Electronic Supplementary Material (ESI) for Catalysis Science & Technology.

Electronic Supplementary Information

Iterative Multitarget Evolution Dramatically Enhances Enantioselectivity and Catalytic Efficiency of *Bacillus subtilis* Esterase towards Bulky Benzoate Ester of *dl*-Menthol

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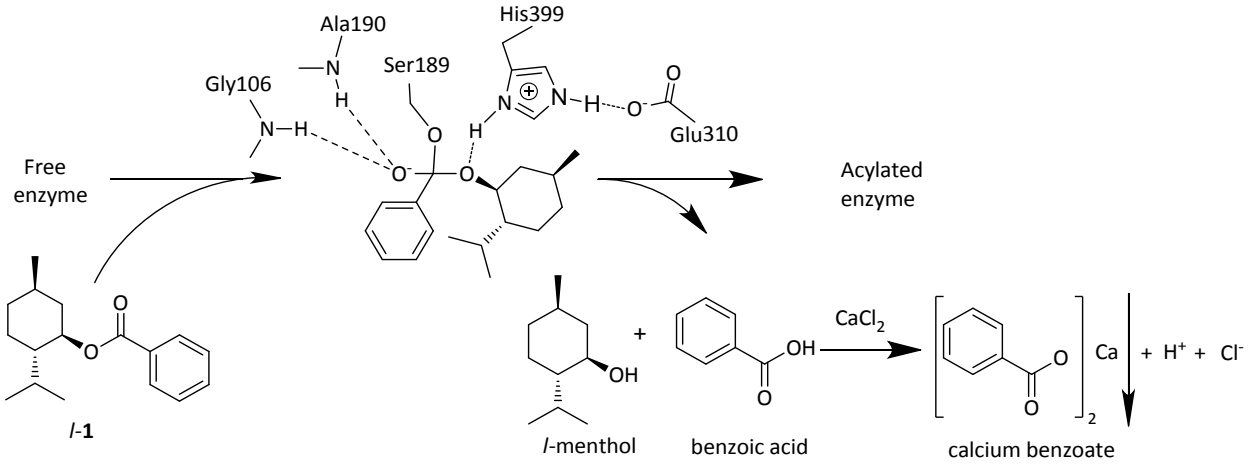

Scheme S1. An abbreviated reaction mechanism for the formation of a tetrahedral intermediate of *l*-**1**. The model used for selection of the amino acid residues for CASTing has docked this intermediate into the active site. The formation of benzoic acid was detected by phenol red with the addition of $CaCl_2$.

Table S1. Primers designed for mutation (all are written 5'–3'). The respective mutant codons are indicated in red, and the restriction sites are underlined. Degenerate nucleotide designation: n = a, t, c, g; k = t, g; m = a, c. Primers were ordered from Sangon, Shanghai.

Primer	Sequence of primer
107/109/110-f	attcacggaggcnnktttnnknnkggagcgggcagtgagcc
107/109/110-r	cactgcccgctccmnnmnnaaamnngcctccgtgaatccac
362/363-f	atgactgatttannknnktggcgccctgccgtcgc
362/363-r	ggcagggcgccamnnmnntaaatcagtcatcatat
400-f	aaagcgtttcacnnkttagagcttccttttgtc
400-r	aggaagctctaamnngtgaaacgctttattgta
68/71-f	ccgtctgatttgnnktcacttnnktatactgagctgccc
68/71-r	cagctcagtatamnnaagtgamnncaaatcagacggctg
188-f	aacagtatttggannktccgccggcggga
188-r	ccgccggcggamnntccaaatactgttac
217/218-f	atggaaagcggcnnknnkcgaacgatgacgaaa
217/218-r	cgtcatcgttcgmnnmnngccgctttccatgat
401/403-f	cgtttcactgcnnkgagnnkccttttgtctttgga
401/403-r	aagacaaaaggmnnctcmnngcagtgaaacgcttta
A107G-f	tggattcacggaggcggtttttatctaggagcg
A107G-r	cgctcctagataaaaaccgcctccgtgaatcca
Y109E-f	tcacggaggcgcttttgagctaggagcgggcag
Y109E-r	ctgcccgctcctagctcaaaagcgcctccgtga
L110V-f	cacggaggcgctttttatgtaggagcggg
L110V-r	cccgctcctacataaaaagcgcctccgtg
A400C-f	aaagcgtttcactgcttagagcttccttttgtc
A400C-r	aggaagctctaagcagtgaaacgctttattgta
A107G/Y109E-f	tggattcacggaggcggttttgagctaggagcgggcagtg
A107G/Y109E-r	cactgcccgctcctagctcaaaaccgcctccgtgaatcca
A107G/L110V-f	gattcacggaggcggtttttatgtaggagcgggcagt

A107G/L110V-r	actgcccgctcctacataaaaaccgcctccgtgaatc
Y109E/L110V-f	gtggattcacggaggcgcttttgaggtaggagcgggca
Y109E/L110V-r	tgcccgctcctacctcaaaagcgcctccgtgaatccac
ep-f	cgcggatccatgactcatcaaatagtaacg
ep-r	cccaagctttattctccttttgaagggaa

Table S2. Substitutions observed over multiple rounds of evolution. The substitution was showed in bold when it was the first time introduced.

Variants	BSE_{V4}	BSE_{V5}	BSE_{V6}	BSE_{V7}	BSE_{V8}	BSE_{V9}	BSE_{V10}	BSE_{V11}
Evolutional target	–	Enantioselectivity			Activity			
14	**R**	R	R	R	R	R	R	R
34	**I**	I	I	I	I	I	I	I
68	L	L	L	L	L	L	**I**	L
73	T	T	T	T	T	T	T	**S**
75	L	L	L	L	L	L	L	**P**
107	A	A	**G**	G	G	G	G	G
109	Y	Y	E	E	E	E	E	**V**
110	L	L	**V**	V	V	V	V	V
270	I	I	I	I	**H**	I	I	I
327	L	L	L	L	L	L	L	**H**
391	**E**	E	E	E	E	E	E	E
400	A	**C**	A	C	C	C	C	C
401	L	L	L	L	L	**M**	M	M
465	**F**	F	F	F	F	F	F	F
Total	4	5	7	8	9	9	10	12

Table S3. Docking analysis.

Mutant	*l*-**1**			*d*-**1**		
	r(O-C) [Å]	*r*(N-O) [Å]	Binding affinity [kcal/mol] [a]	*r*(O-C) [Å]	*r*(N-O) [Å]	Binding affinity [kcal/mol] [a]
BSE_{V4}	3.2	3.4	-7.0	3.6	4.8	-6.9
BSE_{V7}	3.8	3.3	-7.1	4.3	4.4	-6.3
BSE_{V11}	3.1	3.4	-7.1	3.7	4.4	-6.3

[a] The binding affinity is caculated by AutoDock.

Table S4. T_m value (°C) of BSE mutants measured with purified proteins.

Entry	Mutant	T_m (°C)
1	BSE_{V4}	55.2
2	BSE_{V5}	51.0
3	BSE_{V6}	54.2
4	BSE_{V7}	53.3
5	BSE_{V9}	52.9
6	BSE_{V11}	52.6

Table S5. Effects of different cosolvents and surfactants. BSE_{V4} was applied to hydrolyze 50 mM *dl*-menthyl benzoate in 10-mL scale.

Cosolvent	Log*P*	Time (h)	Conv. (%)	ee_p (%)
DMSO	-1.49	5	6.1	69.3
DMF	-0.60	5	10.1	64.3
Methanol	-0.27	5	6.6	70.6
Ethanol	0.07	5	12.5	72.7
Isopropanol	0.38	5	9.9	64.9
n-Butanol	0.97	5	6.6	93.5
Triton X-100	–	0.5	35.7	92.1
Tween 80	–	0.5	47.1	90.2

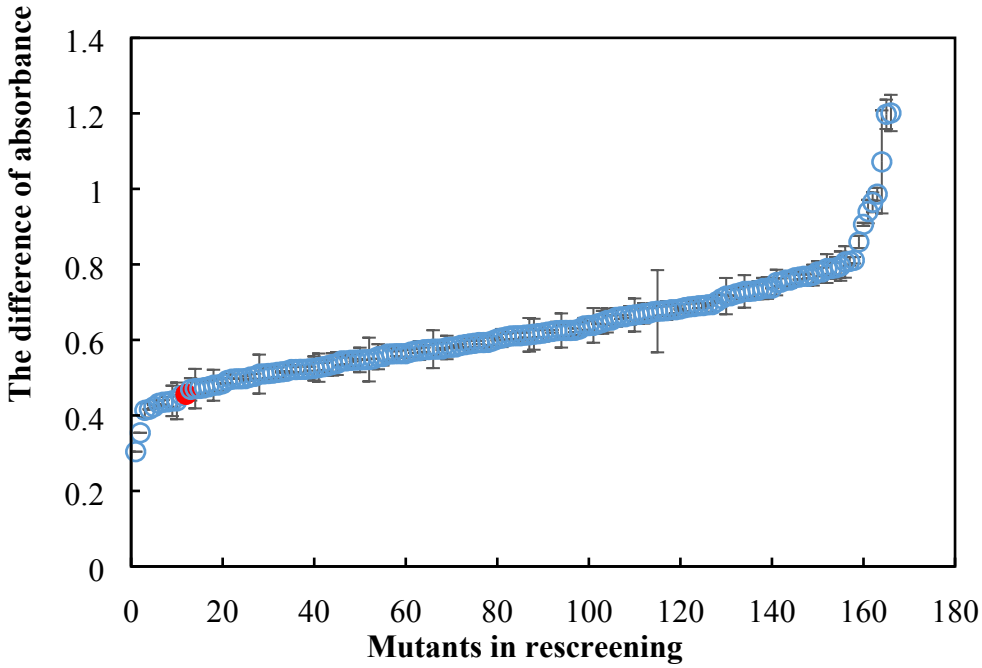

Figure S1. Rescreening results of the random mutagenesis library. The parent enzyme BSE_{V9} was displayed in red point.

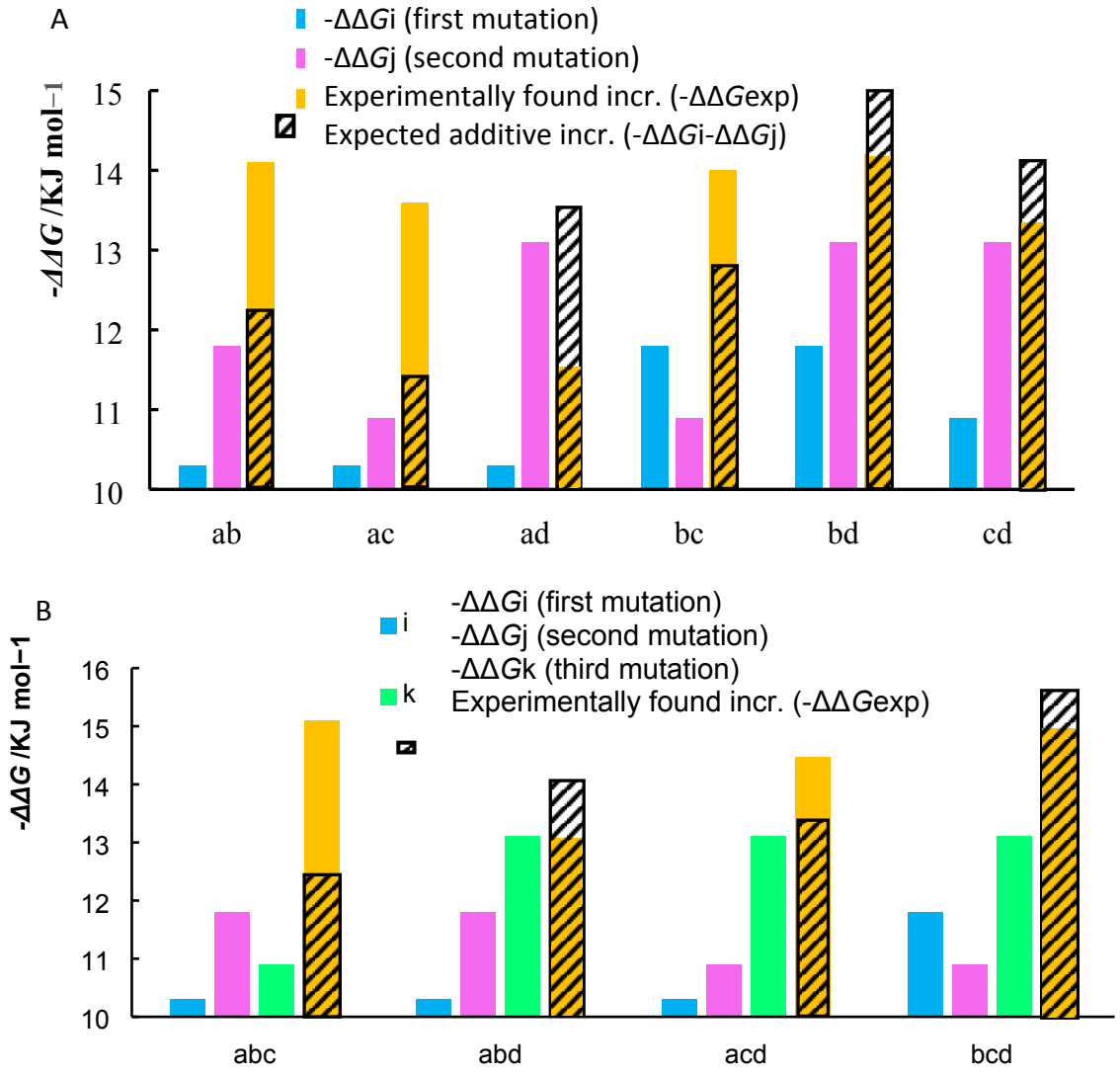

Figure S2. Thermodynamic analysis of deconvolved BSE_{V7} variants in the hydrolysis of substrate *dl*-**1**. **A**) The double mutants: ab, ac, ad, bc, bd and cd; **B**) The triple mutants: abc, abd, acd and bcd.

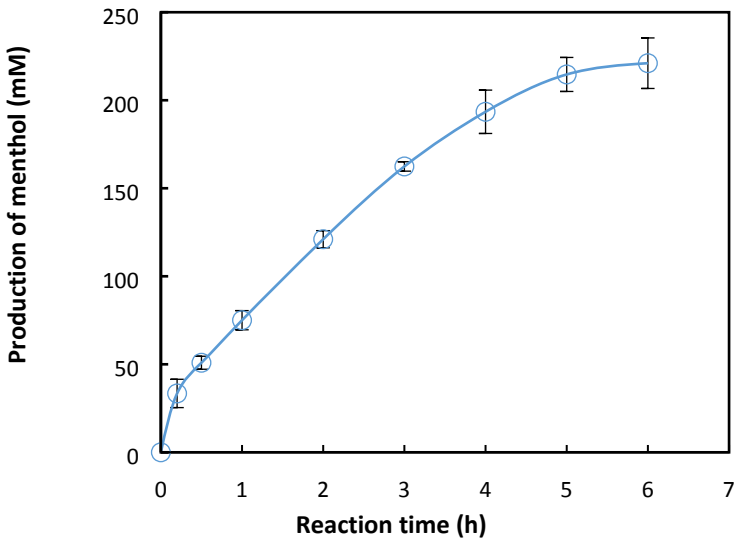

Figure S3. Asymmetric preparation of *l*-menthol from substrate *dl*-**1** at 0.5 M employing BSE_{V11} with a catalyst loading of 0.5 g_{CFE} L^{-1} in 0.1-L scale.