In vitro evolution of an L-amino acid deaminase active on L-1-naphthylalanine

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Supplementary Materials:

Table S1 Analysis of the genetic diversity of the libraries of PmaLAAD variants generated by site-saturation mutagenesis.

Position	Original codon	Number of sequenced clones	Introduced mutation	Encoded residue	Fraction of variants (%)
First-generation, single-site PmaLAAD variants (template gene: *PmaLAAD-00N*)					
L279	TTA	6	GGG TTG GTT GGT GGG ATG	Gly Leu Val Gly Gly Met	100
F318	TTC	6	GGG ACT CCT TTG TTT TGG	Gly Thr Pro Leu Phe Trp	100
V412	GTA	7	CTT GCG GTT GTT GTA* AGG GGG	Leu Ala Val Val Val Arg Gly	86
V438	GTG	6	CCG GCG GAT TCG GGG CAT	Pro Ala Asp Ser Gly His	100

			TAT	Tyr	
			CTG	Leu	
			CAG	Leu	
W439	TGG	7	CTG	Leu	100
			TAT	Tyr	
			CGG	Arg	
			AGT	Ser	
First-generation, double-site PmaLAAD variants (template gene: *PmaLAAD-00N*)					
			TAT/TGT	Tyr/Cys	
			ATT/CAT	Ile/His	
			CTT/AAT	Leu/Asn	
			TTT/TGT	Phe/Cys	
			TTT/AGT	Phe/Ser	
S99/Q100	AGC/CAA	10	TAT/CGT	Tyr/Arg	100
			CAT/CAT	His/His	
			TGT/CAT	Cys/His	
			CAT/AAT	His/Asn	
			GTT/CTT	Val/Leu	
			CAT/GTA*	His/Val	
			TAT/GGT	Tyr/Gly	
			TAT/ATT	Tyr/Ile	
			TAT/ATT	Tyr/Ile	
			TAT/TGT	Tyr/Cys	
F318/V412	TTC/GTA	10	CGT/TGT	Arg/Cys	100
			AAT/TAT	Asn/Tyr	
			TGT/ATT	Cys/Ile	
			TTT/GAT	Phe/Asp	
			AAT/AAT	Asn/Asn	
Second-generation PmaLAAD variants (template gene: V412A/V438P-*PmaLAAD-00N*)					
			CGG	Arg	
			TTA*	Leu	
			AGG	Lys	
			GAT	Asp	
			GTG	Val	
L279	TTA	10	CTT	Leu	90
			TCT	Ser	
			CCG	Pro	
			AGT	Ser	
			CGT	Arg	
			CAG	Gln	
			ATT	Ile	
			CGT	Arg	
			TGG	Trp	
			GTT	Val	
			TGG	Trp	
F318	TTC	12	CAT	His	91.7
			TTT	Phe	
			GAT	Asp	
			TGG	Trp	
			CCG	Pro	
			TTC*	Phe	

* parental DNA

Table S2 Expression and purification of wild-type PmaLAAD and variants. Proteins were purified from cells harvested from 1 L of culture. Activity values were determined on 50 mM L-Phe as substrate. (BD: below detection; ND: not determined).

Variant	Specific activity	Protein yield	
	(U/mg)	mg/g $_{cells}$	mg/L $_{culture}$
Wild-type	2.90	1.0	9.9
First-generation, single-site variants (template gene: *PmaLAAD-00N*)			
L279G	0.35	0.4	4.4
F318A	1.36	0.7	4.0
F318C[1]	0.90	0.5	3.5
F318G[1]	0.97	0.7	5.0
F318I[1]	1.34	1.7	8.9
F318L	0.89	0.5	4.7
F318M	1.04	0.7	5.8
F318V	0.39	0.7	6.4
V412A	2.85	0.6	5.9
V412L	2.99	0.8	6.2
V412A/V438P	0.63	0.8	6.1
V438G	0.45	0.6	4.8
V438P	1.14	1.1	6.4
First-generation, double-site variants (template gene: *PmaLAAD-00N*)			
F318C/V412L	0.82	0.4	3.5
F318C/V412G	0.07	0.5	3.1
F318G/V412G	BD	0.5	3.0
F318I/V412S	0.48	0.7	7.8
F318S/V412S	0.12	0.4	4.2
F318V/V412L[2]	ND	ND	ND
F318V/V412G	0.32	1.1	6.9
F318V/V412I	1.45	0.2	1.6
F318V/V412S	0.79	0.1	1.0
Second-generation variants (template gene: *V412A/V438P PmaLAAD-00N*)			
F318A/V412A/V438P	0.63	1.0	6.7

[1]These variants were identified in the second round of SSM.

[2] This variant was purified as apoprotein.

Table S3 Apparent kinetic parameters of selected PmaLAAD variants on L-Phe and L-1-Nal.

	L-Phe					L-1-Nal				Specificity constant
	V_{max} (U/mg)	k_{cat} (s^{-1})	K_m (mM)	K_i (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	V_{max} (U/mg)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	$(k_{cat}/K_m)_{L\text{-}1\text{-}Nal}/(k_{cat}/K_m)_{L\text{-}Phe}$
Wild-type	3.00 ± 0.04	2.66 ±0.04	1.60 ± 0.09	-	1.66 ± 0.12	1.18 ± 0.24[1]	1.05 ± 0.21	0.79 ± 0.02[1]	1.30 ± 0.29	0.78 ± 0.25
First-generation, single-site variants (template gene: *PmaLAAD-00N*)										
L279G	0.67 ± 0.17	0.59 ± 0.15	40 ± 19	-	0.01 ± 0.01	BD				-
F318A	1.53 ± 0.1	1.36 ± 0.09	3.76 ± 0.98	-	0.36 ± 0.12	1.15 ± 0.06	1.02 ± 0.05	0.24 ± 0.03	4.3 ± 0.8	11.9 ± 2.4
F318C[1]	1.04 ± 0.07	0.92 ± 0.06	5.7 ± 1.5	-	0.16 ± 0.05	0.65 ± 0.02	0.58 ± 0.02	0.35 ± 0.03	1.7 ± 0.2	10.6 ± 1.6
F318G[1]	1.32 ± 0.23	1.17 ± 0.20	1.87 ± 0.94	-	0.63 ± 0.42	0.98 ± 0.09	0.87 ± 0.08	0.21 ± 0.04	4.1 ± 1.2	6.5 ± 2.5
F318I3[1]	1.44 ± 0.03	1.28 ± 0.03	1.8 ± 0.2	-	0.71 ± 0.09	1.10 ± 0.10	0.96 ± 0.09	0.31 ± 0.06	3.1 ± 0.9	4.4 ± 1.4
F318L	0.94 ± 0.02	0.83 ± 0.02	0.98 ± 0.10	-	0.85 ± 0.11	0.85 ± 0.06	0.75 ± 0.05	0.16 ± 0.03	4.7 ± 1.2	5.5 ±1.52
F318M	1.09 ± 0.02	0.97 ± 0.02	1.05 ± 0.09	-	0.92 ± 0.1	1.10 ± 0.06	0.98 ± 0.05	0.41 ± 0.04	2.4 ± 0.4	2.6 ± 0.5
F318V	0.19 ± 0.01	0.17 ± 0.01	5.95 ± 1.02	-	0.03 ± 0.01	0.13 ± 0.02	0.12 ± 0.02	0.13 ± 0.05	0.9 ± 0.5	30 ± 17
V412A	3.04 ± 0.07	2.7 ± 0.06	2.75 ± 0.26	-	0.98 ± 0.11	1.33 ± 0.17[2]	1.18 ± 0.15	0.61 ± 0.13[2]	1.9 ±0.7	1.9 ± 0.7
V412A-V438P	1.59 ± 0.4	1.41 ± 0.35	7.4 ± 2.6	23.5 ± 7.7	0.19 ± 0.11	1.74 ± 0.29	1.55 ± 0.26	0.52 ± 0.10	3.1 ± 1.1	15.8 ± 6.2
V438G	1.53 ± 0.21	1.36 ± 0.19	3.94 ± 0.99	14.2 ± 3.5	0.35 ± 0.14	0.84 ± 0.08	0.75 ± 0.07	0.5 ± 0.09	1.5 ± 0.4	4.3 ± 1.6
V438P	2.34 ± 0.05	2.08 ± 0.04	1.64 ± 0.08	47.6 ± 2.8	1.27 ± 0.09	1.71±0.23[2]	1.52 ± 0.21	1.28 ± 0.25[2]	1.2 ± 0.4	0.9 ± 0.4
First-generation, double-site variants (template gene: *PmaLAAD-00N*)										
F318V/V412G	1.02 ± 0.1	0.90 ± 0.09	7.2 ± 1.2	24.8 ± 3.8	0.12 ± 0.03	1.51 ± 0.02	1.34 ± 0.02	0.27 ± 0.01	5.0 ± 0.26	41.7 ± 2.4
F318I/V412S	0.56 ± 0.03	0.50 ± 0.03	1.77 ± 0.48	-	0.28 ± 0.09	0.76 ± 0.07	0.67 ± 0.06	0.08 ± 0.02	8.4 ± 2.9	30 ± 10.5
F318C/V412L	0.99 ± 0.02	0.88 ± 0.02	9.9 ± 0.7	-	0.09 ± 0.01	0.58 ± 0.06	0.51 ± 0.05	0.67 ± 0.12	0.8 ± 0.2	8.9 ± 2.6
F318C/V412G	0.17 ± 0.01	0.15 ± 0.01	0.77 ± 0.09	32.9 ± 3.7	0.19 ± 0.03	0.39 ± 0.02	0.35 ± 0.02	0.15 ± 0.02	2.3 ± 0.44	12.1 ± 2.5
F318V/V412S	0.97 ± 0.15	0.86 ± 0.13	13.4 ± 5.4	-	0.06 ± 0.03	1.54 ± 0.3[2]	1.37 ± 0.28	0.58 ± 0.13[2]	2.4 ± 1.04	40 ± 18

F318V/V412I	1.64 ± 0.07	1.45 ± 0.06	7.8 ± 1.1	-	0.19 ± 0.04	BD				
Second-generation variants (template gene: V412A/V438P-*PmaLAAD-00N*)										
F318A/V412A/V438P	0.71 ± 0.02	0.63 ± 0.02	6.0 ± 0.9	-	0.11±0.02	1.77 ± 0.05	1.57 ± 0.04	0.17 ± 0.01	9.2 ± 0.8	83.6 ± 7.3

[1]These variants were identified in the first-generation, double-site, SSM.

[2] Values estimated using the Lineweaver-Burk plot.

Table S4. Primers used for site-saturation mutagenesis carrying the NNK or NDT degenerated codon.

Position	Primers with NNK codon
L279	Forward: 5'-CCAACCTTGAATGTTTAC**NNK**TCACAACAACGTGTATC-3'
	Reverse: 5'-GATACACGTTGTTGTGA**MNN**GTAAACATTCAAGGTTGG-3'
F318	Forward: 5'-CTTATGCTGTAGCCCCACGTATC**NNK**ACAAGCTCCATTG-3'
	Reverse: 5'-CAATGGAGCTTGT**MNN**GATACGTGGGGCTACAGCATAAG-3'
V412	Forward: 5'-GAACGTTGGGGTGCAGTT**NNK**AGTCCAACATTTGATG-3'
	Reverse: 5'-CATCAAATGTTGGACT**MNN**AACTGCACCCCAACGTTC 3'
V438	Forward: 5'-CAATACAGCGACA**NNK**TGGGGAATGACAGAAGGTCC-3'
	Reverse: 5'-GGACCTTCTGTCATTCCCCA**MNN**TGTCGCTGTATTG-3'
W439	Forward: 5'-CAATACAGCGACAGTG**NNK**GGAATGACAGAAGGTCC-3'
	Reverse: 5'-GGACCTTCTGTCATTCC**MNN**CACTGTCGCTGTATTG-3'
Position	**Primers with NDT codon**
F318	Forward: 5'-CTTATGCTGTAGCCCCACGTATC**NDT**ACAAGCTCCATTG-3'
	Reverse: 5 -CAATGGAGCTTGT**AHN**GATACGTGGGGCTACAGCATAAG-3'
V412	Forward: 5'-GAACGTTGGGGTGCAGTT**NDT**AGTCCAACATTTGATG-3'
	Revese : 5'-CATCAAATGTTGGACT**AHN**AACTGCACCCCAACGTTC-3'
S99/Q100	Forward: 5'-GAGCAATCAGGCCGCGCATACNDT**NDT**ATCATTAGCTACCAAACGTC-3'
	Reverse: 5'-GACGTTTGGTAGCTAATGATAHN**AHN**GTATGCGCGGCCTGATTGCTC-3'

Figure S1

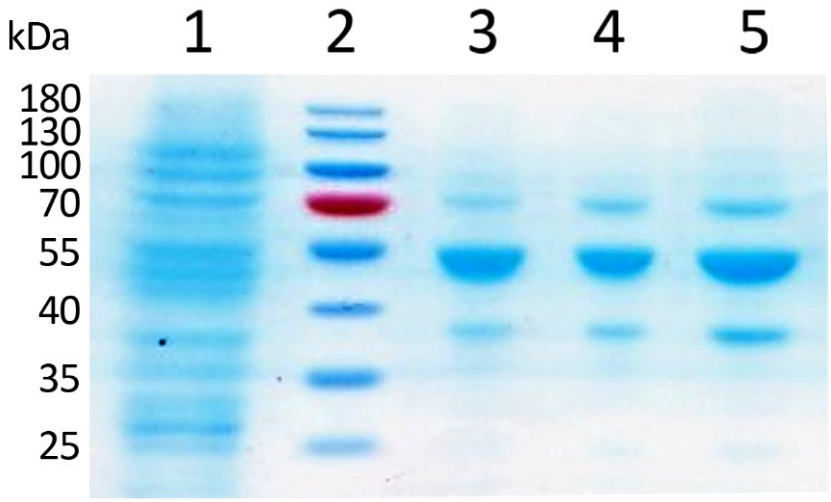

Figure S1 SDS-PAGE analysis of selected purified PmaLAAD variants. Lane 1: crude extract (80 µg); lane 2: LMW markers (Prestained Protein Ladder, PageRuler™); lane 3: wild-type PmaLAAD; lane 4: V412A/V438P variant; lane 5: F318A/V412A/V438P variant. For each lane 5 µg of total proteins were loaded.

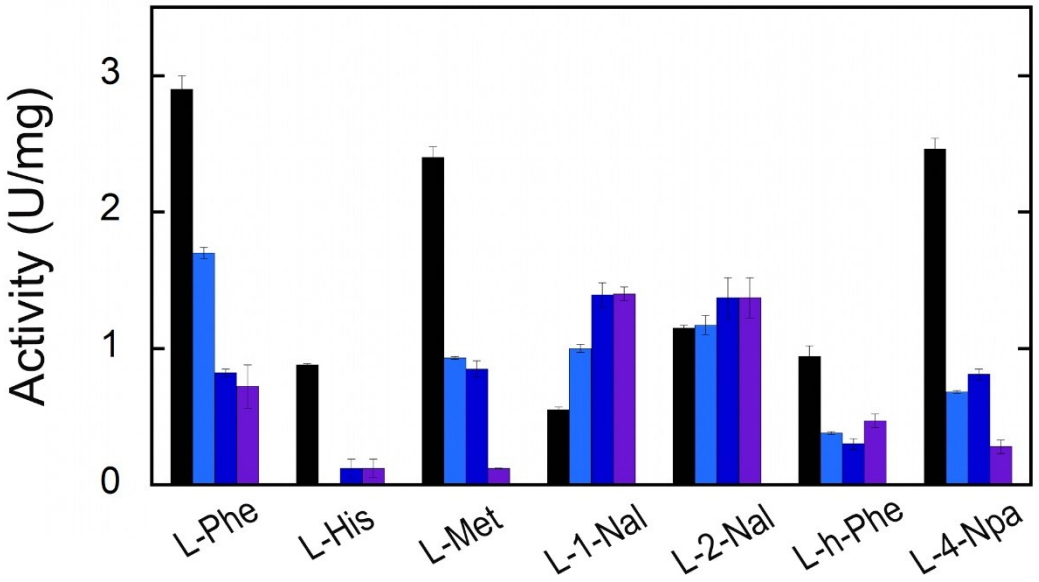

Figure S2

Figure S2 Substrate scope (reported as specific activity) of wild-type (black), F318A (cyan), V412A/V438P (blue), and F318A/V412A/V438P (purple) PmaLAAD variants. The enzymatic activity was measured using the polarographic assay. The enzymatic activity was determined on different natural and synthetic L-amino acids: 50 mM L-Phe, 50 mM L-His, 50 mM L-Met, 1.2 mM D,L-1-naphthylalanine (D,L-1-Nal), 1.2 mM D,L-2-naphthylalanine (D,L-2-Nal), 0.7 mM D,L-1-naphthylglycine (D,L-1-NGly), 0.7 mM D,L-2-naphthylglycine (D,L-2-NGly), 5 mM D,L-homophenylalanine (D,L-h-Phe), and 10 mM L-4-nitrophenylalanine (L-4-Npa) under standard conditions (50 mM potassium phosphate, pH 7.5, 25 °C and at air saturation).

Figure S3

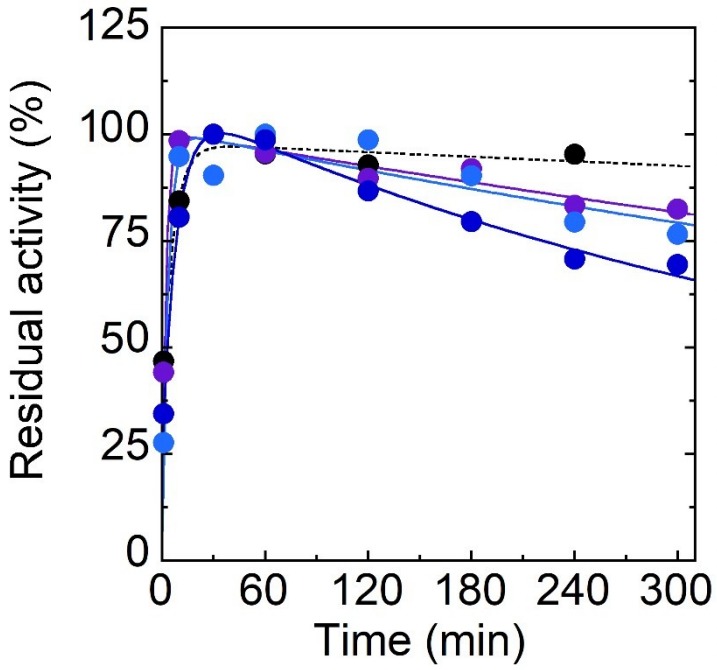

Figure S3 Time course of enzymatic activity of wild-type PmaLAAD and selected variants at 25 °C in presence of *E. coli* membranes added before starting the experiment. Wild-type PmaLAAD, k_{fast}= 9.09 ± 1.65 min^{-1}, k_{slow}= 0.018 ± 0.004 (black); F318A, k_{fast}= 17.3 ± 9.8, k_{slow}=0.06±0.05 min^{-1} (cyan); V412A/V438P, k_{fast}= 7.0 ± 0.7 min^{-1}, k_{slow}=0.14 ± 0.01 min^{-1} (blue); V412A/V438P/F318A, k_{fast}= 25.1 ± 16.3 min^{-1}, k_{slow}=0.04 ± 0.02 min^{-1} (purple). We defined k_{fast} as the rate of PmaLAAD re-activation following membrane addition and k_{slow} as the rate of PmaLAAD inactivation during the subsequent incubation at 25 °C.

Figure S4

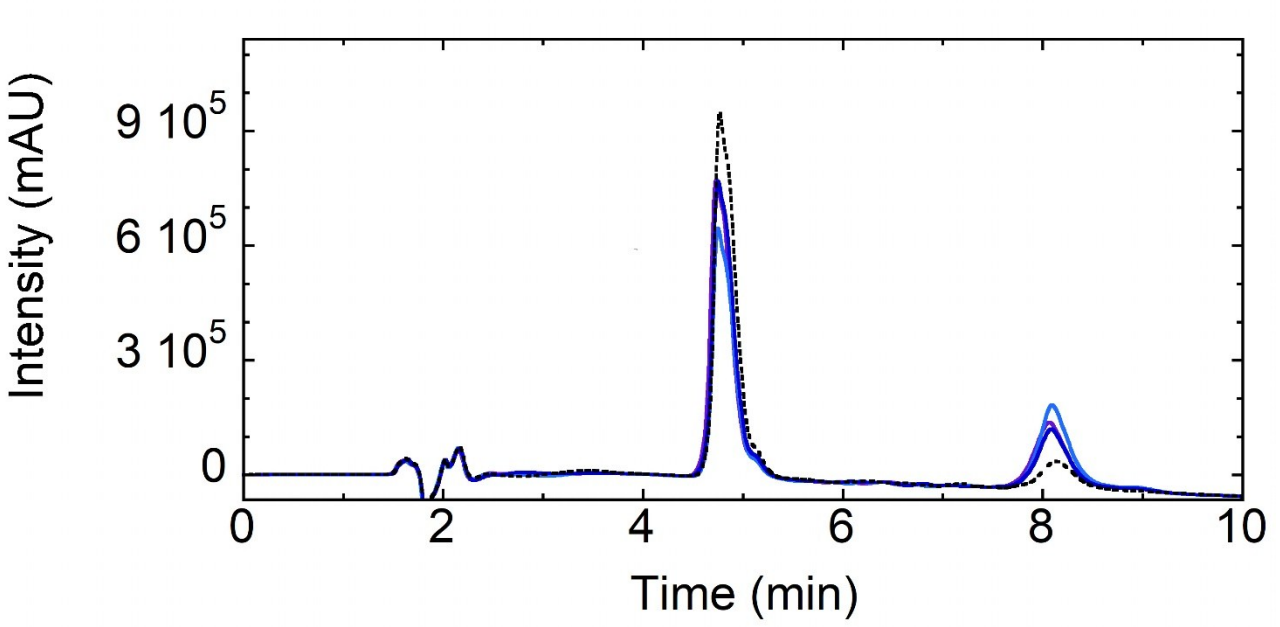

Figure S4. HPLC chromatograms after 10 min of bioconversion of D,L-1-Nal (retention time = 4.7 min) to 1-naphthylpyruvate (retention time = 8.1 min) catalyzed by wild-type PmaLAAD (dashed line) or the F318A (cyan), V412A/V438P (blue), and F318A/V412A/V438P (purple). Conditions: 1.2 mM substrate, 0.05 mg/mL PmaLAAD-00N or different PmaLAAD variants, *E. coli* membranes, pH 7.5, 25 °C (1.0 mL).

Optimization of F318A/V412A/V438P PmaLAAD expression

Optimization of expression conditions

Starter cultures were prepared by growing a single colony of *E. coli* BL21(DE3) cells carrying the recombinant pET11a-PmaLAAD-00N or pET11a-PmaLAAD-F318A/V412A/V438P plasmid overnight at 37 °C in flasks containing TB broth to which 100 μg/mL ampicillin was added. These cultures were diluted with the same media to a starting OD_{600nm} of 0.1 and then incubated at 37 °C on a rotatory shaker at 200 rpm. Experiments were carried out in 500 mL baffled Erlenmeyer flasks containing 125 mL of liquid media at 37 °C and 200 rpm. Both the optical density and the pH value of the medium were assayed every hour. Growth curves were generated by the interpolation of OD_{600nm} values according to the Gompertz equation (Zwietering et al., 1990).

In order to optimize F318A/V412A/V438P PmaLAAD production in *E. coli* BL21(DE3) cells, the effect of adding 0.1 mM IPTG at different phases of the growth curve (corresponding to OD_{600} of 0.5, 2, 5), of the temperature of growth (28 or 15 °C), and of the time of cell harvest (4 h or overnight after IPTG addition) on protein expression was investigated. The LAAD enzymatic activity of crude extracts (50 μL) was determined by using the polarographic assay (see below) on 1.2 mM D,L-1-Nal. Since the F318A/V412A/V438P PmaLAAD showed the most interesting enzymatic properties, the expression conditions of this recombinant enzyme in BL21(DE3) *E. coli* cells were optimized using a *semi*-factorial design approach. The growth curve of the variant was almost superimposable to the one of the PmaLAAD-00N wild-type with almost identical Gompertz equation parameters (Figure S5a). The temperature, induction growth phase, and the interval between IPTG addition and cell harvesting were evaluated. The best expression condition was: adding 0.1 mM IPTG at $OD_{600nm} = 2.0$ (corresponding to a mid-exponential growth phase) and collecting cells after overnight growth at 28 °C (condition 4). Under these conditions, a 4.5-fold higher volumetric yield (235 U/L) was reached than the one obtained under standard conditions (i.e., induction at $OD_{600nm} = 0.5$ and collecting cells

after 4 hours at 28 °C). This result is due to a 2-fold increase in enzyme activity value per gram of cells and of the biomass accumulation (Table S5 and Figure S5b).

M.H. Zwietering, I. Jongenburger, F.M. Rombouts, a nd K. Van't Riet, *App. Env. Microbiol.*, 1990, **56**, 1875-1881.

Table S5

Conditions tested for the recombinant expression of F318A/V412A/V438P PmaLAAD variant.

Conditions	g of cells
1; $OD_{600\ nm}$ = 0.5, 28 °C, harvest time = 4 h	1.15
2; $OD_{600\ nm}$ = 0.5, 28 °C, harvest time = overnight	2.52
3; $OD_{600\ nm}$ = 2, 28 °C, harvest time = 4 h	2.04
4; $OD_{600\ nm}$ = 2, 28 °C, harvest time = overnight	2.18
5; $OD_{600\ nm}$= 5, 28 °C, harvest time = 4 h	2.26
6; $OD_{600\ nm}$ = 5, 28 °C, harvest time = overnight	2.73
7; $OD_{600\ nm}$ = 0.5, 15°C, harvest time = 4 h	0.41
8; $OD_{600\ nm}$ = 0.5, 15 °C, harvest time = overnight	2.06
9; $OD_{600\ nm}$ = 2, 15 °C, harvest time = 4 h	1.09
10; $OD_{600\ nm}$ = 2, 28 °C, harvest time = overnight	2.32
11; $OD_{600\ nm}$= 5, 15 °C, harvest time = 4 h	1.99
12; $OD_{600\ nm}$ = 5, 15 °C, harvest time = overnight	2.66

Experiments were carried out in 500 mL baffled Erlenmeyer flasks containing 125 mL of TB broth.

Figure S5

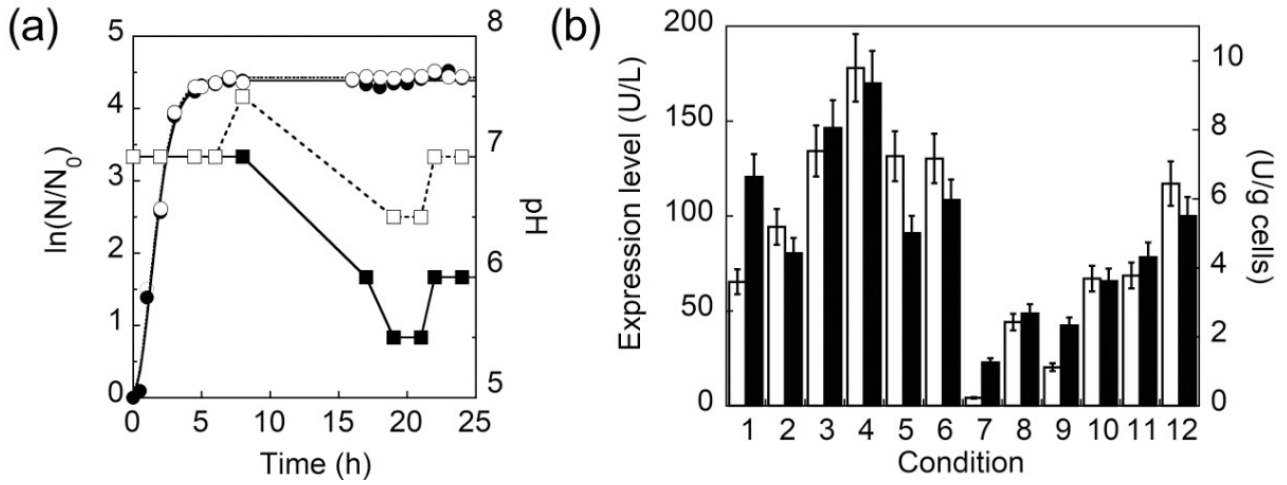

Figure S5: (a) Growth curve of BL21(DE3) *E. coli* cells carrying the pET11b-PmaLAAD-00N (ஜ) or the pET11b-PmaLAAD-F318A/V412A/V438P (ಞ) plasmid in Terrific broth. pH of the growing medium (PmaLAAD-00N, ❍; F318A/V412A/V438P variant, ❍). (b) Expression yields of F318A/V412A/V438P PmaLAAD under different conditions (conditions reported in Table S5). The activity values were determined under standard conditions on 1.2 mM D,L-1-Nal as a substrate (i.e. in the presence of *E. coli* membranes at pH 7.5, 25 °C).