Fig. S1 Hydrolytic activities of the A141S and wild-type (WT) CALB on *p*-NPs with varying carbon chain lengths (C2–C12). Relative enzyme activities depicted are the averages of three independent experiments, with error bars as indicated. Substrates: 4-nitrophenyl acetate (*p*-NPA), 4-nitrophenyl butyrate (*p*-NPB), 4-nitrophenyl octanoate (*p*-NPO), 4-nitrophenyl decanoate (*p*-NPDe), and 4-nitrophenyl dodecanoate (*p*-NPDo). **Substrate screening for characterizing the volume of the substrate pocket of CALB.** When the substrate was *p*–NPB, the gap of the relative activity between the A141S mutant and CALB (W-T) was larger than that of the others, which indicates that the cavity volume of the A141S mutant was reduced by 35.19 %. *p*-NPB was the best substrate for characterizing the volume of the substrate pocket of CALB.

Fig. S2 Hydrolytic activity of CALB double mutants and liquid chromatographic analysis of the product of the A141S-A283V mutant. (A) Hydrolytic activity of CALB double mutants. (B) The liquid chromatograph of R-J_6 production by the A141S-A283V mutant.

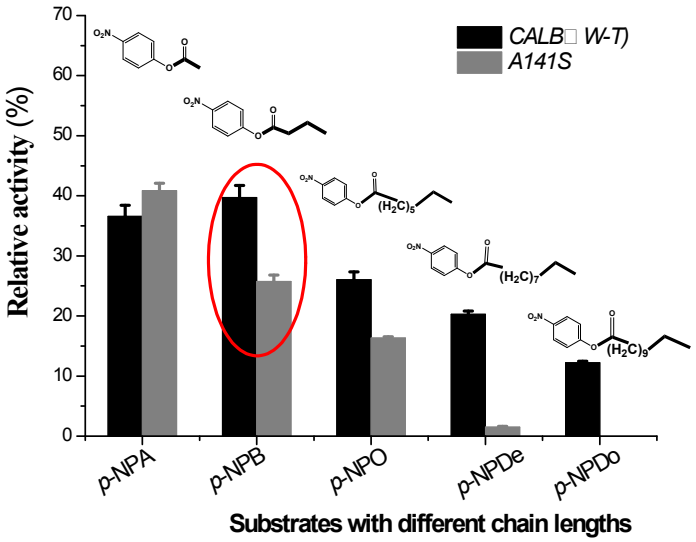
Fig.S3 Effect of initial pH, glucose concentration, fermentation time, and induction temperature on EF5 expression.

Fig.S4 Effect of inducers on EF5 protein expression. (A) Effect of different inducers on EF5 protein expression. (B) Enhancing the protein expression by feeding 25 g/L glucose.

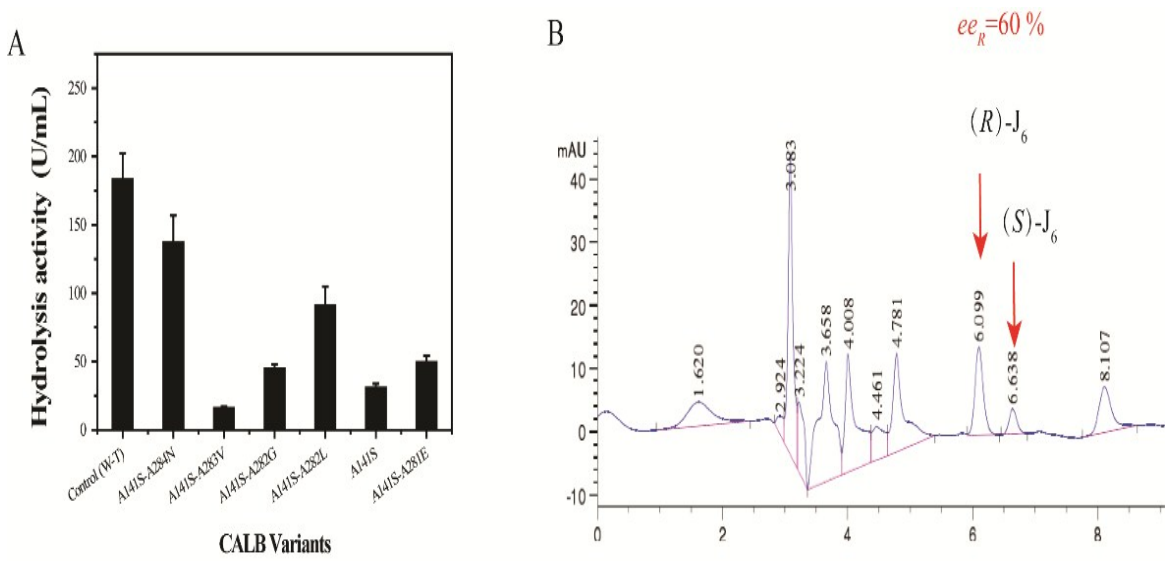
Fig.S5 Optimization of the EF5 immobilization conditions. (A) CALB immobilization on carriers of differing particle size; immobilization rate (%)= activity of immobilized enzyme / total enzyme activity × 100%. (B) Effect of pH on EF5 immobilization; (C,D) Effect of temperature and time on EF5 immobilization; (E) Effect of enzyme: carrier ratio on EF5 immobilization

Fig.S6 The liquid chromatograph of R-J6 producted by the EF5 mutant. (A) The Standard of (R)-3-TBDMSO glutaric acid methyl monoester; (B) The racemate of 3-TBDMSO glutaric acid methyl monoester, the retention times of R-J6 and S-J6 were 6.6 and 7.0 min, respectively; (C) The liquid chromatograph of R-J6 (concentrated reaction solution) by the EF5; (D) The liquid chromatograph of R-J6 (purified product) by the EF5 mutant, with ee of 98.5%.

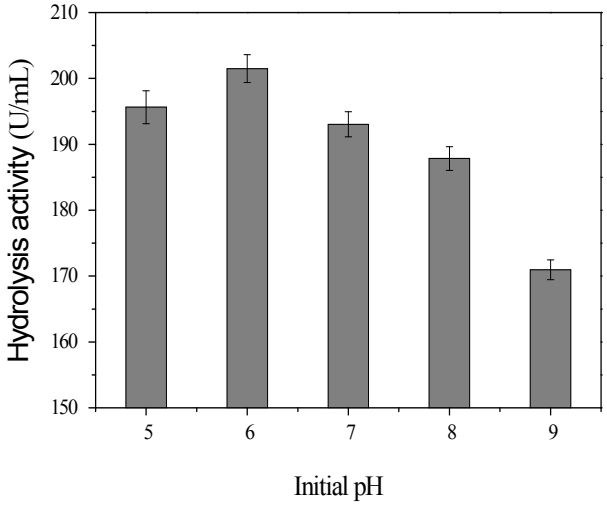
Fig.S7 The HPLC data of the purified R-J6 (final product) by the EF5. HPLC conditions: C18 column (250mm×4.6mm, 5mm), acetonitrile: water =1:9, UV 210 nm, 1 ml/min, 25 ℃. Product purification process: an equal volume aqueous solution (pH 8.0) was added to the reaction solution, discarded the organic phase; Next, the aqueous phase was washed with MTBE three times; Then, the pH of the aqueous phase was adjusted to 6.0 with 0.5 M HCl, extracted with MTBE (three times), dried over anhydrous Na_2SO_4; The organic phase was concentrated by rotary evaporation and the target product has been harvested.

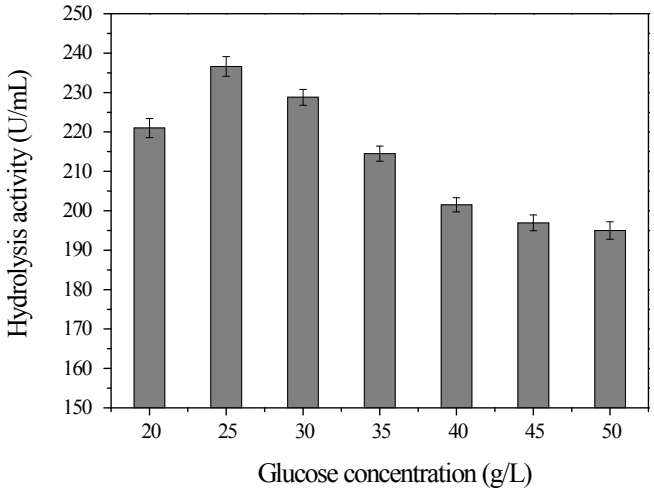

CALB□ W-T)
A141S
Relative activity (%)
70
60
50
40
30
20
10
0
p-NPA
p-NPB
p-NPO
p-NPDe
p-NPDo
Substrates with different chain lengths

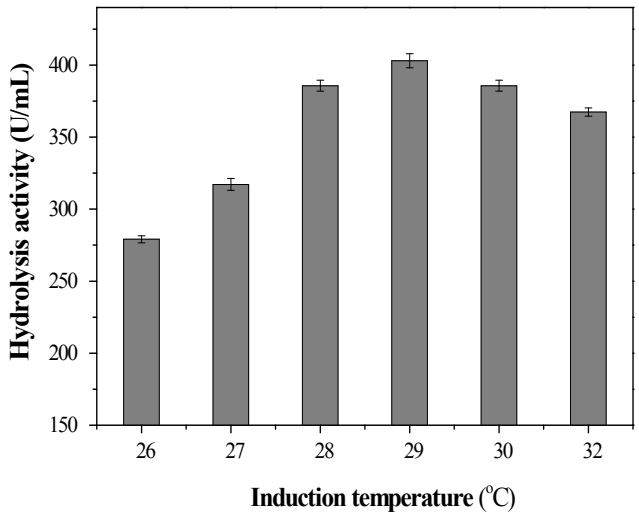
(Fig. S1)

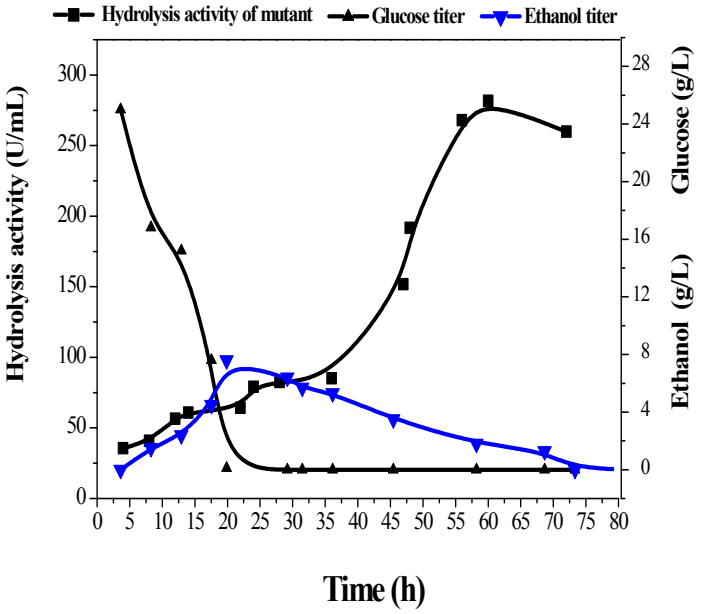

A
Hydrolysis activity (U/mL)
250
200
150
100
50
0
Control (WT)
A141S-A284N
A141S-A283V
A141S-A282G
A141S-A282L
A141S
A141S-A281E
CALB Variants
B
ee_R=60 %
(R)-J6
(S)-J6
mAU
40
30
20
10
0
-10
1.620
2.924
3.083
3.224
3.658
4.008
4.461
4.781
6.099
6.638
8.107
0
2
4
6
8

(Fig. S2)

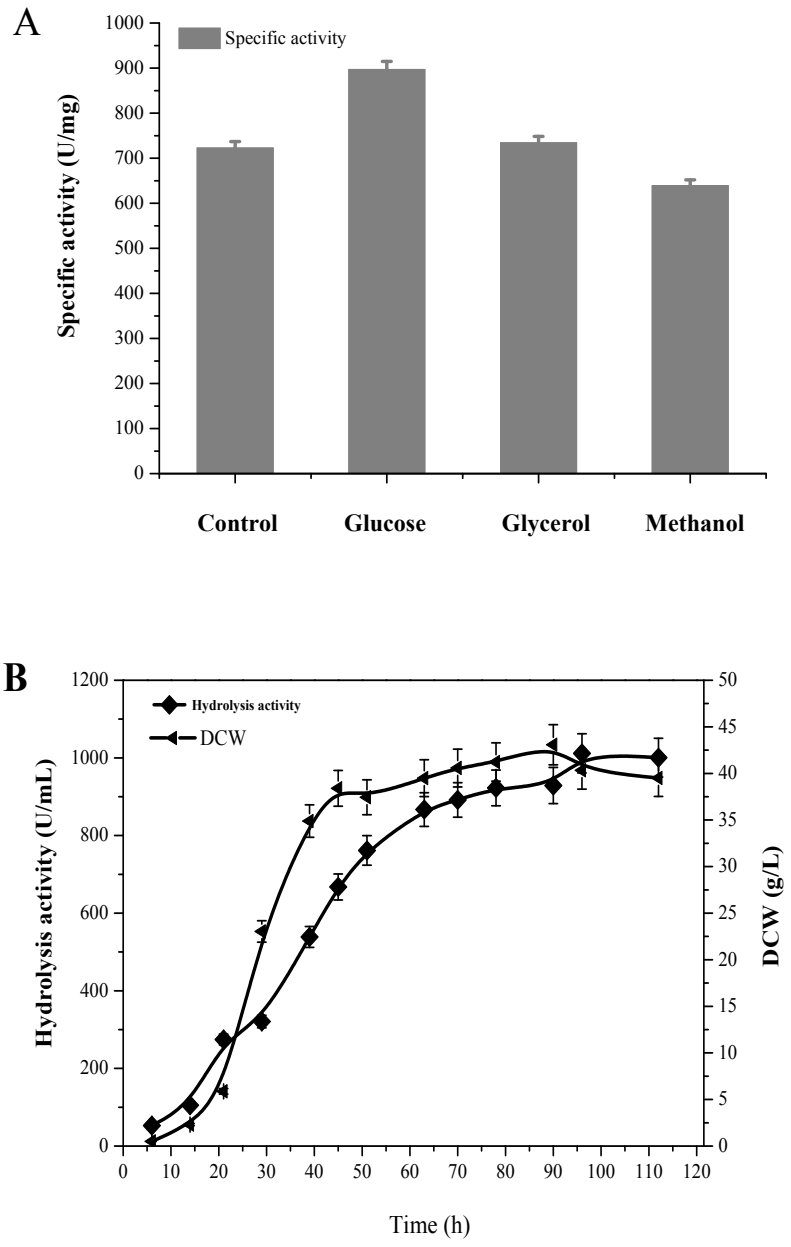

Hydrolysis activity (U/mL)
210
200
190
180
170
160
150
5
6
7
8
9
Initial pH


Hydrolysis activity (U/mL)
250
240
230
220
210
200
190
180
170
160
150
20
25
30
35
40
45
50
Glucose concentration (g/L)


Hydrolysis activity (U/mL)
400
350
300
250
200
150
26
27
28
29
30
32
Induction temperature (°C)

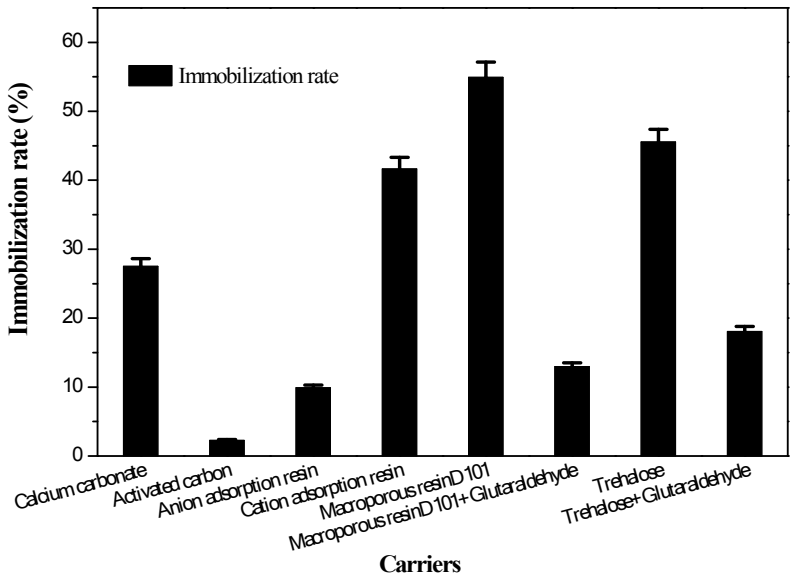

Hydrolysis activity of mutant
Glucose titer
Ethanol titer
Hydrolysis activity (U/mL)
Glucose (g/L)
Ethanol (g/L)
Time (h)

(Fig.S3)

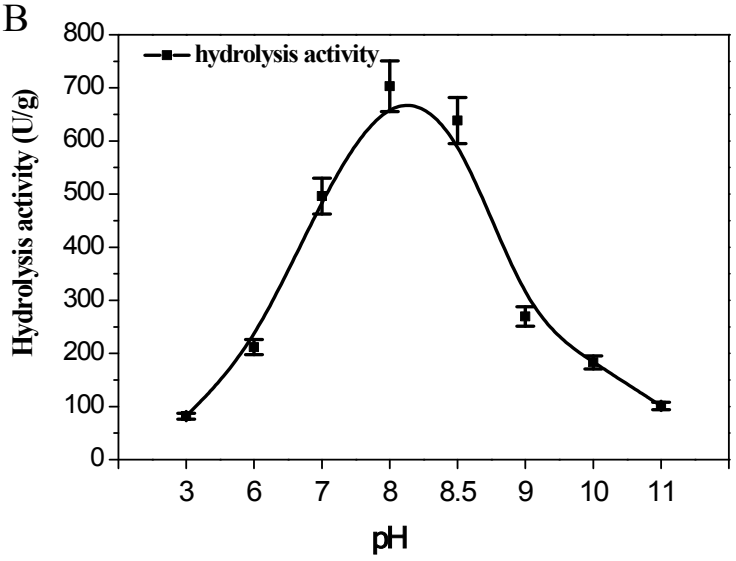

A
Specific activity
Specific activity (U/mg)
1000
900
800
700
600
500
400
300
200
100
0
Control
Glucose
Glycerol
Methanol
B
Hydrolysis activity
DCW
Hydrolysis activity (U/mL)
DCW (g/L)
1200
1000
800
600
400
200
0
50
45
40
35
30
25
20
15
10
5
0
0
10
20
30
40
50
60
70
80
90
100
110
120
Time (h)

(Fig. S4)

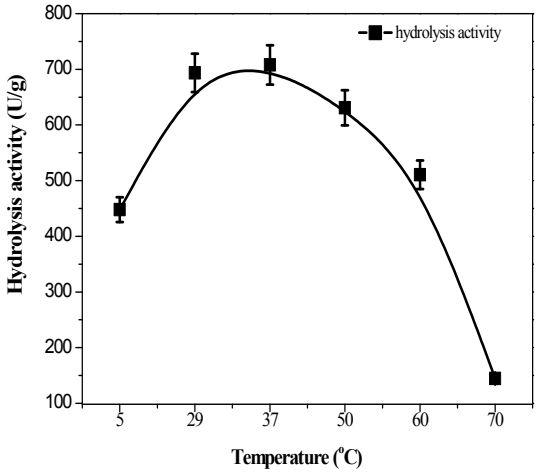
A


Immobilization rate
Immobilization rate (%)
0
10
20
30
40
50
60
Calcium carbonate
Activated carbon
Anion adsorption resin
Cation adsorption resin
Macroporous resin D101
Macroporous resin D101+Glutaraldehyde
Trehalose
Trehalose+Glutaraldehyde
Carriers

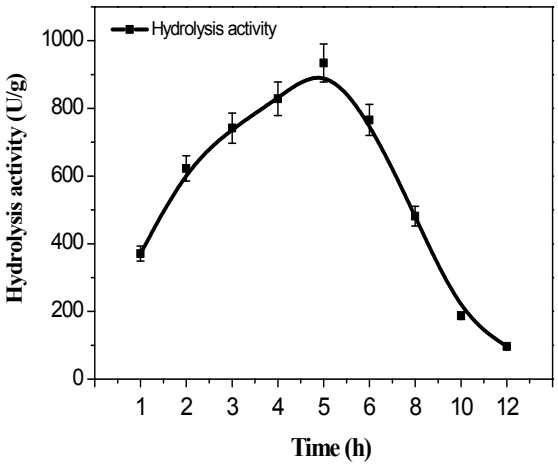
B


hydrolysis activity
Hydrolysis activity (U/g)
0
100
200
300
400
500
600
700
800
3
6
7
8
8.5
9
10
11
pH

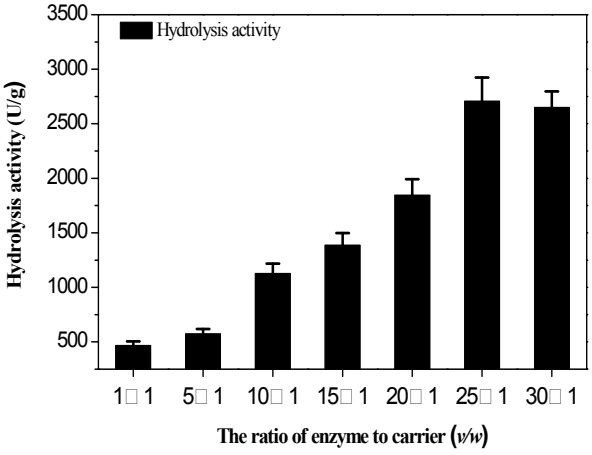
C


hydrolysis activity
Hydrolysis activity (U/g)
800
700
600
500
400
300
200
100
5
29
37
50
60
70
Temperature (°C)

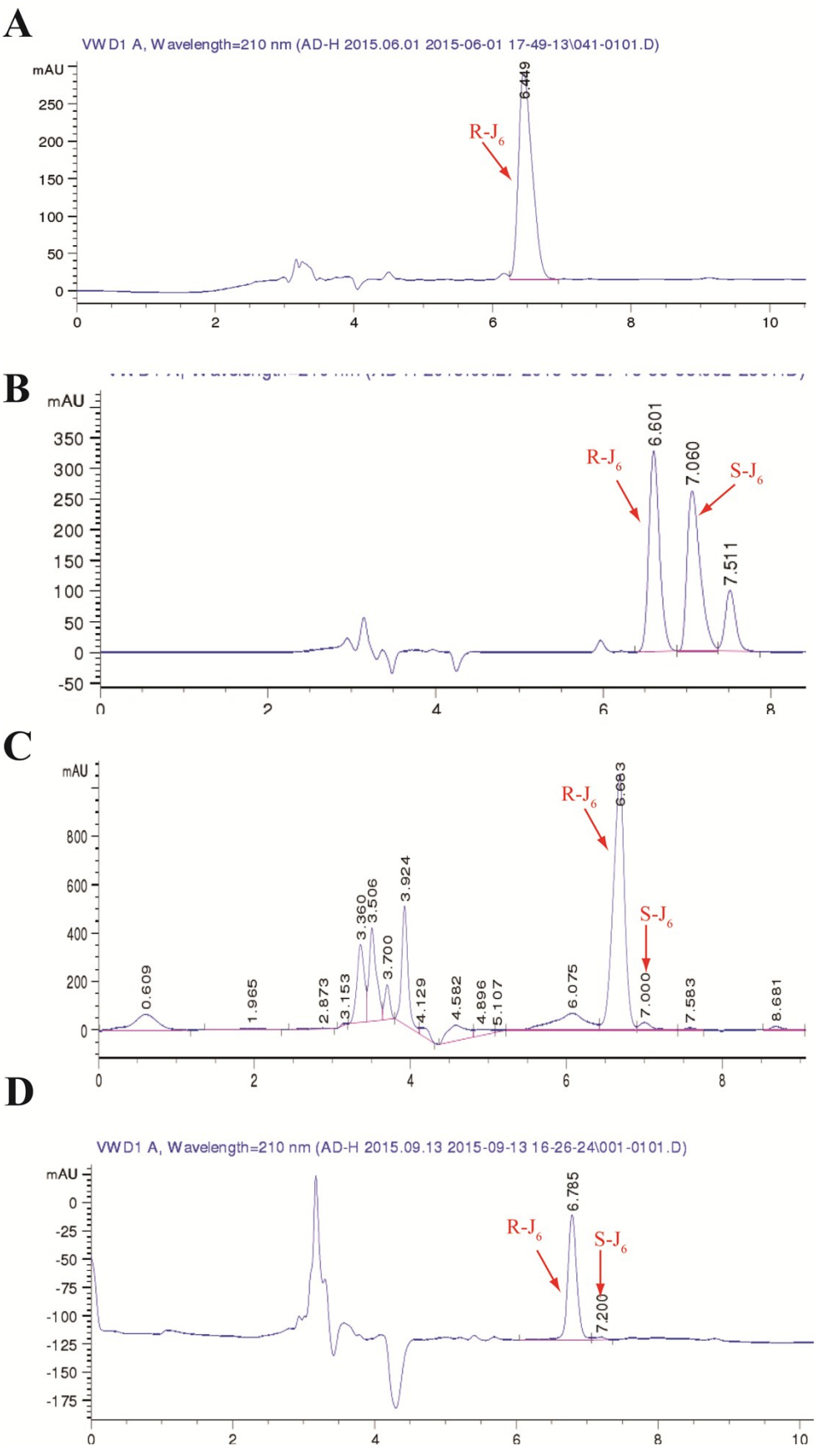
D


Hydrolysis activity
Hydrolysis activity (U/g)
1000
800
600
400
200
0
1
2
3
4
5
6
8
10
12
Time (h)

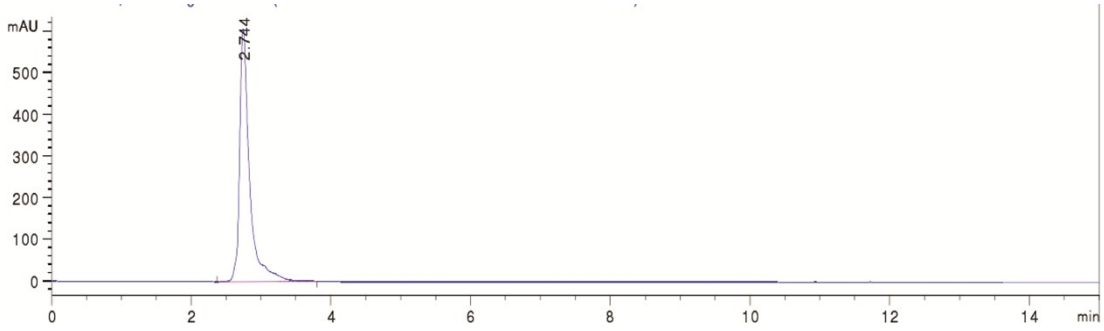
E


Hydrolysis activity
Hydrolysis activity (U/g)
3500
3000
2500
2000
1500
1000
500
1□1
5□1
10□1
15□1
20□1
25□1
30□1
The ratio of enzyme to carrier (w/w)

(Fig. S5)


A
VWD1 A, Wavelength=210 nm (AD-H 2015.06.01 2015-06-01 17-49-13\041-0101.D)
mAU
6.449
R-J6
B
mAU
6.601
7.060
7.511
R-J6
S-J6
C
mAU
0.609
1.965
2.873
3.153
3.360
3.506
3.700
3.924
4.129
4.582
4.896
5.107
6.075
6.683
7.000
7.583
8.681
R-J6
S-J6
D
VWD1 A, Wavelength=210 nm (AD-H 2015.09.13 2015-09-13 16-26-24\001-0101.D)
mAU
6.785
7.200
R-J6
S-J6

(Fig. S6)



(Fig. S7)

Table S1 Highly frequent amino acids, as revealed by a multiple sequence alignment

Candidate amino acids	**Occurrence frequency (%)**			
W104	S	**D**	A	T
	63.8	**10.7**	7.9	6.2
Q106	Q	**H**	A	K
	93.7	**4.8**	1.1	1
S47	S	**N**	T	A
	30.7	**27.4**	24.7	5.3
D134	D	**S**	P	T
	84.2	**11.1**	10.6	3.2
A141	A	**S**	G	E
	67.7	**7.1**	5.1	2
G137	G	**R**	-	-
	99.5	**0.5**	-	-
L278	L	**I**	V	A
	51.7	**21.2**	11.9	3.4
T138	T	**A**	L	S
	89.9	**10**	1	8.1
I189	I	**V**	L	S
	58.6	**32.3**	6.1	1

Note: Candidate amino acids was selected by docking; Occurrence frequency was calculated by multiple sequence alignment.

Table S2 Primers used for site-directed mutagenesis of the CALB gene

Primer	**Sequence (5'- 3')**
T138A-For	CCGACTACAAGGGC**GCC**GTCCTCGCCGGCCC
T138A-Rev	GGGCCGGCGAGGAC**GGC**GCCCTTGTAGTCGG
S47N-For	CACCACAGGTCCACAG**AAC**TTCGACTCGAACTGGA
S47N-Rev	TCCAGTTCGAGTCGAA**GTT**CTGTGGACCTGTGGTG
I189V-For	CGGCGACCGACGAG**GTC**GTTCAGCCTCAGGT
I189V-Rev	ACCTGAGGCTGAAC**GAC**CTCGTCGGTCGCCG
A141S-For	AGGGCACCGTCCTC**TCC**GGCCCTCTCGATGC
A141S-Rev	GCATCGAGAGGGCC**GGA**GAGGACGGTGCCCT
D134S-For	TGGCCTTTGCGCCC**TCG**TACAAGGGCACCG
D134S-Rev	CGGTGCCCTTGTA**CGA**GGGCGCAAAGGCCA
W104D-For	ACTCGCCCCTCGAC**GAC**TCGTACCTCTTC
W104D-Rev	GAAGAGGTACGA**GTC**GTCGAGGGGCGAGT
Q106H-For	CTTACGTGGTCT**CAC**GGTGGGCTGGTGGC
Q106H-Rev	GCCACCAGCCCACC**GTG**AGACCACGTAAG
A283V-For	CGCCCCTGCGGCT**GTG**GCCATCATCGCGG
A283V-Rev	CGCGATGATGGC**CAC**AGCCGCAGGGGCG

Note: The mutated amino acids codons are underlined in bold.

Table S3 Primers used for A141S-S283V semi-saturation/saturation mutagenesis

Libraries		**The primers**
Library I	189-190-S	GGCCACCGACGAG**NNNNNN**CAGCCCCAGGTGTC
	189-190-A	GACACCTGGGGCTG**NNNNNN**CTCGTCGGTGGCC
Library II	134-138-S	GCGCCC**NNN**TACAAGGGC**NNN**GTCCTCTCCGGC
	134-138-A	GCCGGAGAGGAC**NNN**GCCCTTGTA**NNN**GGGCGC
Library III	278-282-S	GCGCTG**NDT**GCCCCTGC**NDT**TGCGGCCATCAT
	278-282-A	ATGATGGCCGCA**AHN**GCAGGGGC**AHN**CAGCGC
Library IV	42-47-S	CAGGC**NDK**ACGGGTCCGGGA**NDK**TTCGACTCGAAC
	42-47-A	GTTCGAGTCGAA**MHN**TCCCGGACCCGT**MHN**GCCTG

Mutations have been marked in bold.

Table S4 Docking free energy and ee values of the variants

Variants	**Docking Energy**[a]		E_R/E_S[a]	***R*-J$_6$ Titer(g/L)** [b]	ee_R[b]
	***R*-J$_6$**(kcal/mol)	**S-J$_6$** (kcal/mol)			***(%)***
S47N	−88.49	−77.76	1.138	-	-
A141S	−92.01	−82.22	1.119	10.1	17.6
W104D	−88.9	−81.70	1.089	-	-
Q106H	−105.93	−98.41	1.076	-	-
D134S	−92.03	−88.44	1.041	-	-
T138A	−92.09	−89.08	1.034	-	-
G137R	−96.02	−93.59	1.023	-	-
WT[b]	−91.22	−93.477	0.975	4.4	−63
L278I	−84.13	−89.16	0.944	-	-
I189V	−85.03	−96.55	0.881	-	-

Note: E_R/E_S represents the ratio of the docking energy of the *R*-isomer to that of the *S*-isomer. WT, WT CALB; ee_R indicates the ee for *R*-J_6; ee_S indicates the ee for *S*-J_6. a. the result was from molecular docking; b. it was experimental result.