Supporting Information

Engineering a *Bacillus subtilis* esterase for selective hydrolysis of D, L-menthyl acetate in an organic solvent-free system

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Supplementary tables

Table S1 The primers for site-directed mutagenesis of the esterase pnbA-BS in alanine scanning

Primer		Sequence
G105A	F	5’-ATGGTTTGGATTCATGCTGGTGCATTTTATCTG-3’
	R	5’-CACCCAGATAAAATGCACCAGCATGAATCCAA-3’
G106A	F	5’-ATGGTTTGGATTCATGGTGCTGCATTTTATCTG-3’
	R	5’-CACCCAGATAAAATGCAGCACCATGAATCCAA-3’
A107G	F	5’-CATGGTGGTGGTTTTTATCTGGGTGCAGGTAGCG-3’
	R	5’-CAGATAAAAACCACCACCATGAATCCAAACCATAAC-3’
A107V	F	5’-ATGGTTTGGATTCATGGTGGTGTATTTTATCTG-3’
	R	5’-CACCCAGATAAAATACACCACCATGAATCCAA-3’
A190G	F	5’-TGGTGAAAGCGTAGGTGGTATGAGTAT-3’
	R	5’-ATACTCATACCACCTACGCTTTCACCA-3’
A190V	F	5’-GGTGAAAGCGTTGGTGGTATGAGTATTGCTGCACTG-3’
	R	5’-CATACCACCAACGCTTTCACCAAAAACGGTAACATT-3’
E188A	F	5’-CGTTTTTGGTGCAAGCGCAGGTGGTATGA-3’
	R	5’-TCATACCACCTGCGCTTGCACCAAAAACG-3’
S189A	F	5’-CCGTTTTTGGTGAAGCAGCAGGTGGTATG-3’
	R	5’-CATACCACCTGCTGCTTCACCAAAAACGG-3’
M193A	F	5’-GGTGGTGCAAGTATTGCTGCACTGCTGGCA-3’
	R	5’-AATACTTGCACCACCTGCGCTTTCACCA-3’
T326A	F	5’-GCATTCTCAGGAAGCATTTGATGCAGCACT-3’
	R	5’-AGTGCTGCATCAAATGCTTCCTGAGAATGC-3’
A330G	F	5’-ATTTGATGCAGTACTGGAATATCTGCTGG-3’
	R	5’-CCAGCAGATATTCCAGTACTGCATCAAAT-3’
A330V	F	5’-GATGCAGTGCTGGAATATCTGCTGGGTCAGC-3’
	R	5’-TTCCAGCACTGCATCAAATGTTTCCTGAGAATGC-3’
L331A	F	5’-ACATTTGATGCAGCAGCAGAATATCTGCTG-3’
	R	5’-CAGCAGATATTCTGCTGCTGCATCAAATGT-3’
M358A	F	5’-CATATGGCAACCGATCTGCTGTTTTGGCGT-3’
	R	5’-ATCGGTTGCCATATGAATCTGGCTTTCCAGGC-3’
A400G	F	5’-GCATTTCATGGACTGGAACTGCCGTTTGTTT-3’
	R	5’-AGTTCCAGTCCATGAAATGCTTTATTATACGGCG-3’
A400V	F	5’-GCATTTCATGTACTGGAACTGCCGTTTGTTT-3’
	R	5’-AGTTCCAGTACATGAAATGCTTTATTATACGGCG-3’

L403A	F	5’-TTCATGCACTGGAAGCACCGTTTGTTTTTG-3’
	R	5’-CAAAAACAAACGGTGCTTCCAGTGCATGAA-3’

Table S2 The primers for site-saturation mutagenesis of the residue Ala400 of the esterase pnbA-BS

Primer		Sequence
A400P	F	5’-GCATTTCATCCTCTGGAACTGCCGTTTGTTT-3’
	R	5’-AGTTCCAGAGGATGAAATGCTTTATTATACGGCG-3’
A400K	F	5’-GCATTTCATAAACTGGAACTGCCGTTTGTTT-3’
	R	5’-AGTTCCAGTTTATGAAATGCTTTATTATACGGCG-3’
A400Q	F	5’-GCATTTCATGAACTGGAACTGCCGTTTGTTT-3’
	R	5’-AGTTCCAGTTCATGAAATGCTTTATTATACGGCG-3’
A400R	F	5’-GCATTTCATCGACTGGAACTGCCGTTTGTTT-3’
	R	5’-AGTTCCAGTCGATGAAATGCTTTATTATACGGCG-3’
A400H	F	5’-GCATTTCATCACCTGGAACTGCCGTTTGTTT-3’
	R	5’-AGTTCCAGGTGATGAAATGCTTTATTATACGGCG-3’
A400L	F	5’-GCATTTCATCTACTGGAACTGCCGTTTGTTT-3’
	R	5’-AGTTCCAGTAGATGAAATGCTTTATTATACGGCG-3’
A400E	F	5’-GCATTTCATCAGCTGGAACTGCCGTTTGTTT-3’
	R	5’-AGTTCCAGCTGATGAAATGCTTTATTATACGGCG-3’
A400T	F	5’-GCATTTCATACCCTGGAACTGCCGTTTGTTT-3’
	R	5’-AGTTCCAGGGTATGAAATGCTTTATTATACGGCG-3’
A400I	F	5’-GCATTTCATATCCTGGAACTGCCGTTTGTTT-3’
	R	5’-AGTTCCAGGATATGAAATGCTTTATTATACGGCG-3’
A400N	F	5’-GCATTTCATAACCTGGAACTGCCGTTTGTTT-3’
	R	5’-AGTTCCAGGTTATGAAATGCTTTATTATACGGCG-3’
A400F	F	5’-GCATTTCATTTCCTGGAACTGCCGTTTGTTT-3’
	R	5’-AGTTCCAGGAAATGAAATGCTTTATTATACGGCG-3’
A400M	F	5’-GCATTTCATTAACTGGAACTGCCGTTTGTTT-3’
	R	5’-AGTTCCAGTTAATGAAATGCTTTATTATACGGCG-3’
A400D	F	5’-GCATTTCATGACCTGGAACTGCCGTTTGTTT-3’
	R	5’-AGTTCCAGGTCATGAAATGCTTTATTATACGGCG-3’
A400V	F	5’-GCATTTCATGTACTGGAACTGCCGTTTGTTT-3’
	R	5’-AGTTCCAGTACATGAAATGCTTTATTATACGGCG-3’
A400C	F	5’-GCATTTCATTGCCTGGAACTGCCGTTTGTTT-3’
	R	5’-AGTTCCAGGCAATGAAATGCTTTATTATACGGCG-3’
A400S	F	5’-GCATTTCATTCACTGGAACTGCCGTTTGTTT-3’

	R	5’-AGTTCCAGTGAATGAAATGCTTTATTATACGGCG-3’
A400G	F	5’GCATTTCATGGACTGGAACTGCCGTTTGTTT-3’
	R	5’-AGTTCCAGTCCATGAAATGCTTTATTATACGGCG-3’
A400Y	F	5’-GCATTTCATTATCTGGAACTGCCGTTTGTTT-3’
	R	5’-AGTTCCAGATAATGAAATGCTTTATTATACGGCG
A400W	F	5’-GCATTTCATTGGCTGGAACTGCCGTTTGTTT-3’
	R	5’-AGTTCCAGCCAATGAAATGCTTTATTATACGGCG-3’

Supplementary figures

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Figure S1 The codon-optimized nucleotide sequence encoding then esterase pnbA-BS.

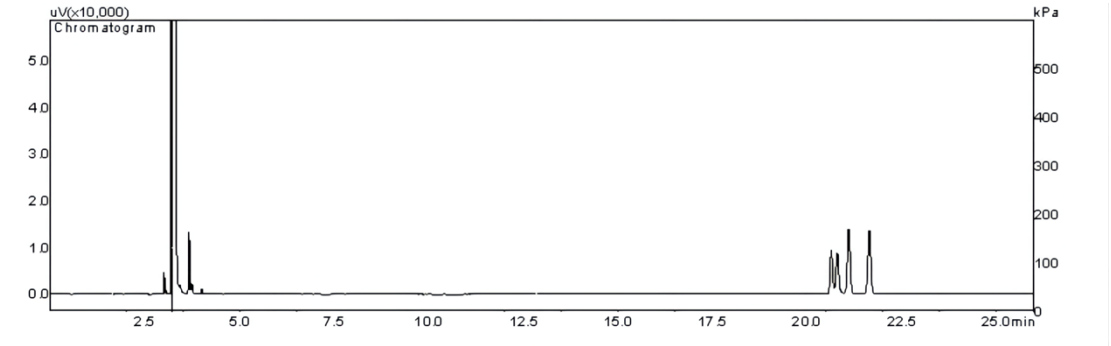
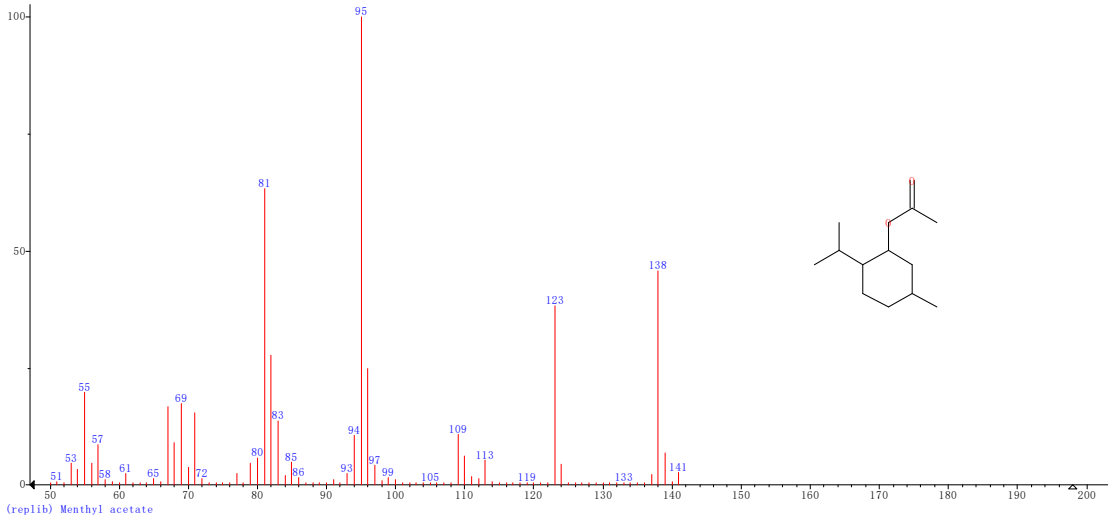
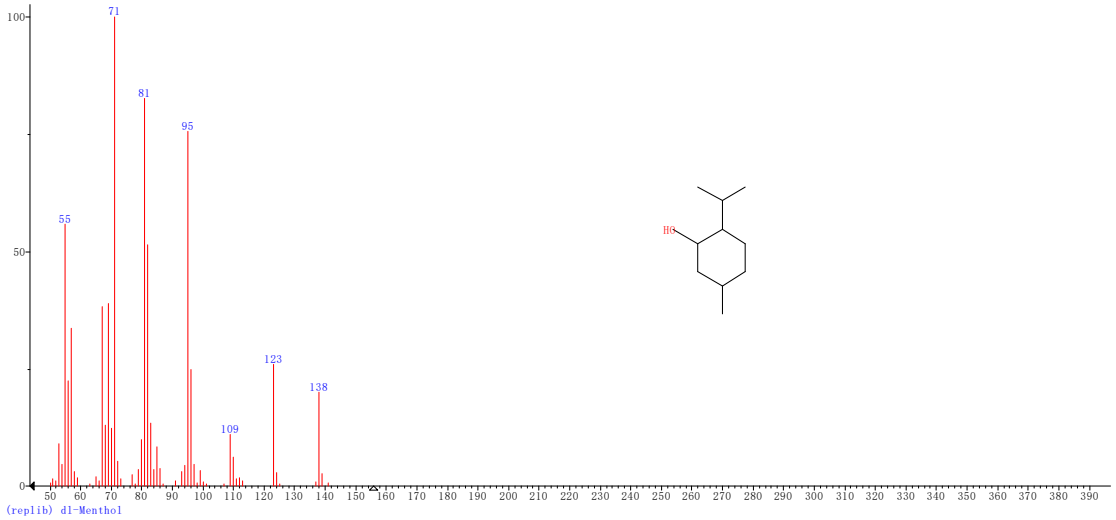


Figure S2 The GC chromatogram of the standards of substrate and product. The retention times of D-menthol, L-menthol, L-menthyl acetate and D-menthyl acetate were 20.615 min, 20.774 min, 21.097 min and 21.638 min, respectively.

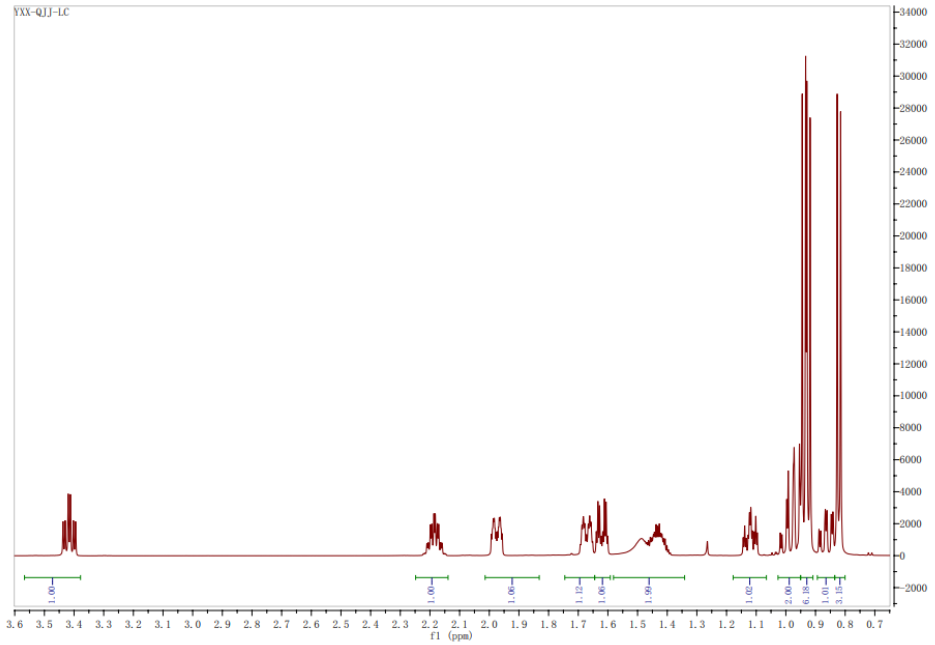


(a)

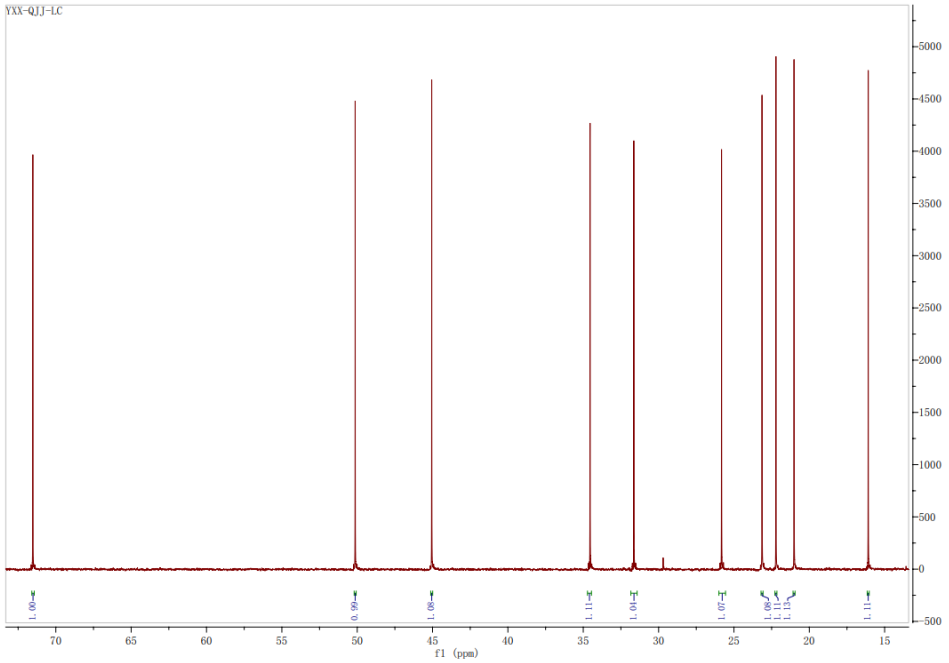


(b)

Figure S3 GC-MS analyses of substrate and product. (a), GC-MS chromatogram for menthyl acetate (MW 198). (b), GC-MS chromgatogram for menthol (MW 156).



(a)



(b)

Figure S4 The ^{1}H NMR (a) and ^{13}C NMR (b) analyses of the product L-menthol. (a), ^{1}H NMR (600 MHz, $CDCl_3$) δ 3.42 (td, *J*=10.5, 4.3 Hz, 1H), 2.25–2.14 (m, 1H), 2.01–1.83 (m, 1H), 1.75–1.64 (m, 1H), 1.64–1.59 (m, 1H), 1.58–1.34 (m, 2H), 1.18–1.07 (m, 1H), 1.03–0.95 (m, 2H), 0.93 (dd, *J*=9.3, 6.8 Hz, 6H), 0.89–0.84 (m, 1H), 0.82 (d, *J*=7.0 Hz, 3H). (b), ^{13}C NMR (151 MHz, $CDCl_3$) δ 71.52 (s), 50.14 (s), 45.06 (s), 34.55 (s), 31.64 (s), 25.82 (s), 23.14 (s), 22.21 (s), 21.01 (s), 16.09 (s).

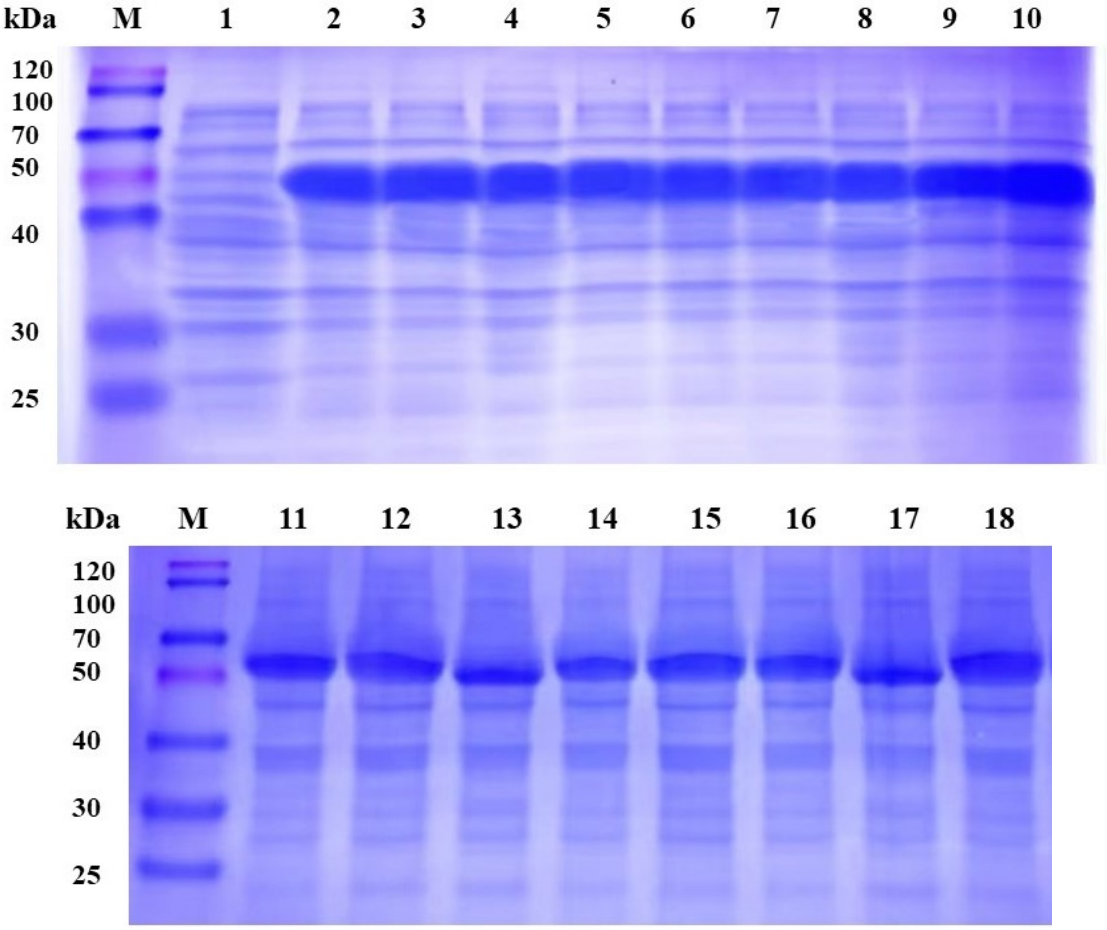


Figure S5 SDS-PAGE (12%) analysis of pnbA-BS and its variants in alanine scanning. Lane M, standard molecular mass proteins; lane 1, the host train *E. coli* BL21(DE3)； lane 2 , wild type pnbA-BS. Other lanes from 3 to 18 represent the pnbA-BS variants (from left to right): G105A, G106A, A107G, A107V, E188A, A190G, A190V, M193A, T326A, A330G, A330V, L331A, M358A, A400G, A400V and L403A . The proteins were visualized by staining with Coomassie brilliant blue R-250.

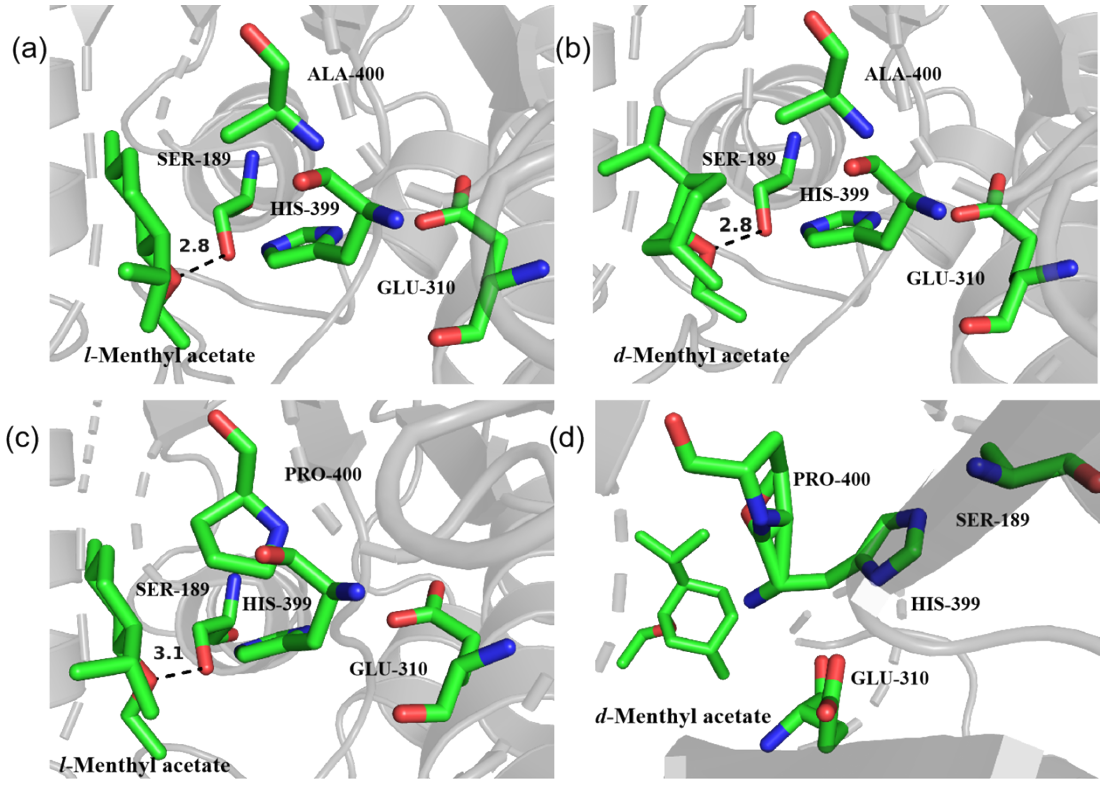


Figure S6 The docking analysis of the esterase pnbA-BS and its variant A400P on the selective hydrolysis of D, L-menthyl acetate. (a) pnbA-BS and L-menthyl acetate; (b) pnbA-BS and D-menthyl acetate; (c) pnbA-BS A400P and L-menthyl acetate; (d) pnbA-BS A400P and D-menthyl acetate. Green, carbon; blue, nitrogen; orange, oxygen.

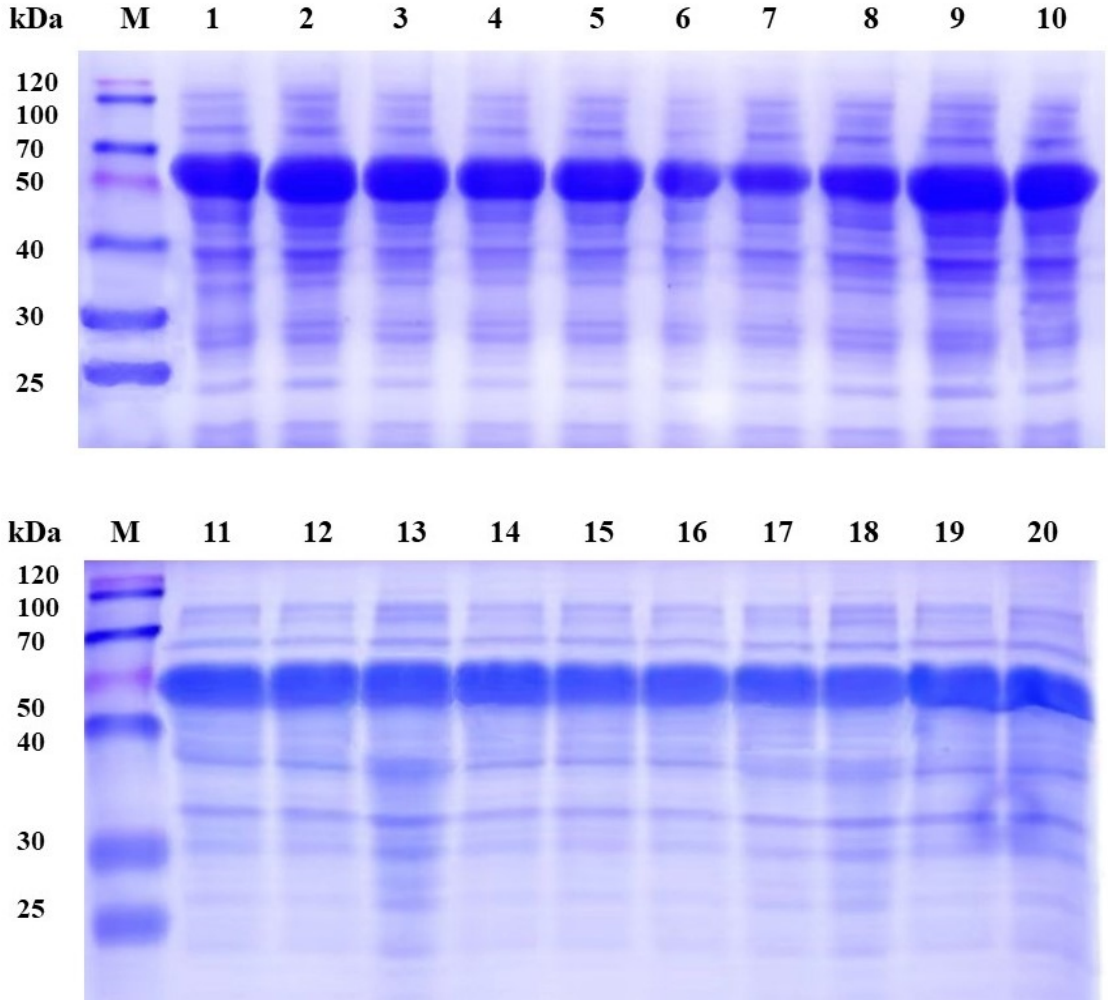


Figure S7 SDS-PAGE (12%) analysis of the esterase pnbA-BS and its variants in the saturation mutagenesis of the residue A400. Lane M, standard molecular mass proteins; lane 1, wild type pnbA-BS. Other lanes from 2 to 20 represent the pnbA-BS variants (from left to right): A400P, A400K, A400Q, A400R, A400H, A400M, A400L, A400E, A400T, A400I, A400N, A400F, A400D, A400V, A400C, A400S, A400G, A400Y and A400W. The proteins were visualized by staining with Coomassie brilliant blue R-250.

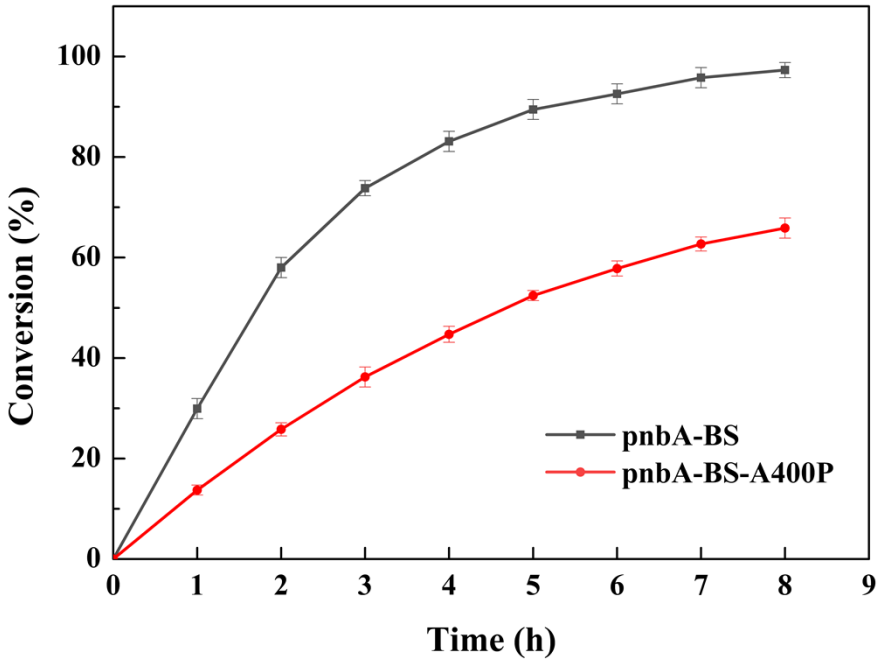
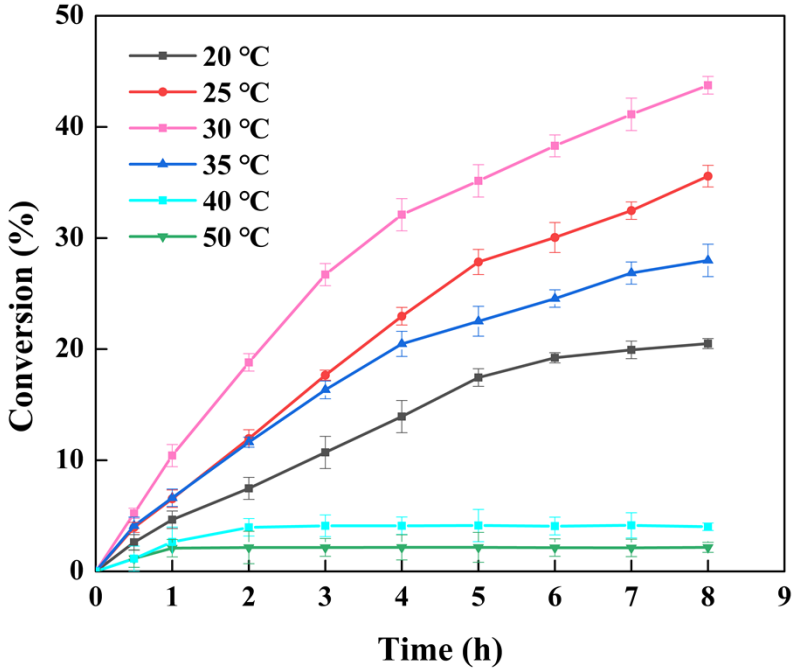
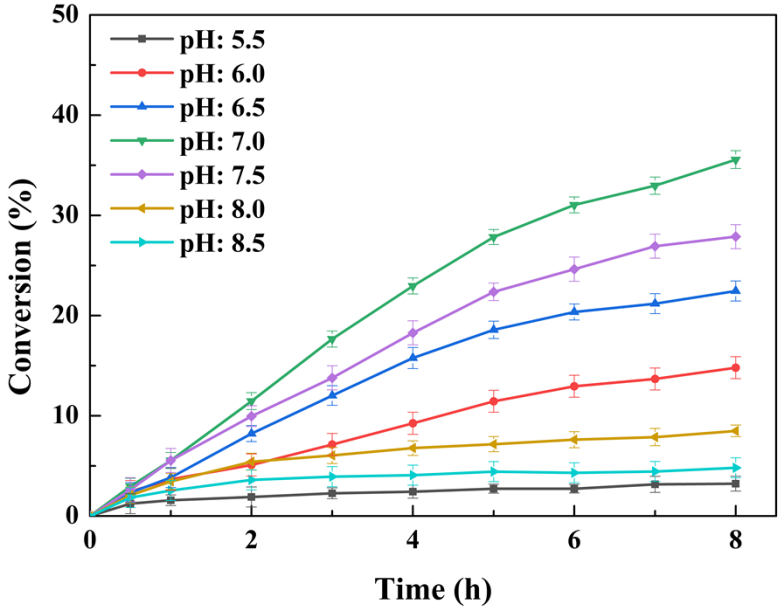


Figure S8 Catalytic performances of the purified esterase pnbA-BS and its variant A400P in the hydrolysis of D, L-menthyl acetate to L-menthol. The reaction mixture (10 ml) contained 500 mM L-menthyl acetate, 70 mg purified pnbA-BS or its variant A400P, and 100 mM PBS buffer (pH 8.0). The reaction was conducted in an orbital shaker (600 rpm, 30 °C) for 8 h meanwhile pH 8.0 was constantly maintained by the titration of 1 M NaOH. The experiments were conducted in triplicate.



(a)



(b)

Figure S9 Key factors affecting whole-cell catalyzed hydrolysis of D, L-menthyl acetate to L-menthol. The standard reaction mixture (10 ml) contained 500 mM D, L-menthyl acetate, 0.2 g lyophilized cells expressing pnbA-BS or its variant, and 100 mM PBS buffer (pH 7.0). The reaction was conducted in a reactor with a pH auto-titration system at 600 rpm for 8 h. The temperature was investigated from 20 to 45 °C (a) and the pH values were explored from 5.5 to 8.5 (b). Data present mean values ± SD from three independent experiments.