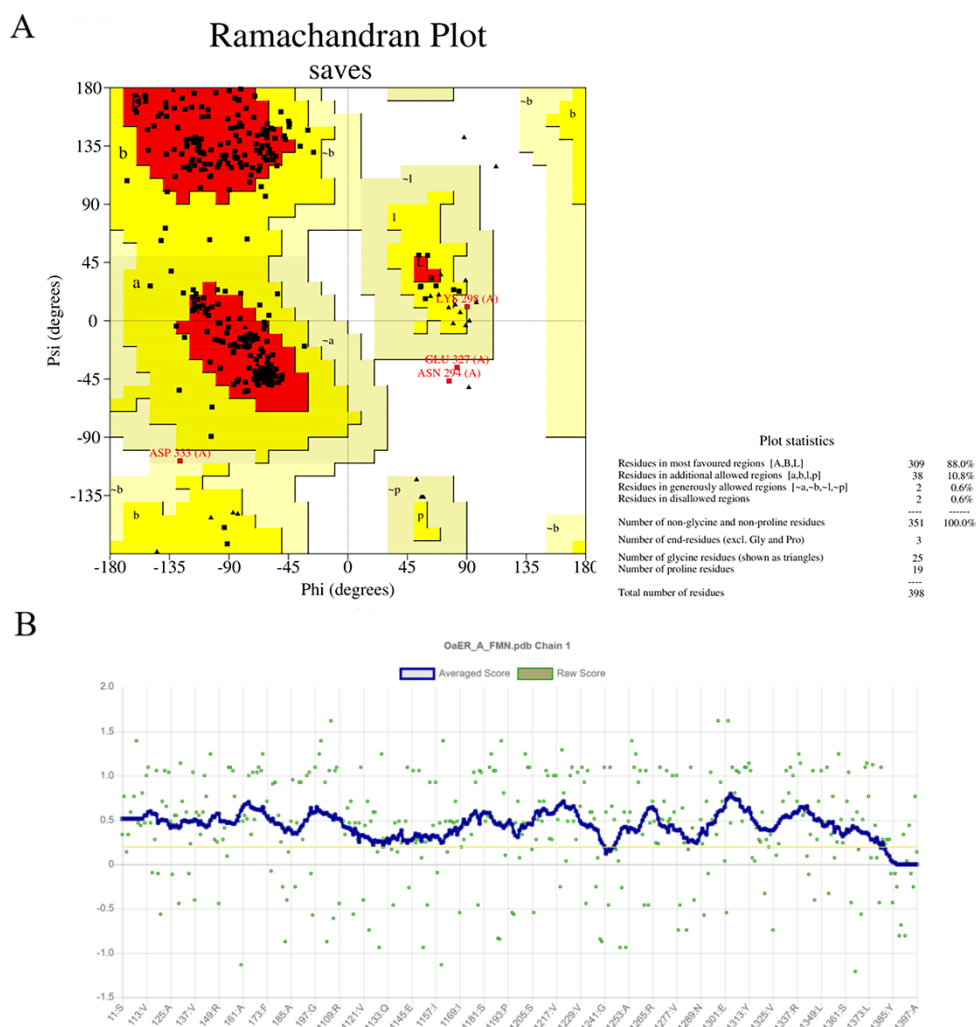**Fig. S3** (A) Standard curve of size-exclusion chromatography, (B) The size-exclusion chromatography of *Pa*ER.

Fig. S4 (A) The Ramachandran plot of *Pa*ER model evaluation. (B) The Verify-3D analysis of *Pa*ER model evaluation.
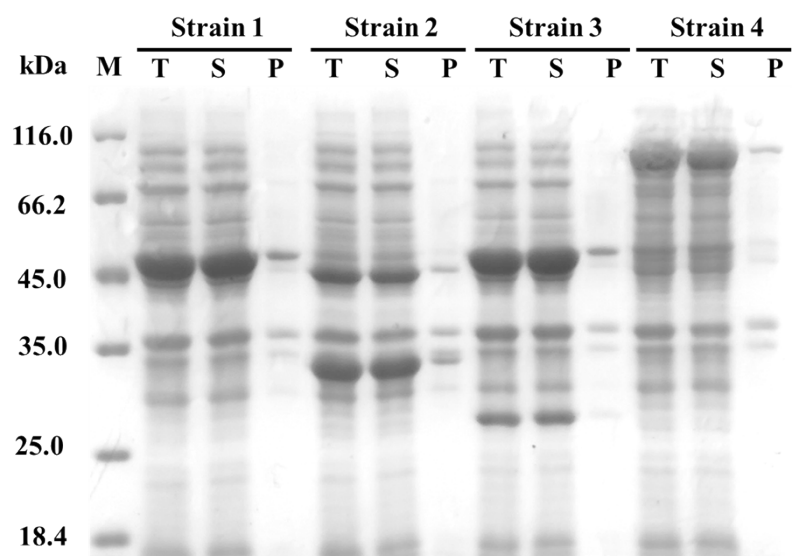
Fig. S5 SDS-PAGE analysis of engineered *E. coli* cells. M: Molecular weight marker; T: whole cell lysate; S: supernatant; P: precipitate.
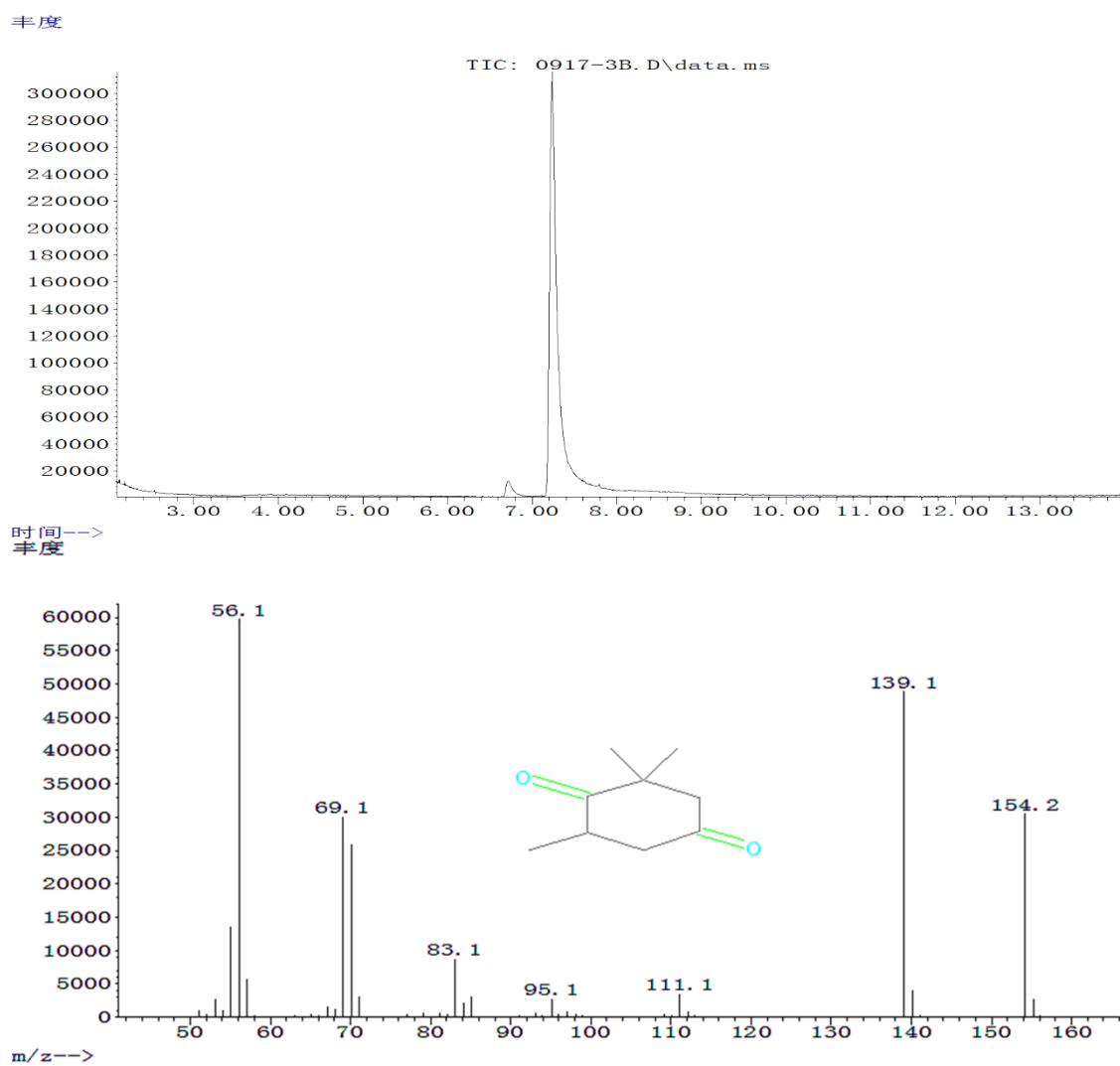
Fig. S6 GC-MS spectrum of (*R*)-levodione prepared by *Pa*ER. GC-EI-MS m/z (M+ 154 for $C_9H_{14}O_2$)

139, 111, 95, 83, 69, 56.
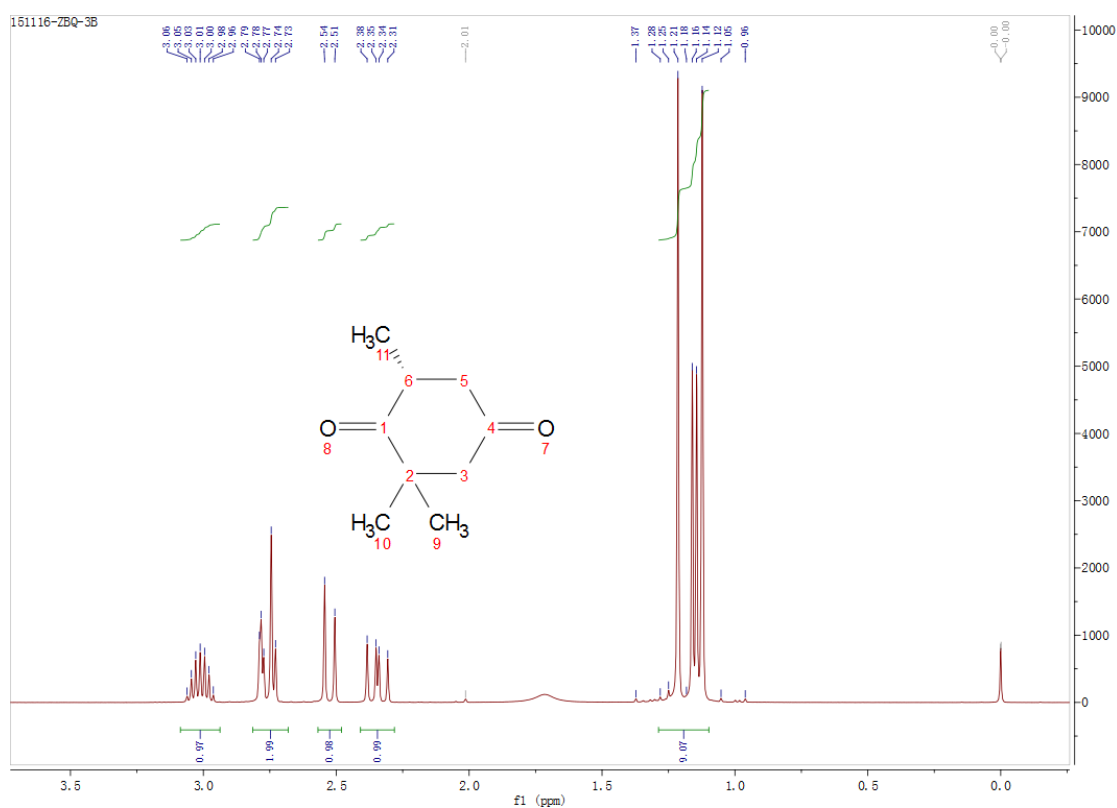
Fig. S7 NMR of product (*R*)-levodione prepared by *Pa*ER.

¹H NMR (400 MHz, Chloroform-d) δ 3.01 (dp, J = 13.2, 6.5 Hz, 1H), 2.81 – 2.68 (m, 2H), 2.52 (d, J = 15.4 Hz, 1H), 2.34 (dd, J = 17.7, 13.3 Hz, 1H), 1.29 – 1.10 (m, 8H).

Table S1 List of primers sequences.

Primer	Sequence (5'→3')
PaER-F	TGGACAGCAAATGGGTCGC<u>GGATCC</u>ATGACCCCAAGCACTTCTCT
PaER-R	GGTGCTCGAGTGCGGCCGC<u>AAGCTT</u>CTACGCAAGGGCCTTTGGCT
rbs-GDH-F	AGCCAAAGGCCCTTGCGTAG<u>AAGCTT</u>GAAGGAGATATACCATGGGC
GDH-R	TGGTGCTCGAGTGCGGCCGC<u>AAGCTT</u>TTAACCGCGGCCTGCCTG
MCS1-PaER-F	TTTAAGAAGGAGATATA<u>CCATGG</u>TTATGACCCCAAGCACTTCTCTT
MCS1-PaER-R	TGTCGACCTGCAGGCGCGCC<u>GAGCTC</u>CTACGCAAGGGCCTTTGGCT
MCS2-GDH-F	GTATAAGAAGGAGATATA<u>CATATG</u>GCAGATCTCATGTATCCGGATTTAAAAGG
MCS2-GDH-R	CCGGCCGATATCCAATTG<u>AGATCT</u>TTAACCGCGGCCTGCCTGGA
GDH-ERK-F	ATTCCAGGCAGGCCGCGGTGAAGAAGAGGAAAAAAGAAACAG
PaER-ERK-R	CAAGAGAAGTGCTTGGGGTCATGGATCCTTTTTTCTTACGTTTTTC

Table S2 Comparison of *Pa*ER with other OYEs regarding half-lives.

Enzyme	Species	Temperature (°C)	half-life	Reference
OYE*Ro*2	*Rhodococcus opacus*	32	28 min	1
OYE*Ro*2a	*Rhodococcus opacus*	32	87 min	1
FOYE-1	*Ferrovum* sp. JA12	50	5 h	2
OYE2p	*Saccharomyces cerevisiae*	40	11 h	3
*Cl*ER	*Clavispora lusitaniae*	40	36 h	4
*Mg*ER	*Meyerozyma guilliermondii*	40	60 h	5
*Cr*OYE3	*Chlamydomonas reinhardtii*	40	46 h	6
*Cr*OYE1	*Chlamydomonas reinhardtii*	40	137 h	6
*Cr*OYE2	*Chlamydomonas reinhardtii*	40	134 h	6
*Pa*ER	*Pichia angusta*	40	89 h	This study

Table S3 Kinetic parameters of *Pa*ER.
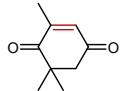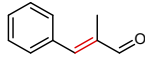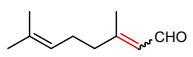
	Substrate	K_m (mM)	k_{cat} (s⁻¹)	k_{cat}/K_m (s⁻¹ mM⁻¹)
3a		0.33	3.57	10.64
10a		0.064	2.42	37.66
12a		0.038	0.54	14.45

Table S4 Comparison of different ene-reductases for asymmetric reduction of ketoisophorone

Catalyst	Source	Concentration (mM)	Cofactor	Conversion (%)	ee (%)/config.	Reference
XenA	*Pseudomonas putida*	5	NADP$^+$/ 0.2 mM	98.9	2.25 (*S*)	7
PETNR	*Enterobacter cloacae* st. PB2	5	NADP$^+$/0.01 mM	>99	57 (*R*)	8
*Gox*0502[a]	*Gluconobacter. oxydans*	10	NADP$^+$/ 0.5 mM	>99	>99 (*R*)	9
*Syn*ER	*Synechoccocus* sp. PCC 7942	10	NADP$^+$/0.5 mM	93%	97 (*R*)	10
*Ts*ER C25D/I67T	*Thermus scotoductus* SA-01	125	NADP$^+$/0.41 mM	>99	98 (*R*)	11
*Cl*ER	*Clavispora lusitaniae*	500	NADP$^+$/ 0.5 mM	>99	98 (*R*)	4
CYE	*Candida macedoniensis*	658	NADP$^+$/ 0.784 mM	95.4	n.d.	12
Geobacillus ene reductase	*Geobacillus* sp. 30	1000	NADH/1.0 mM	63.2	89.2 (*R*)	13
*Pa*ER	*Pichia angusta*	1000	NADP$^+$/0.2 mM	>99%	>99 (*R*)	This study

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