Protein Engineering

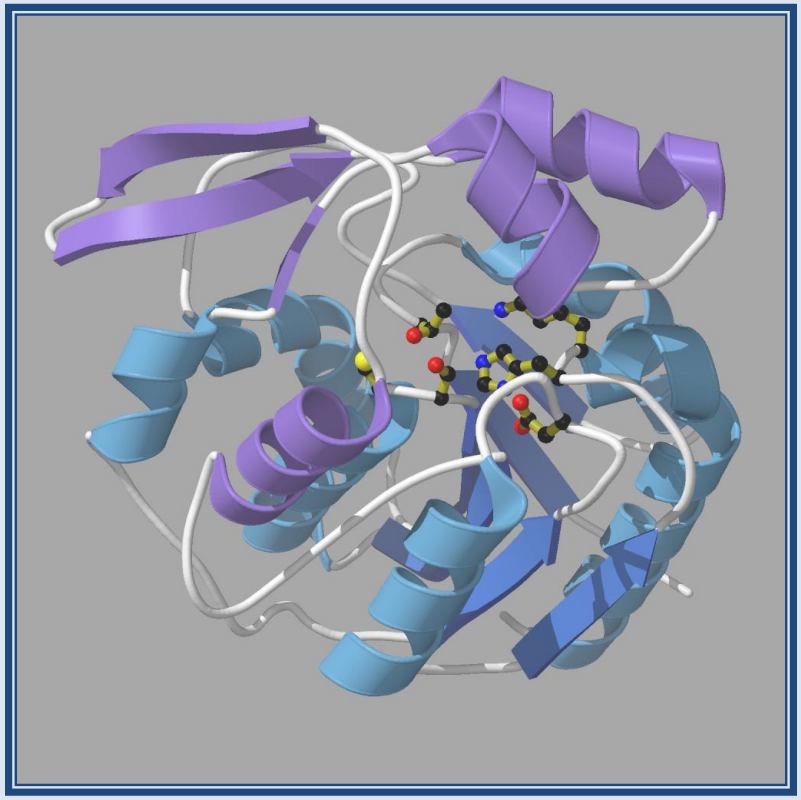
Enzyme Engineering

Examples

Engineering of *Hb*-Hnl

Rational Design

Structure



Example for Engineering of Substrate Specificity by Rational Design

Hydroxynitrile lyases

(S)-Hnl of *Hevea brasiliensis*

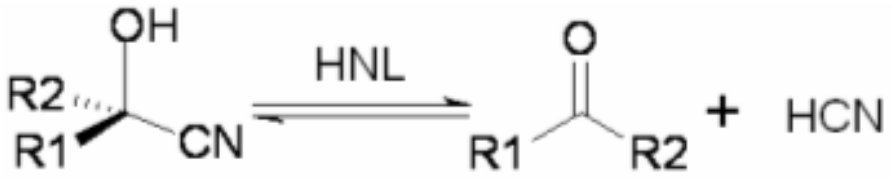
*Hb*_Hnl

- Type II Hnl
- intracellular protein
- 29.2 kDa
- Homology to esterases
- α/β hydrolase fold protein
- catalytic triad
- (S)-selective

(R)-Hnl of *Prunus amygdalus*

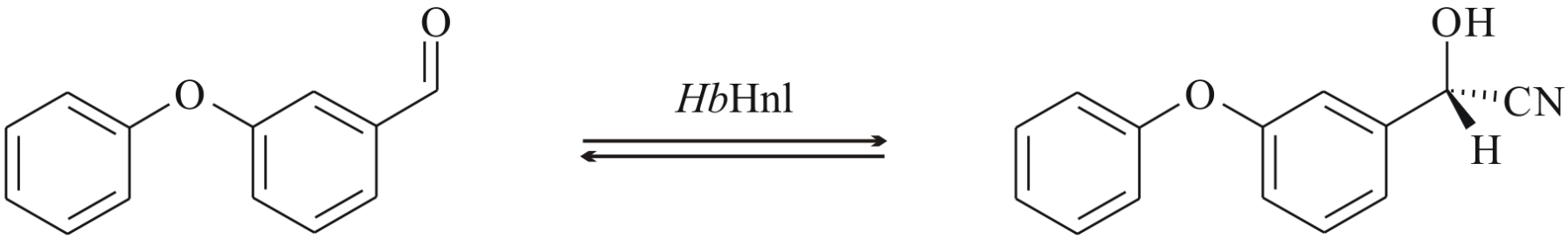
*Pa*_Hnl

- Type I Hnl
- secretory protein
- 61 kDa (57.9 kDa)
- Homology to oxidases
- FAD
- N-glycosylated
- isoenzymes
- (R)-selective



Application of HNL technology on a large industrial scale

(several hundred tons/y):



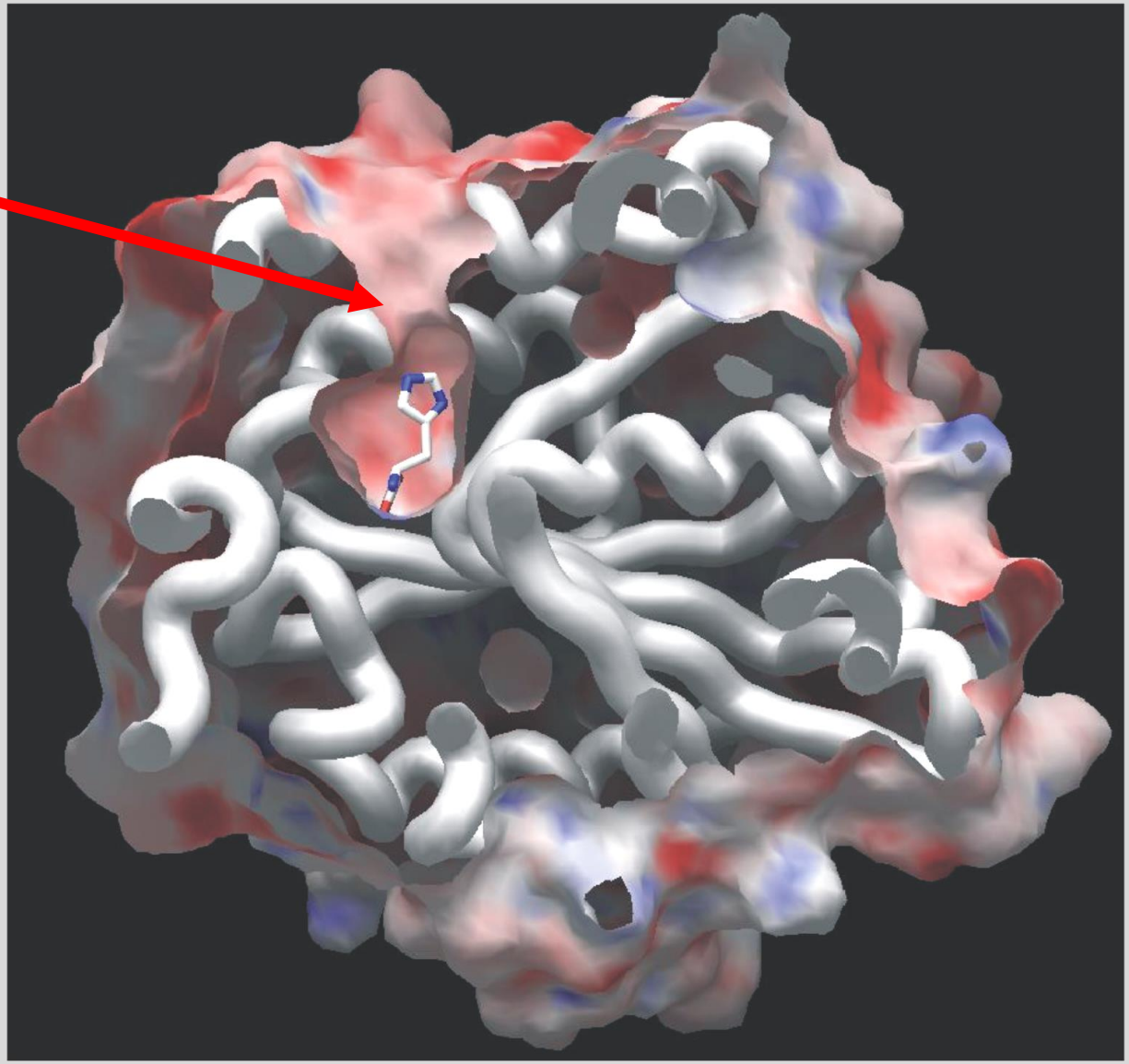
(*S*)-m-phenoxybenzaldehyde cyanohydrin

→ Intermediate in the production of synthetic pyrethroids

→ Biocatalytic production employing *S*-selective *Hb*_HNL provides product in **enantiopure quality**

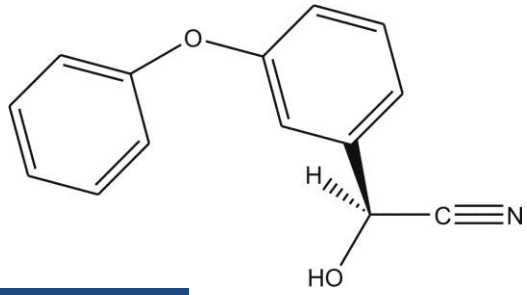
*Hb*_Hnl – tunnel to active site

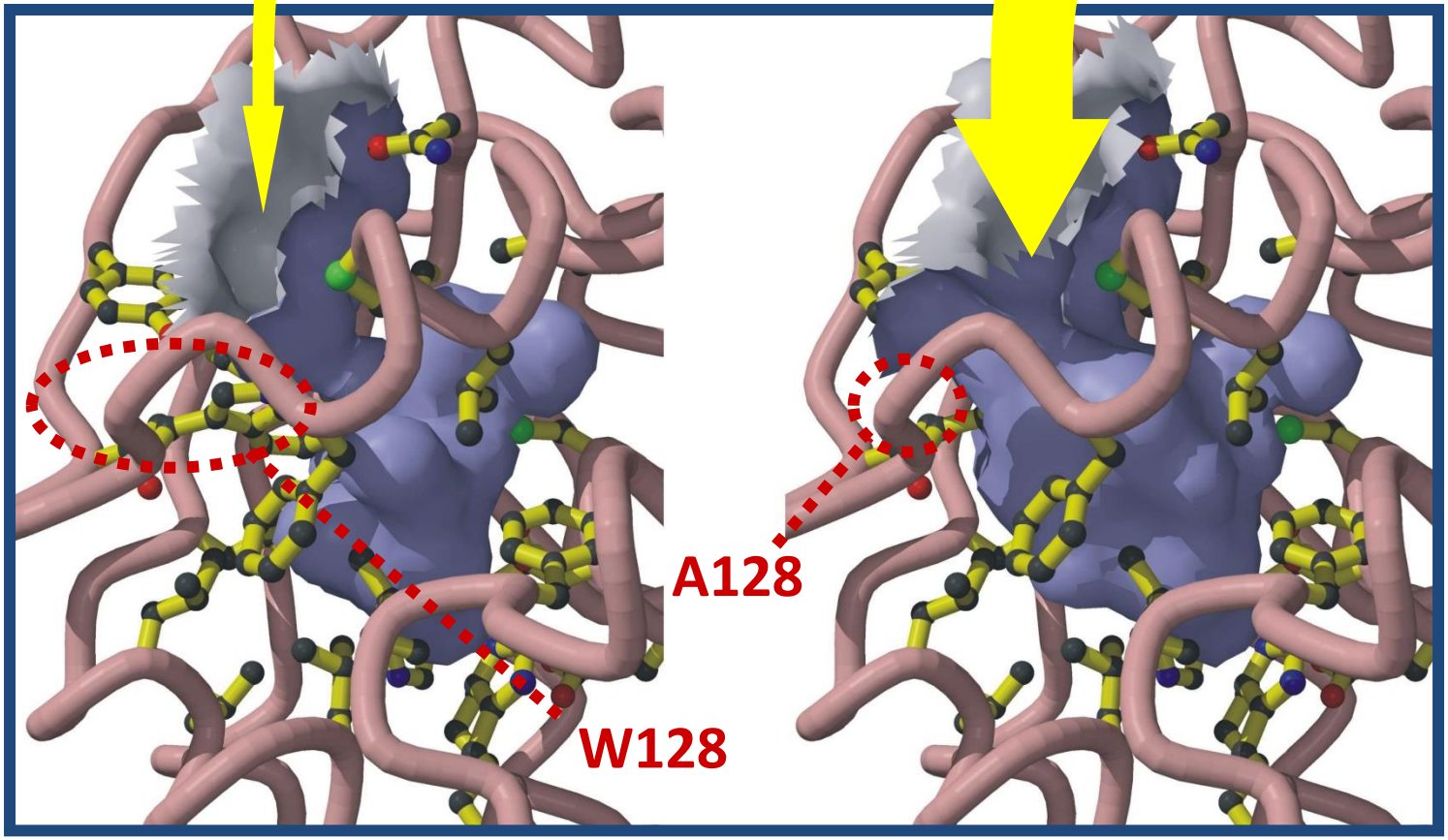
Tunnel to active site



*Hb*_HNL tunnel mutant

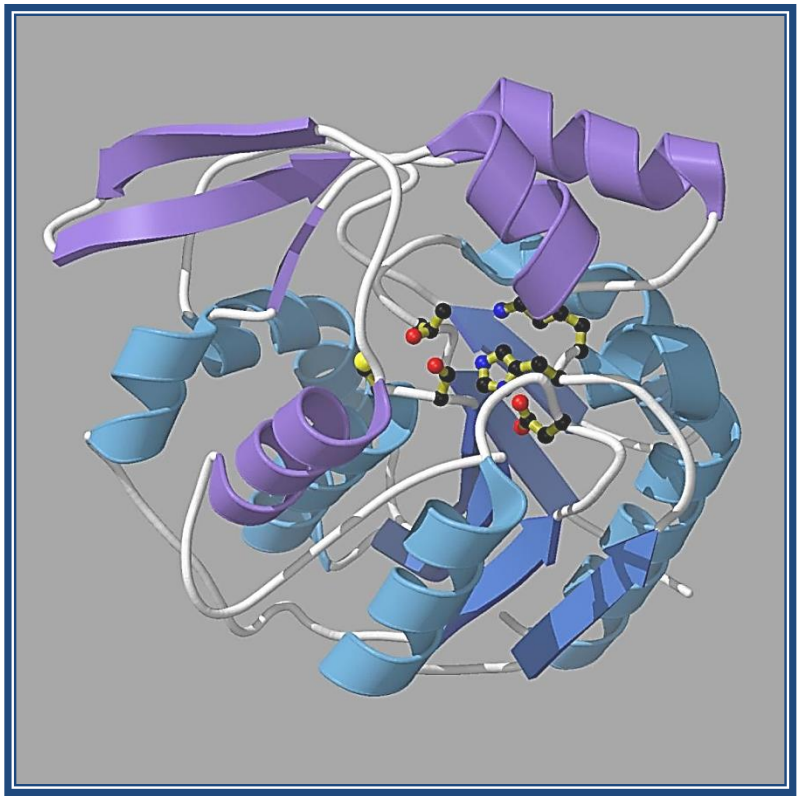
wild-type





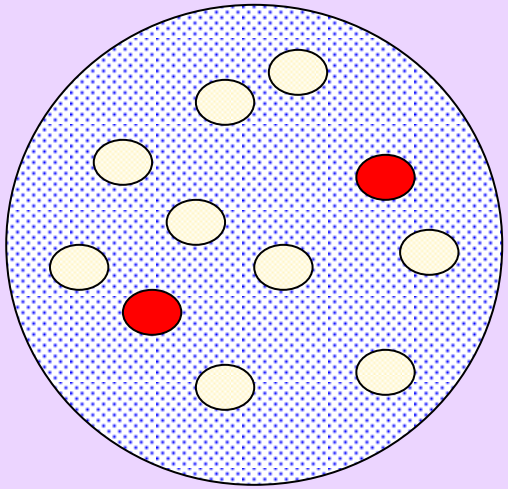
W128A

Engineering of *Hb*-Hnl



Directed Evolution

Screening-Assay (colony level)



Any substrate possible
10^3 colonies per filter
Clear signal-no background
Time as measure for activity

Directed Evolution of *Hb*_Hnl

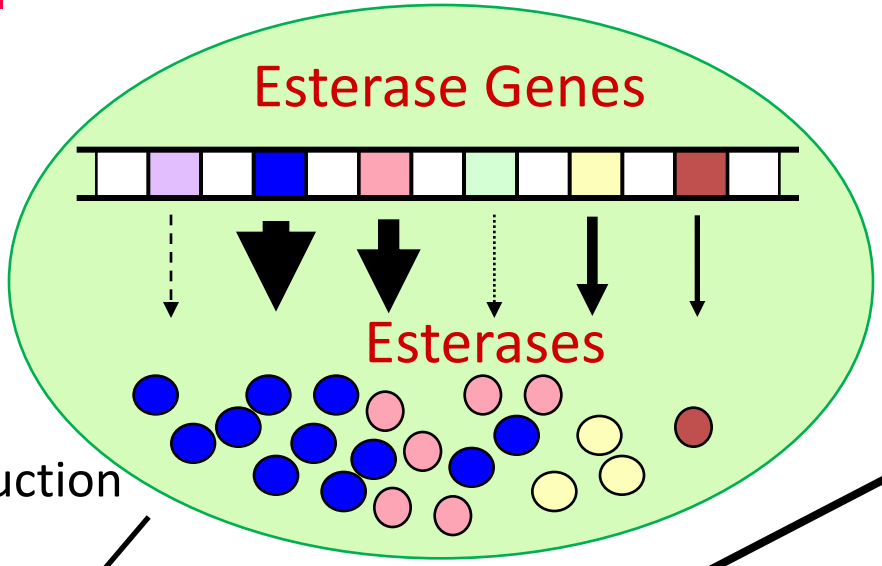
Substrate acceptance for m-Phenoxybenzaldehyde cyanhydrine

Mutant Library	Mutagenesis conditions	Hits selected/ top / parental
Hb-Hnl-wt	B	0
Hb-Hnl-wt	C	1 / **1** (Evo9) / 0
Hb-Hnl-W128F	C	3 / **2** / 0
Hb-Hnl-W128A	C	13 / **2** / 3
Hb-Hnl-Evo	B	5 * / **1** / 2

Evo9: Mutation W128A

*** 1 Clone Mutation F128C**

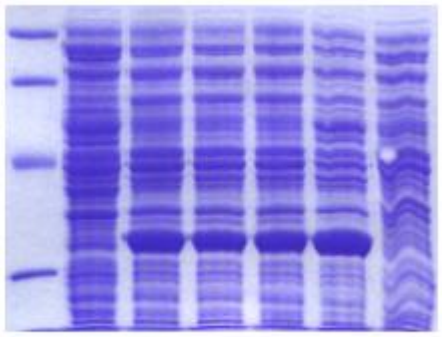
Wild type organism



Induction

Screening

Expression

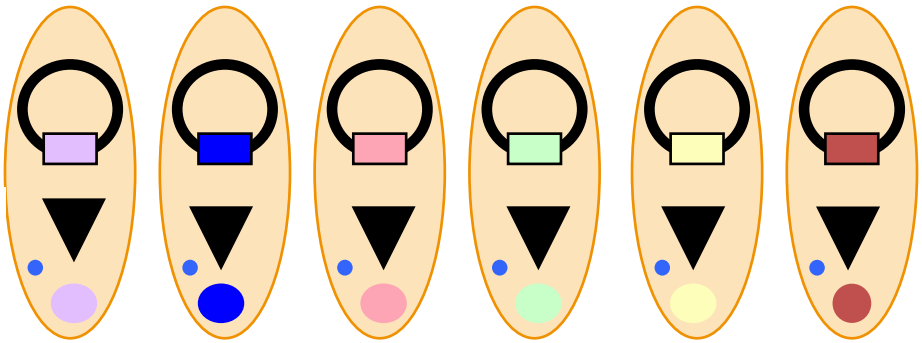


New Esterases

Genome screening

Cloned Esterase Genes

heterologous host (*E.coli*)



Screening

Novel bacterial Esterases

```
Xv_EstD    GGDPTRVAVMGHSAGAHIAGLLVTDRRWLQAQG
Rrh_EstA   GGDPTRIVLAGDSAGGNLAASVAIAARDGGGPA
Rru_EstA   AAADAPLIVGGDSAGGNLAAVVAQRAVRENGPE
Rrh_EstC   GGDPDRVTIAGESAGAMSVVSLLAMPAARGLFR
Xv_EstA    GIDAQRVGVMGFSAGGHVAASLGTRYAAQVYPA
Xv_EstB    GGDAGNVTVFGQSGGGAKIATLMAMPAARGLFH
```

GxSxG Family

```
Bs_EstA    LHPDRPFVLFGHSMGGMVAFRLAQKLEREGIYP
Bg_EstC    ALGHPRVVLVGHSMGGVAITAAAERAPERIAAL
Bg_EstD    TLGLEKPLLVGHSLGGAIALAVGLDHPDSVSRI
Bg_EstE    QLGAGPVHLVGHSRGGCVAFYMAHRYPELVRSL
```

```
Rrh_EstB   FGIERLALVTGGSMGAQQTYEWAVRFPDKVLRA
Rrh_EstD   ECPLTTYVLTGFSQGAVIVGDVAAQIGAGNGPV
```

```
Xv_EstE    DSAFDQTVFFGDSLTDSGYYNPLLPAASRAVTG
Bg_EstA    AGVQKQIVSFGDSLSDAGTYSPQILLGFGGGRF
Xv_EstC    PLAASKIVLVGDSTTAVHGGWGPSFCAQHVTSF
```

GDSL Family

```
Bg_EstB    RPMREDTLFRLASVTKPIVALAVLRLVARGELA
```

SxxK Family

Esterase Platform

Established **Set of Key Enzymes**:

Basic families: GXSXG SXXK GDSL

1 Esterase from *Bacillus subtilis*
4 Esterases from *Burkholderia gladioli*
4 Esterases from *Xanthomonas vesicatoria*
5 Esterases from *Rhodococcus spp*.
5 Esterases from plant endophytic bacteria

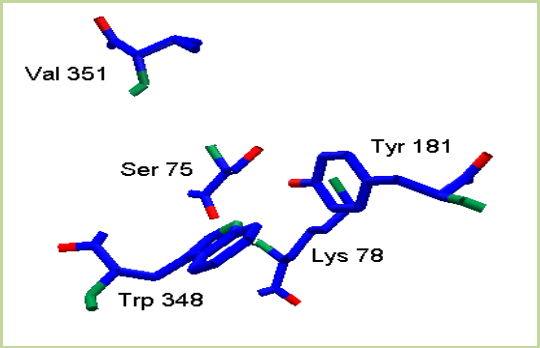
Libraries

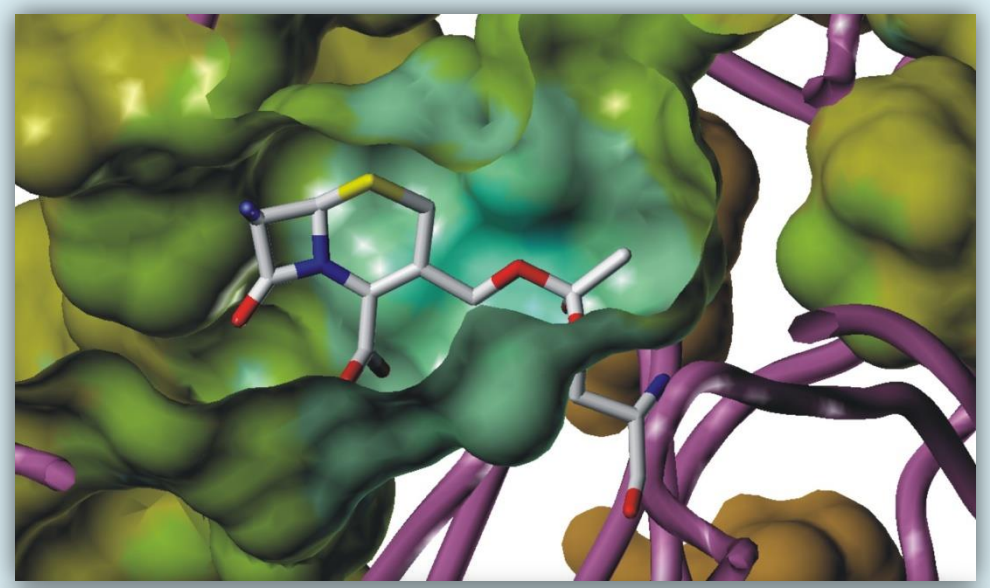
~100 isolated primary esterase active clones
~ 50 Gene bacterial gene libraries
~ Libraries of Metagenomic DNA (> 10^6)

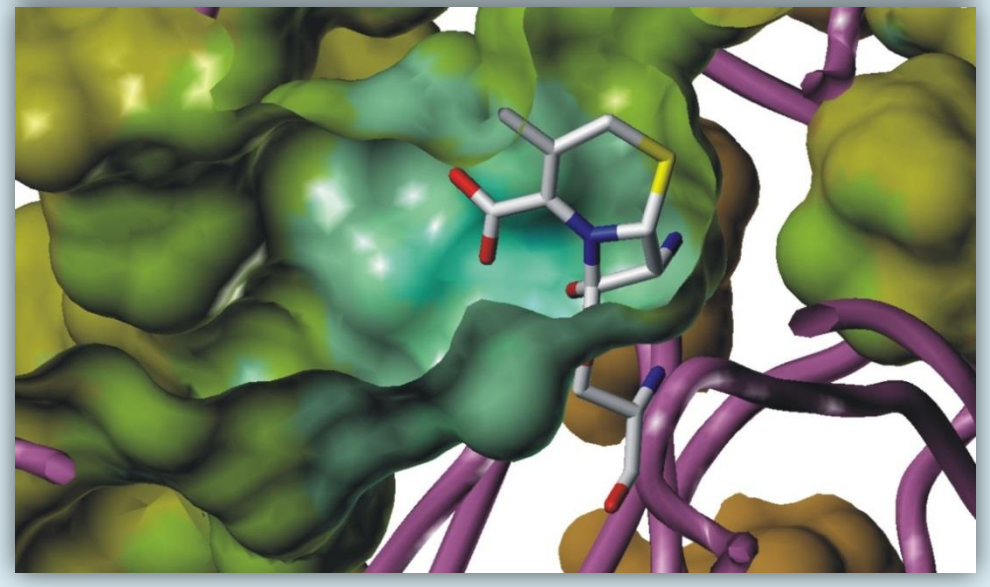
Esterase *Bg*_EstB Designed Evolution

Natural Substrate
Cephalosporin ???

Active Site



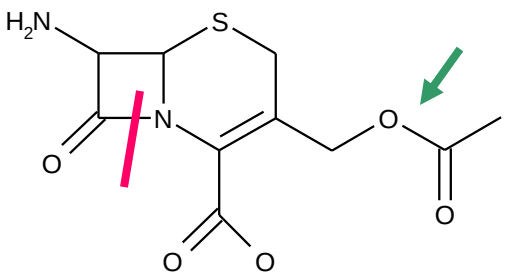




Esterase *Bg*_EstB

Enzyme	ß-Lactamase Activity (U/mg) - Substrates					
	Penicillin G	Penicillin V	Nitrocephin	Cephalotin	Cephaloridine	Cephalosporin C
EstB	0.02	< 0.01	< 0.1	< 0.01	< 0.01	0.01
Bla	0.28	1.0	21.4	1.7	0.6	2.9
RNaseA	<0.01	0.02	n.d.	0.03	<0.01	0.01

Enzyme	Esterase Activity (U/mg) - Substrate			
	Cephalotin	Cephaloridine	Cephalosporin C	7-ACA
EstB	67.5	<0.01	75	70.5



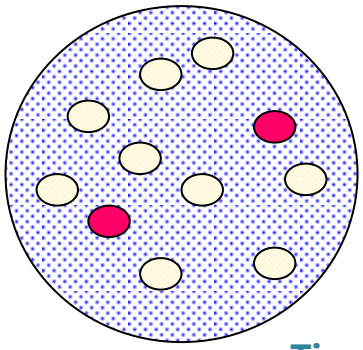
Burkholderia gladioli Esterase EstB

Directed Evolution: stability in vinyl acetate

Directed Evolution

Error-Prone PCR
Mutation rate: 0.5%
5.10^4 Clones screened

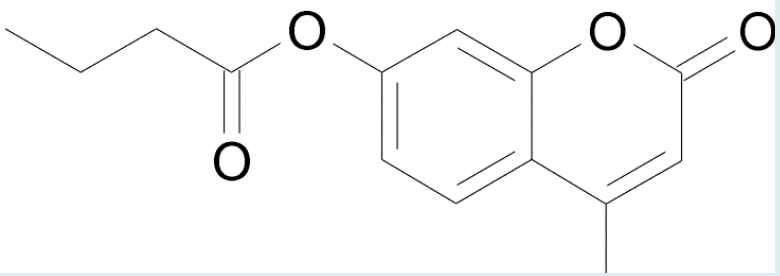
Screening-Assay at Colony-Level



Substrate:
4-Methylumbelli-
feron-Butyrat
pH-Indikator
Organic solvent(VA)
10^3 Colonies per filter
Time as indicator for Activity

Primary sceening: 100 Clones isolated
3 best clones analysed
1 Clone with significant enhanced stability

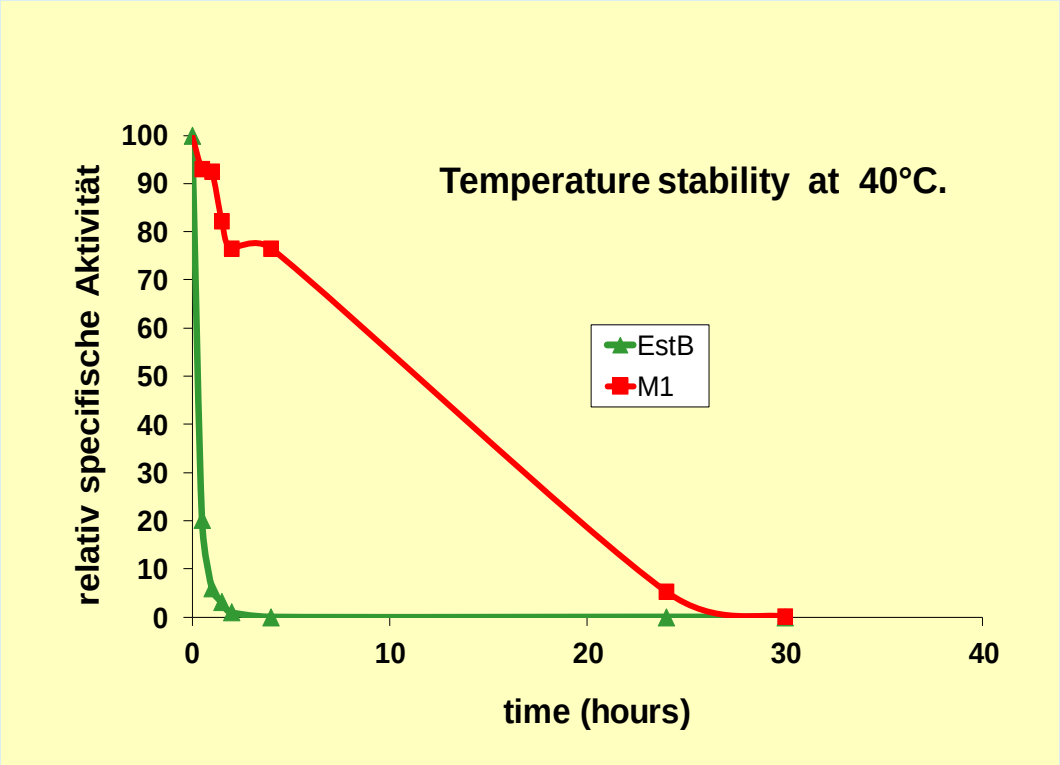
4-methylumbelliferon-butyrate



Surrogate substrate

Burkholderia gladioli Esterase EstB

Stability in Vinyl acetate - Mutation W348G



Substrate: Cephalosporin C
kinetic data

	Vmax	Km
	µmol/min/mg	mM
EstB	79	1,3
M1	19	0,8

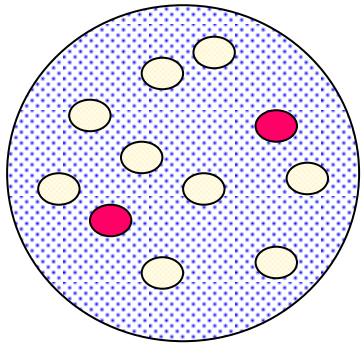
→ Much lower activity on Cephalosporin

Esterase *Bg*_EstB: Engineering of Stability

Directed Evolution

Error-Prone PCR
Mutation rate: 0.5%
10^6 Clones screened

Screening-Assay at Colony-Level



Substrate: Cephalosporin

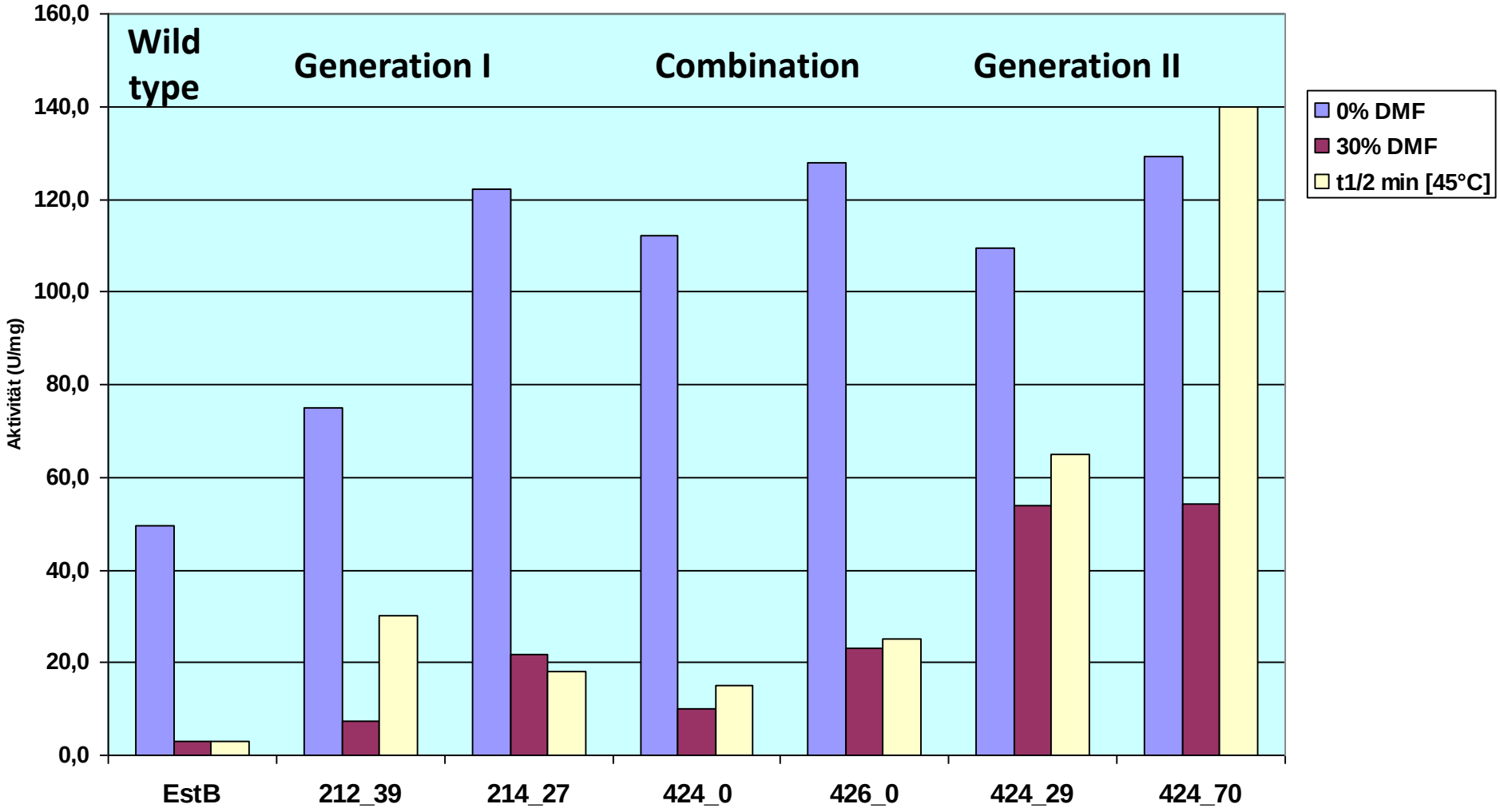
pH-Indicator
Organic solvent (36% DMF)
10^3 Colonies per filter
Time as measure for activity

25 Clones isolated and sequenced
19 different clones identified
11 Clones with different aminoacid sequence

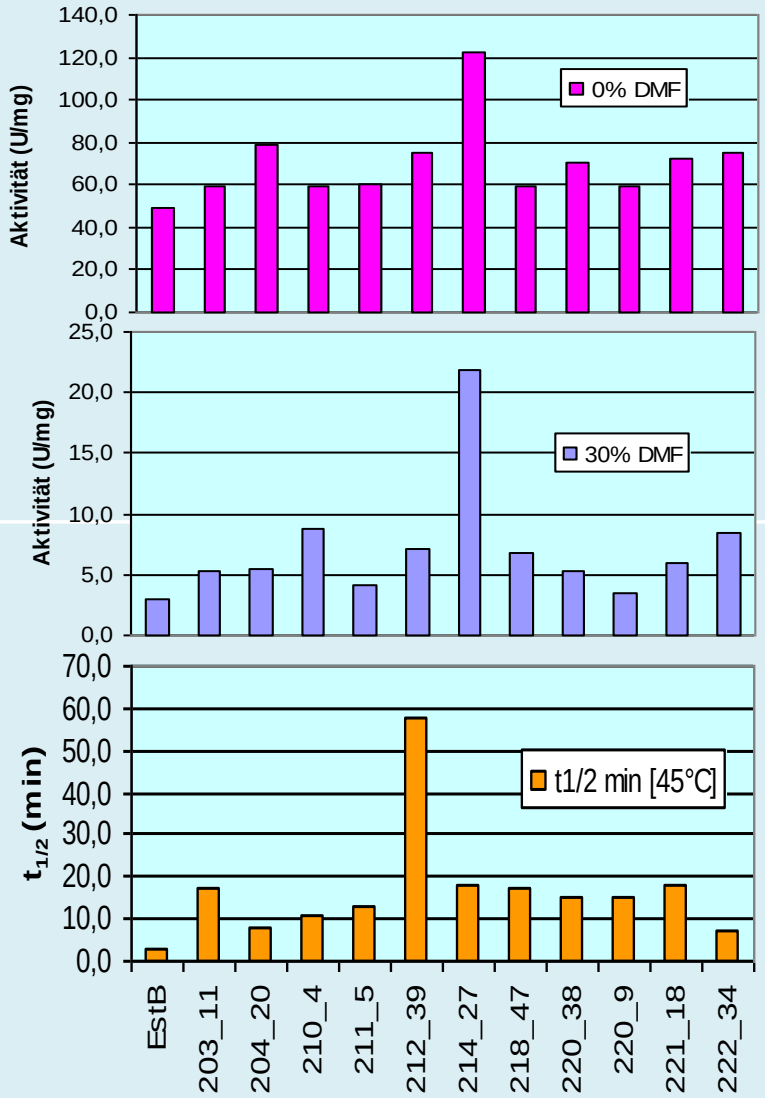
Esterase *Bg*_EstB
Mutants with enhanced Stability (DMF)

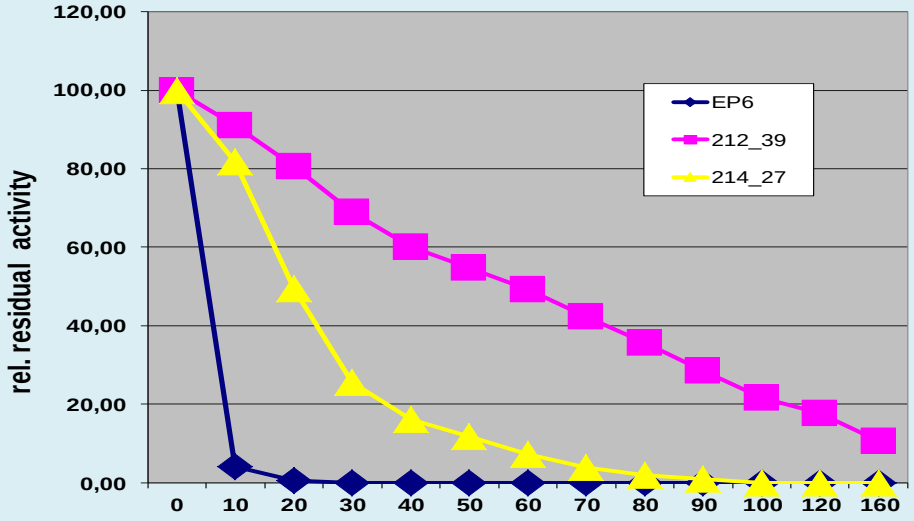
Nr.	Mutations in amino acid sequence																
Generation I																	
203_11														A311V			
204_20							L111Q			W134R				A311V			
210_4		P8L							G132S		R155C						
211_50				E93G										A311V			
212_39			S17L								R155C			A311V			
214_27			S17L						G132S				E251G		A311V		E313K
218_47	I2T													A311V	V313A		
220_38						A95P								A311V			
220_9													T301M	A311V			
221_18												E246V			A311V		
222_34								S115G		W134R					A311V		
Combination																	
424_0		P8L							G132S		R155C		E251G		A311V		E313K
426_0			S17L						G132S		R155C		E251G		A311V		E313K
Generation II																	
426_29		P8L			T77S				G132S	W134R	R155C		E251G		A311V		E313K
426_70		P8L							G132S	W134R	R155C		E251G		A311V		E313K

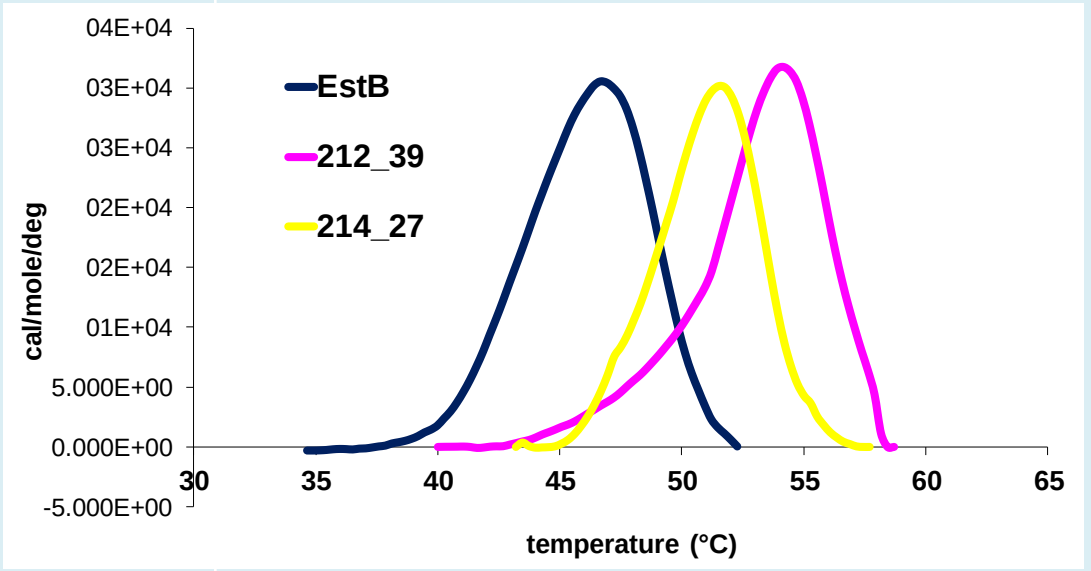
Esterase *Bg*_EstB
Mutants with enhanced Stability (DMF)

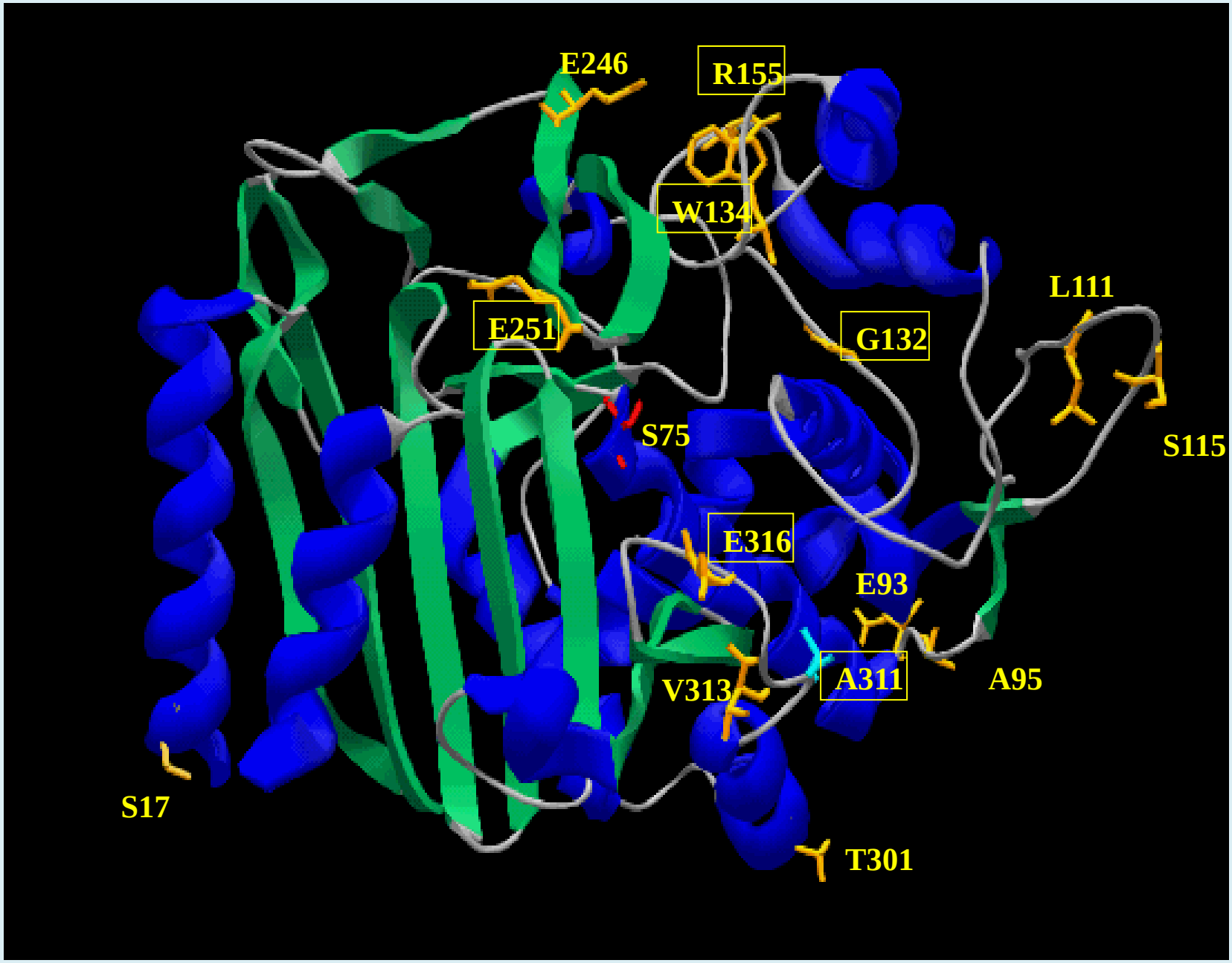


Esterase *Bg*_EstB
Mutants with erhöhter Stabilität (DMF)

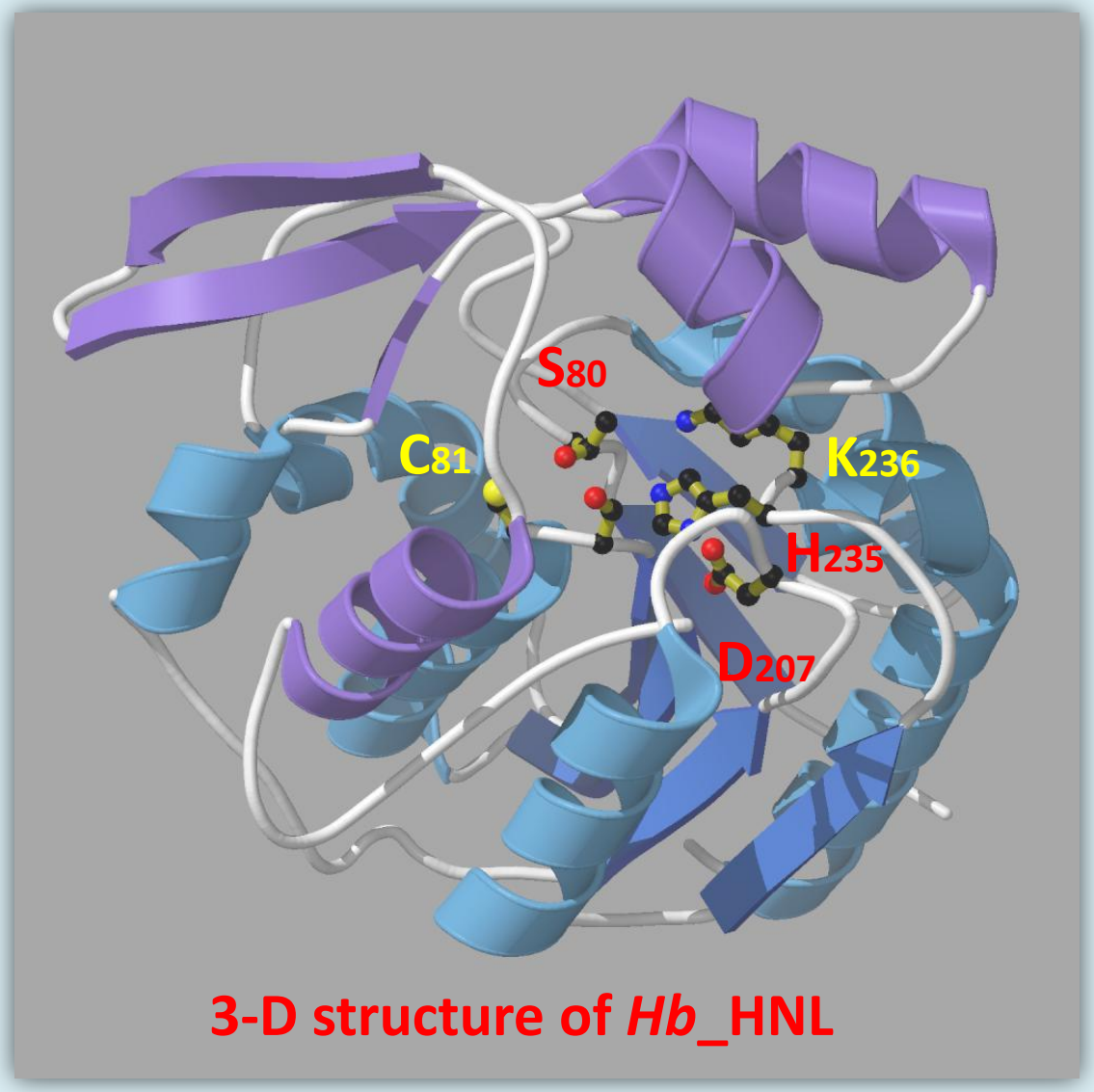







E246
R155
W134
L111
E251
G132
S75
S115
E316
E93
A311
A95
V313
S17
T301

Directed Evolution - Engineering



3-D structure of *Hb*_HNL

Example for Family shuffling by *in vivo* Recombination

Hevea brasiliensis
Hb_Hnl

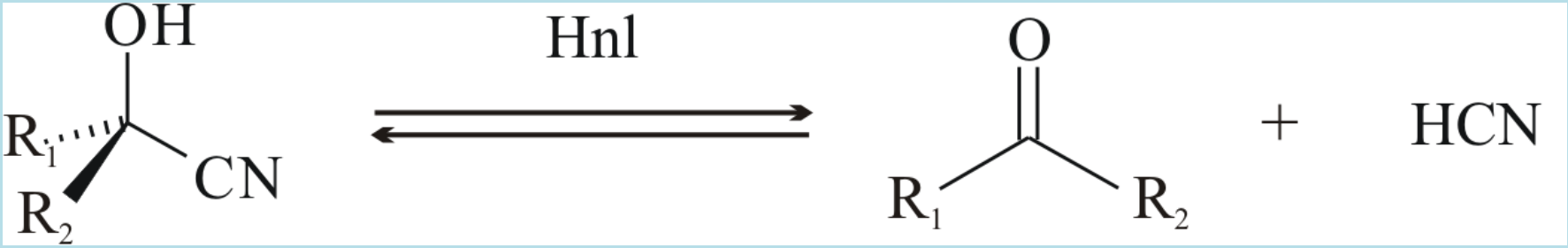
Manihot esculenta
*Me*_Hnl

High homology at sequence
and structure levels

77% identity at the amino acid level

Hydroxynitrile lyases: chiral cyanohydrin synthesis

Improvement of Stability and Activity at low pH



S-selective: *Hevea brasiliensis, Manihot esculenta*

Problems:

higher pH (6-7) → **good enzyme activity**
→ **high chemical background reaction, low selectivity**

lower pH (< 6) → **lower enzyme activity, lower enzyme stability**
→ **low chemical background reaction, high selectivity**

Recombination of MeHNL and HbHNL-W128A

Hb_Hnl W128A: good reactivity with mPBA, low stability at low pH

Me_Hnl wt: better stability at low pH

Aims:

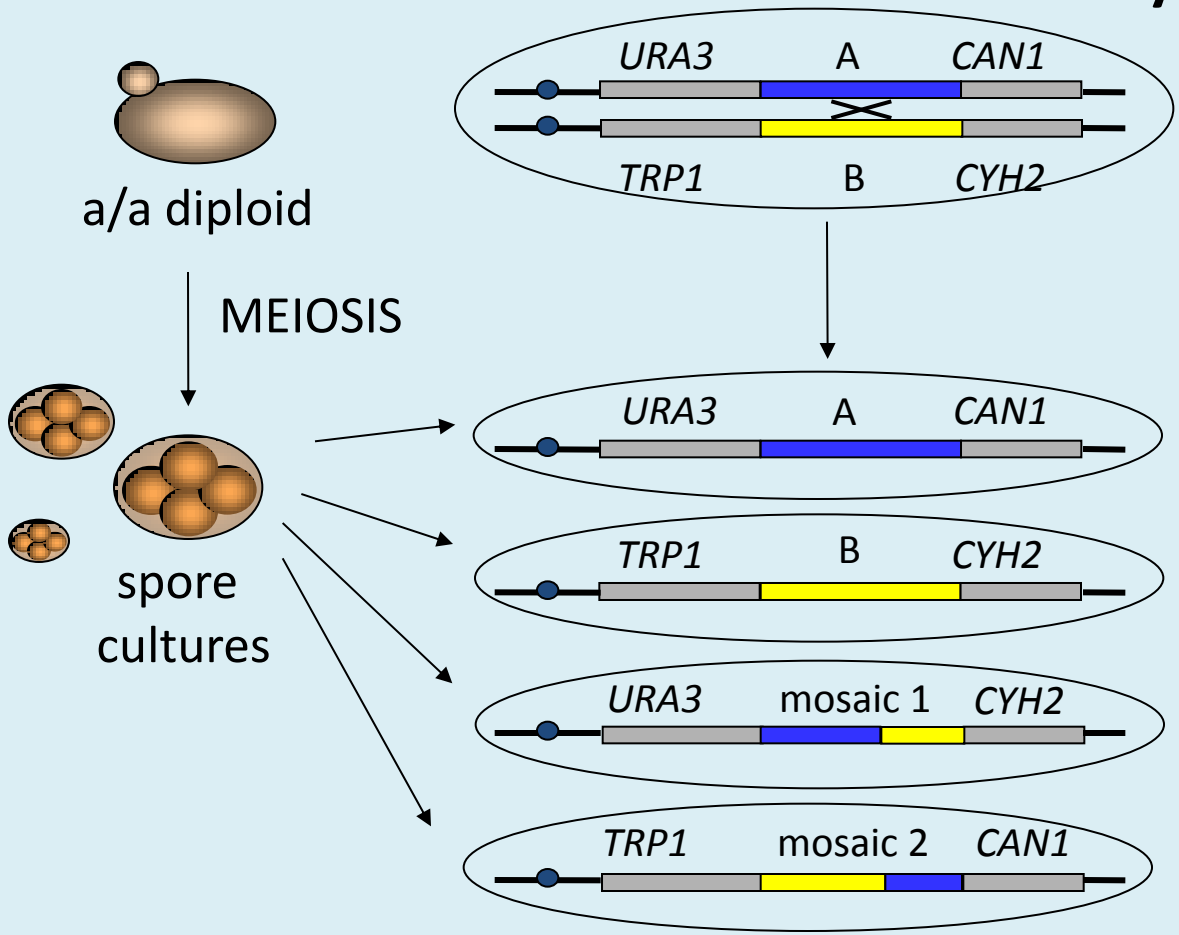
- Improved stability and activity at low pH
- High activity with bulky substrates

→ **Recombine features of both enzymes**

Enzyme Engineering → Recombination

Saccharomyces cerevisiae

yeast shuffling strategy



Meiosis :
high levels of genome-wide recombination

MSH2 **: key player in homeologous recombination, mismatch repair and mutagenesis**

Enzyme Engineering →Recombination

Recombination of MeHNL and HbHNL-W128A

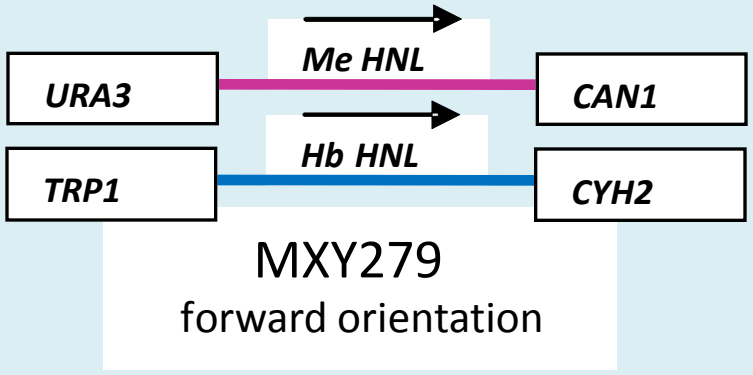
Hb_Hnl W128A: good reactivity with mPBA, low stability at low pH

Me_Hnl wt: better stability at low pH

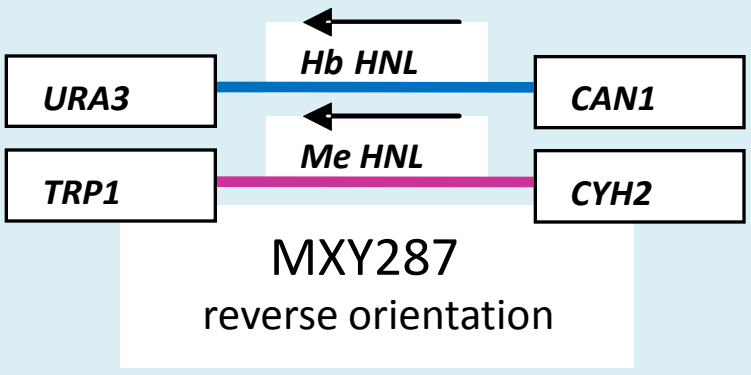
Aims:

Improved stability and activity at low pH

High activity with bulky substrates



MXY279
forward orientation

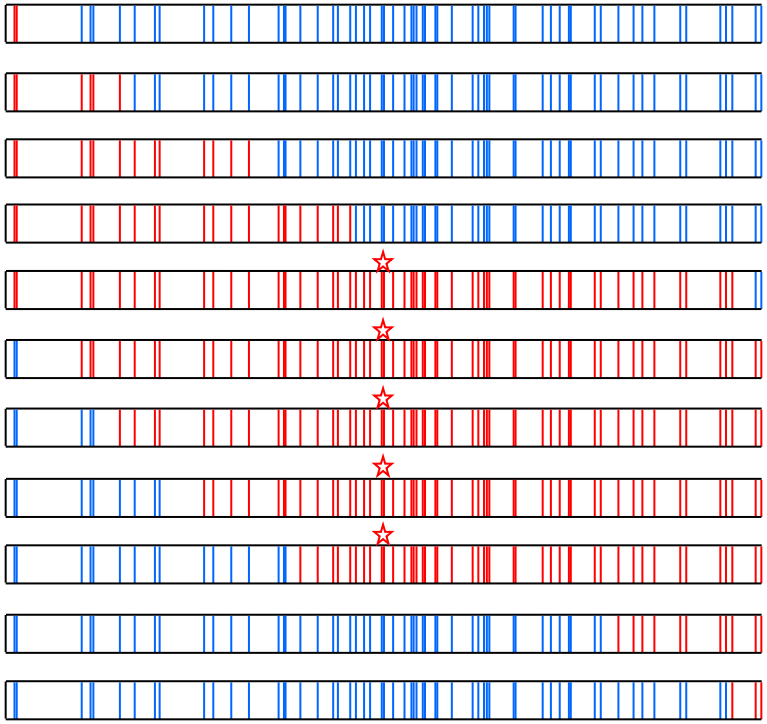


MXY287
reverse orientation

Enzyme Engineering →Recombination

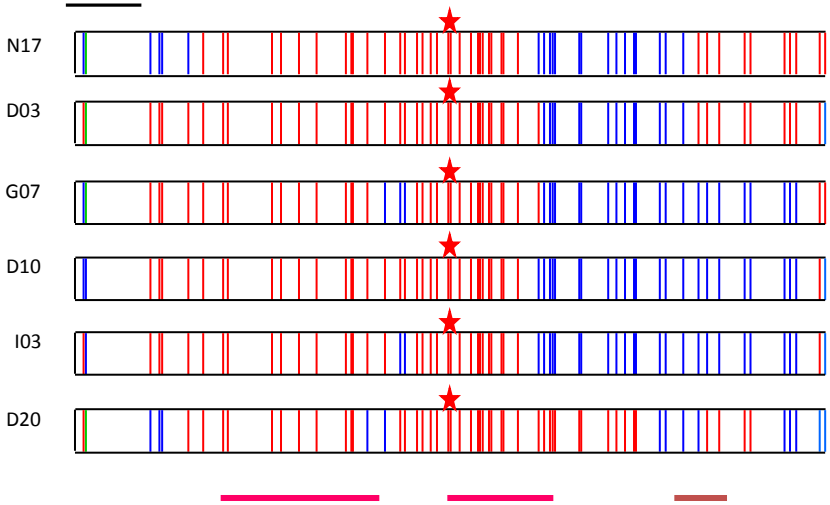
Novel *Hb*_HNLW128A / *Me*_Hnl recombinant proteins

Bipartite proteins



Complex proteins

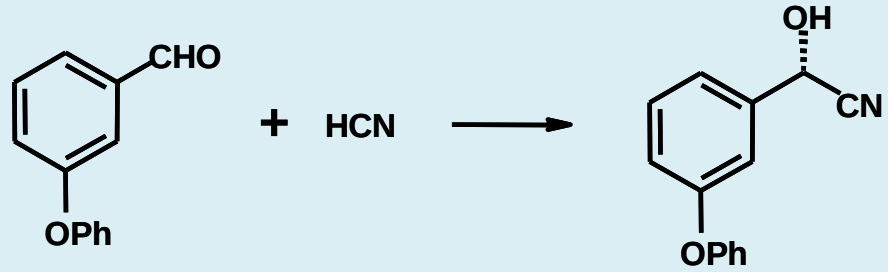
Hits

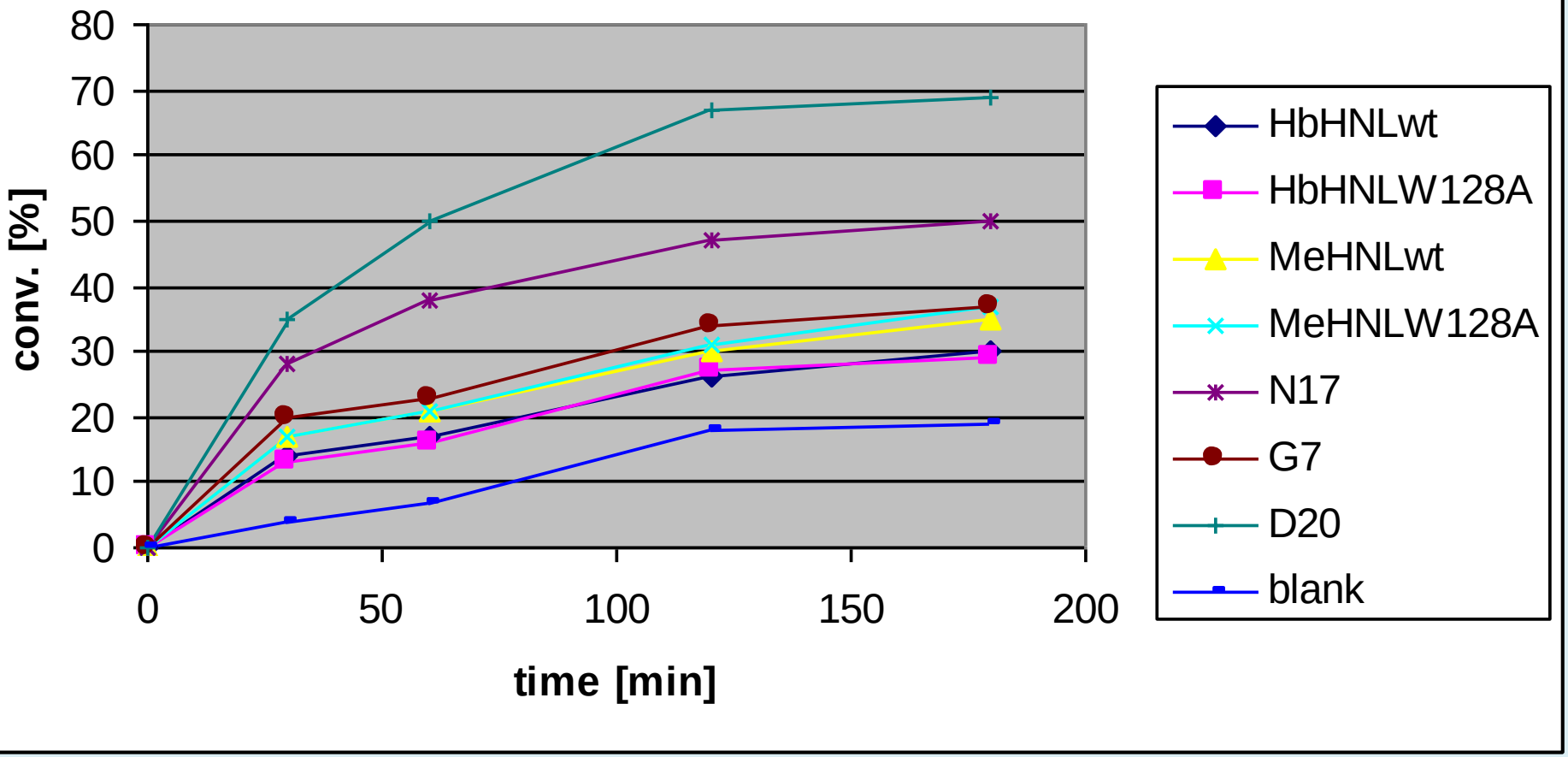


- Hb-specific amino acids
- Me-specific amino acids
- novel amino acids
- ★ Hb W128A alteration

Enzyme Engineering →Recombination

Conversion at pH 5.4





Enzyme Engineering →Recombination

```
HbHNL_W128A      (1)  MAFAHFVLIHTICHGAWIWHKLKPLLEALGHKVTALDLAAS
Hnl_N17          (1)  MVIAHFVLIHTICHGAWIWHKLKPALERAGHKVTALDMAAS
HNL_D20          (1)  MAIAHFVLIHTICHGAWIWHKLKPALERAGHKVTALDLAAS
MeHnl_WT (D7)    (1)  MVTAHFVLIHTICHGAWIWHKLKPALERAGHKVTALDMAAS

HbHNL_W128A     (42)  GVDPRQIEEIGSFDEYSEPLLTFLEALPPGEKVILVGESCG
Hnl_N17         (42)  GVDPRQIEEIGSFDEYSEPLLTFLEALPPGEKVILVGESCG
HNL_D20         (42)  GVDPRQIEEIGSFDEYSEPLLTFLEALPPGEKVILVGESCG
MeHnl_WT        (42)  GIDPRQIEQINSFDEYSEPLLTFLEKLPQGEKVIIVGESCA

HbHNL_W128A     (83)  GLNIAIAADKYCEKIAAAVFHNSVLPDTEHCPSYVVDKLME
Hnl_N17         (83)  GLNIAIAADKYCEKIAAAVFHNSVLPDTEHCPSYVVDKLME
HNL_D20         (83)  GLNIAIAADKYCEKIAAGVFHNSLLPDTEHCPSYVVDKLME
MeHnl_WT        (83)  GLNIAIAADRYVDKIAAGVFHNSLLPDTVHSPSYTVEKLLE

HbHNL_W128A    (124)  VFPDAKDTTYFTYT-KDGKEITGLKLGFTLLRENLYTLCGP
Hnl_N17        (124)  VFPDAKDTTYFTYT-KDGKEITGLKLGFTLLRENLFTKCTD
HNL_D20        (124)  VFPDAKDTTYFTYT-KDGKEITGLKLGFTLLRENLYTLCGP
MeHnl_WT       (124)  SFPDWRDTEYFTFTNITGETITTMKLGFVLLRENLFTKCTD
```

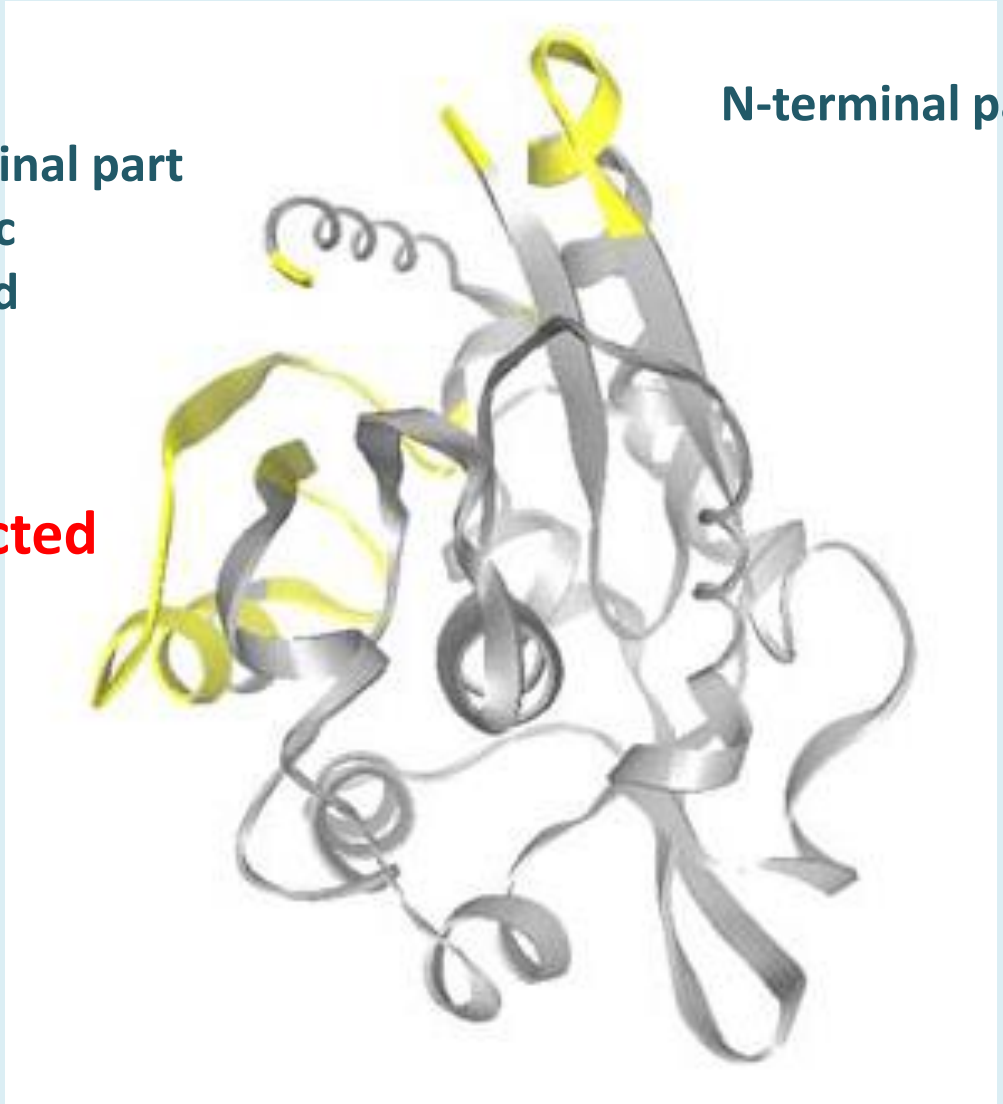
Enzyme Engineering →Recombination

```
HbHNL_W128A   (164)  EEYELAKMLTRKGSLFQNILAKRPFFTKEGYGSIKKIYVWT
Hnl_N17       (164)  GEYELAKMVMRKGSLFQNVLAQRPKFTEKGYGSIKKVYIWT
HNL_D20       (164)  EEYELAKMLTRKGSLFQNILAKRPFFTKEGYGSIKKVYIWT
MeHnl_WT      (165)  GEYELAKMVMRKGSLFQNVLAQRPKFTEKGYGSIKKVYIWT

HbHNL_W128A   (205)  DQDEIFLPEFQLWQIENYKPDKVYKVEGGDHKLQLTKTKEI
Hnl_N17       (205)  DQDKIFLPEFQLWQIENYKPDKVYKVEGGDHKLQLTKTKEI
HNL_D20       (205)  DQDKIFLPDFQLWQIENYKPDKVYKVEGGDHKLQLTKTEEV
MeHnl_WT      (206)  DQDKIFLPDFQRWQIANYKPDKVYQVQGGDHKLQLTKTEEV

HbHNL_W128A   (246)  AEILQEVADTYN
Hnl_N17       (246)  AEILQEVADTYN
HNL_D20       (246)  AHILQEVADAYA
MeHnl_WT      (247)  AHILQEVADAYA
```

Enzyme Engineering →Recombination

C-terminal part of basic scaffold

N-terminal part



Domains affected

Improvement and inversion of the enantioselectivity of esterase EstB

Engineering Concept

Initial Round: Error Prone PCR Library of Entire Gene

first positions affecting selectivity identified

Further rounds of designed evolution:

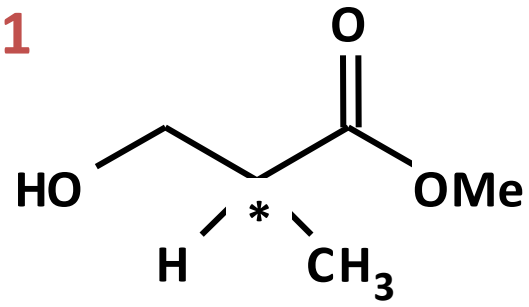
saturation mutagenesis at specific positions
region-specific random PCR libraries
region-specific randomized oligonucleotide mutagenesis
designed construction of specific muteins

Screening: Differential assays using enantiopure substrates

Esterase *Bg*_EstB

Mutants showing improved selectivity

S1



Hydroxyisobutyric acid methyl ester

Directed Evolution

Primary screening:

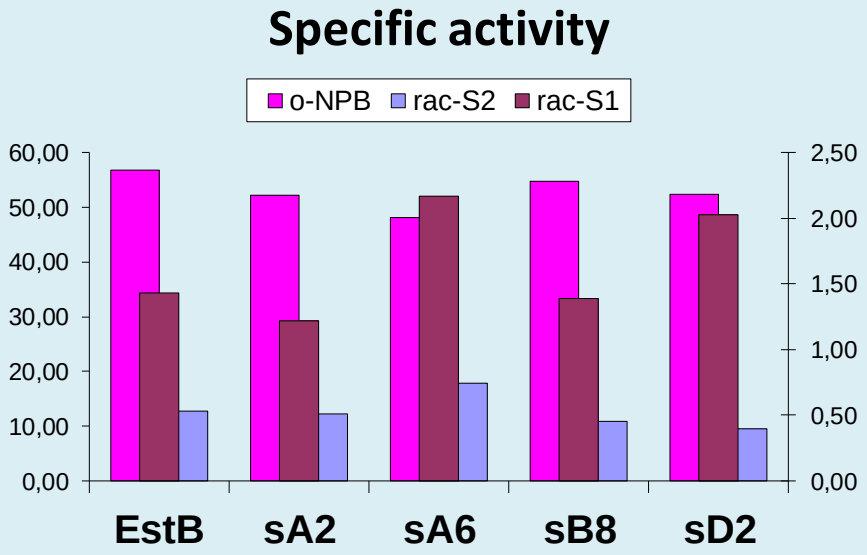
Colony-Filter Assay, pH-shift
high activity on rac-S1
75.000 clones screened
6.000 clones → good activity

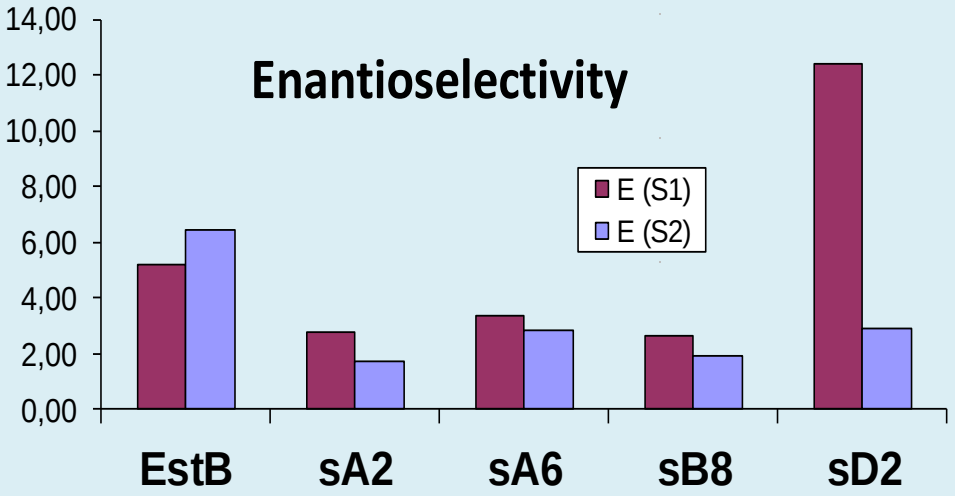
Secondary screening

6000 clones screened
Colonie-Filter assay, pH-shift
Differential screen → (R) und (S)-S1
4 clones showing altered selectivity
1 clone showing enhanced (S)-selectivity

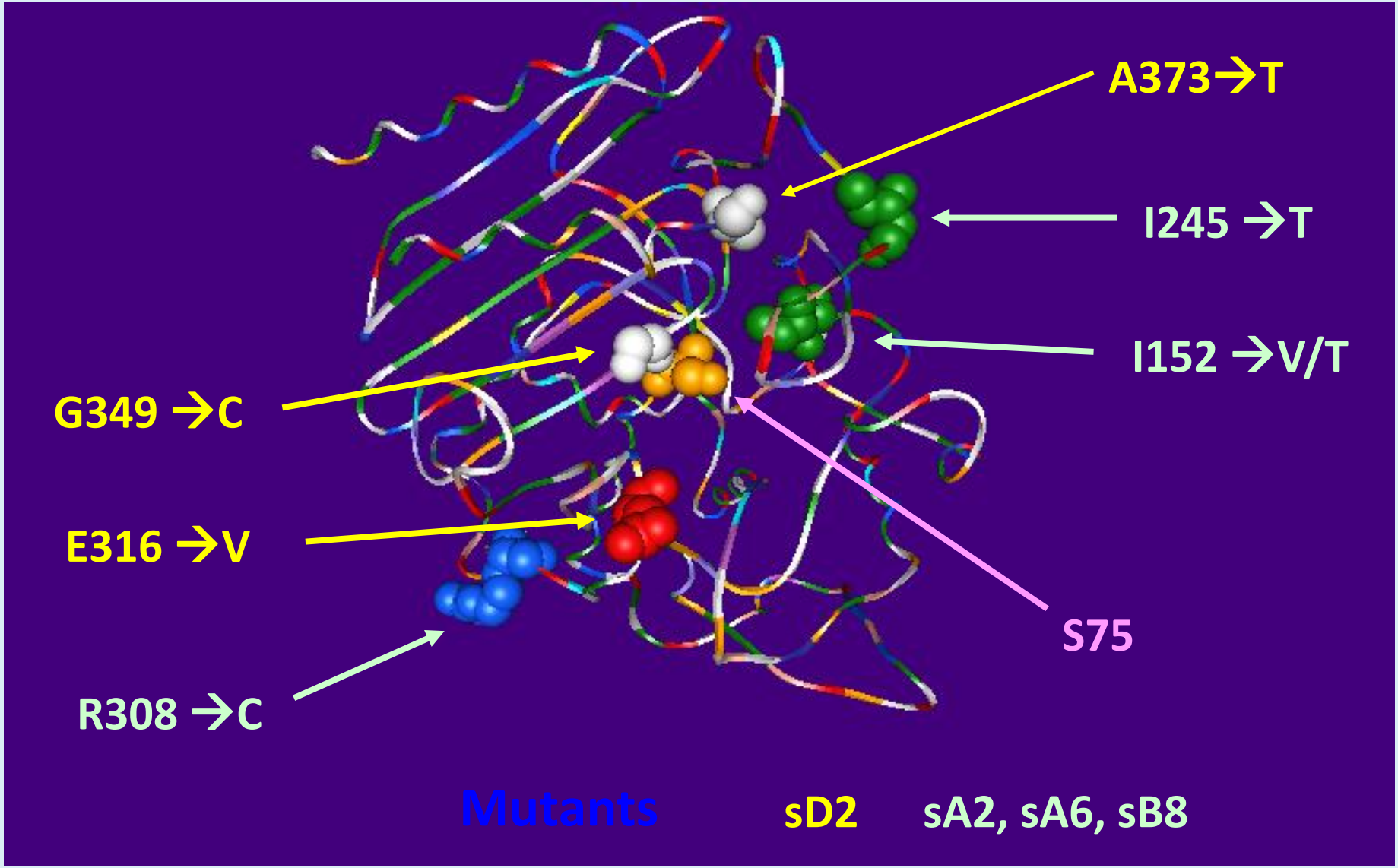
Esterase *Bg*_EstB
Mutants showing altered selectivity

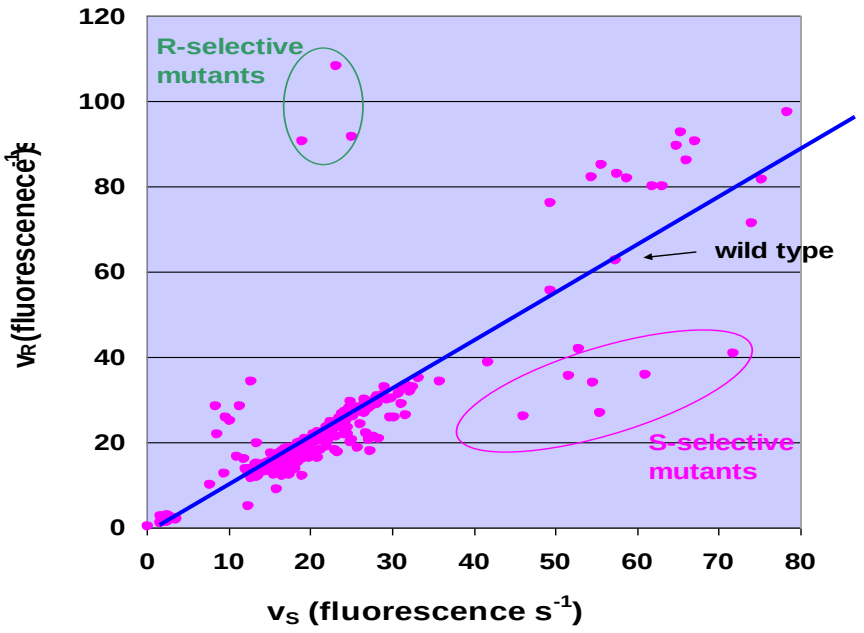
Mutant	Mutation	Properties
sA2	I 152 T I 245 T	higher activity on (R)-S1
sA6	I 152 V	higher activity on(S)-S1 and (R)-S1
sB8	I 152 T R 308 C	higher activity on(R)-S1
sD2	E 316 V G 349 C A 373 T	higher activity on(S)-S1



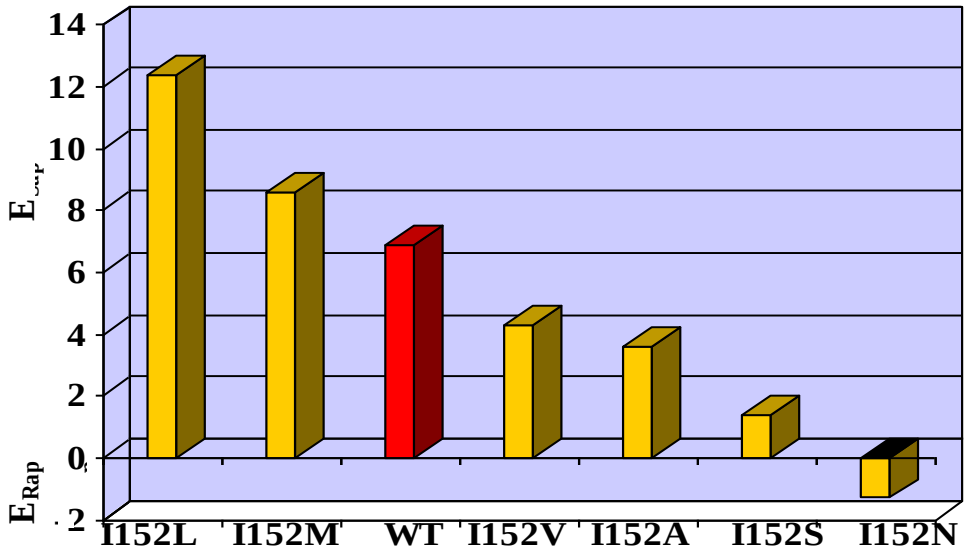


Esterase *Bg*_EstB
Mutants with improved selectivity



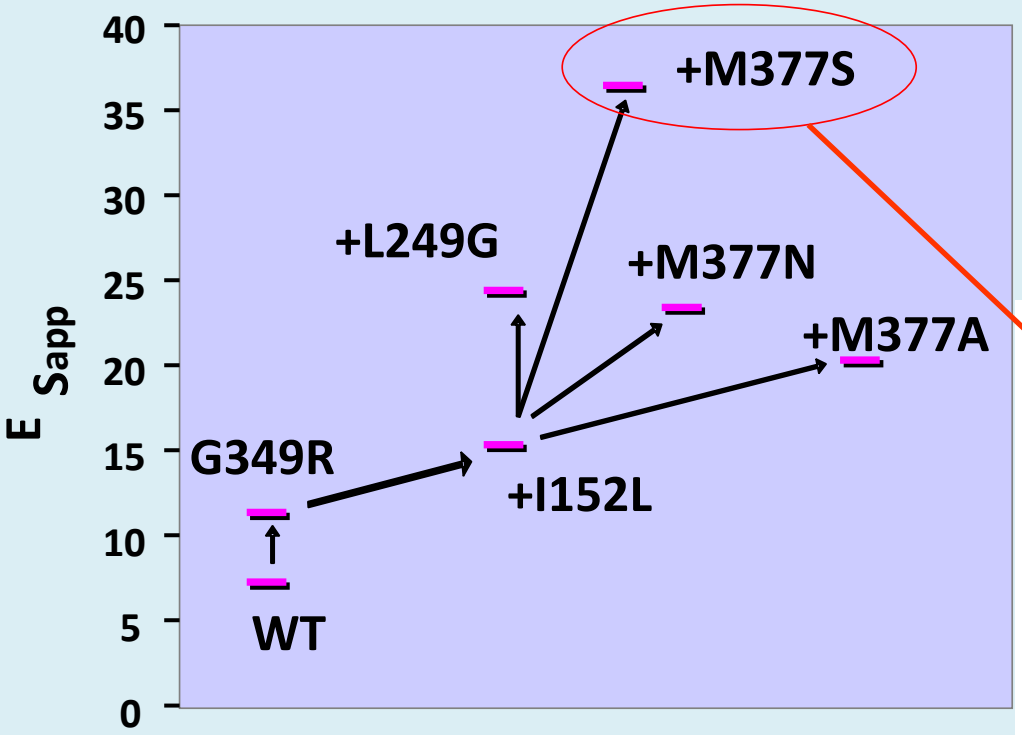


Saturation mutagenesis at position 152

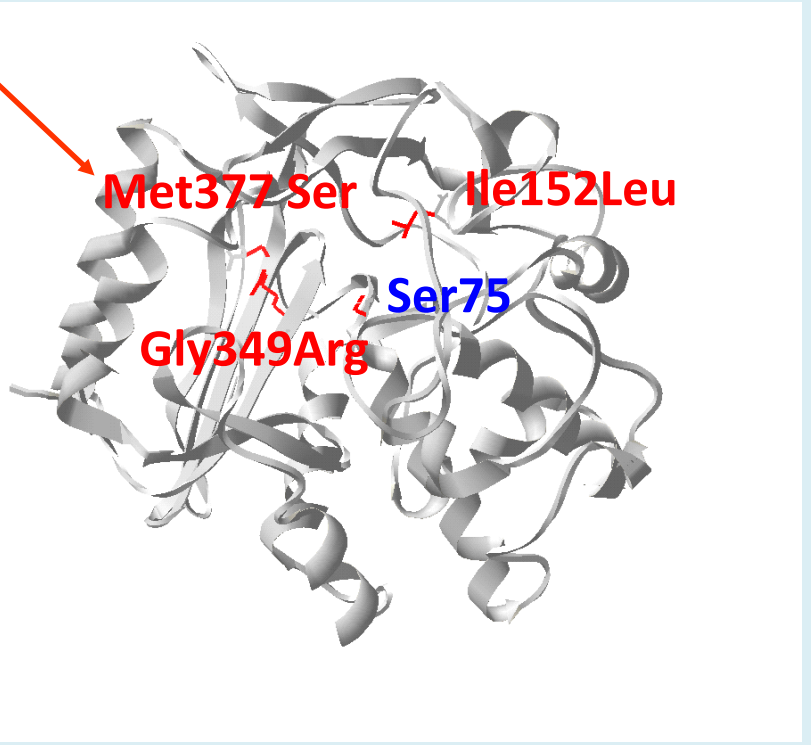


Improvement of S-enantioselectivity by semi-random approach

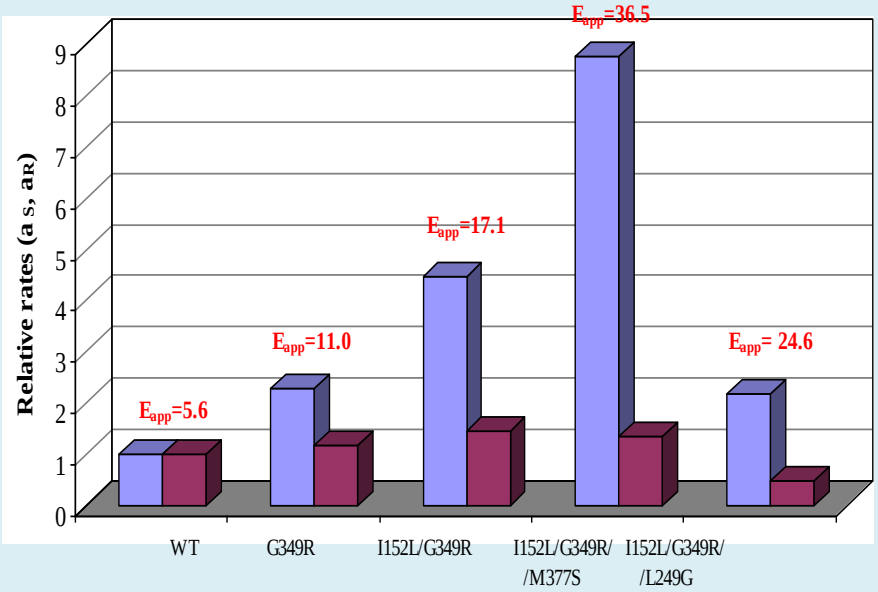
- Amino acid residues in active site influence enantioselectivity
- Semi-random approach – substitution of chosen 20 aa residues in the vicinity of Ser-75



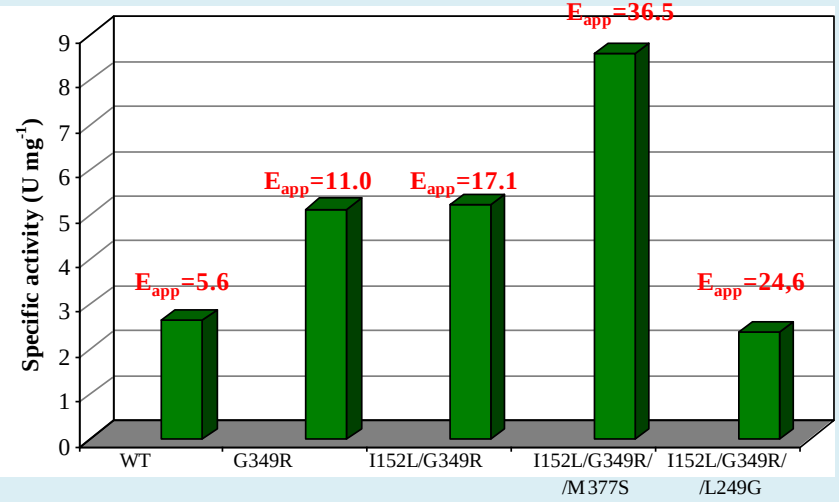
Positions **152, 249, 349 and 377** found to enhance S-selectivity



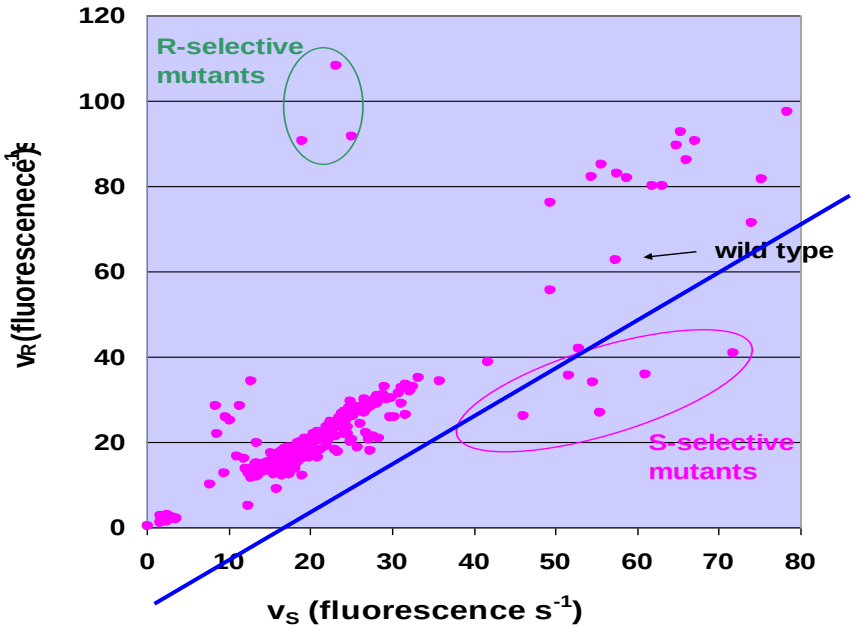
Esterase variant showing the highest S-enantioselectivity (E_{Sapp}= 36) harbors mutations I152L, G349R and M377S



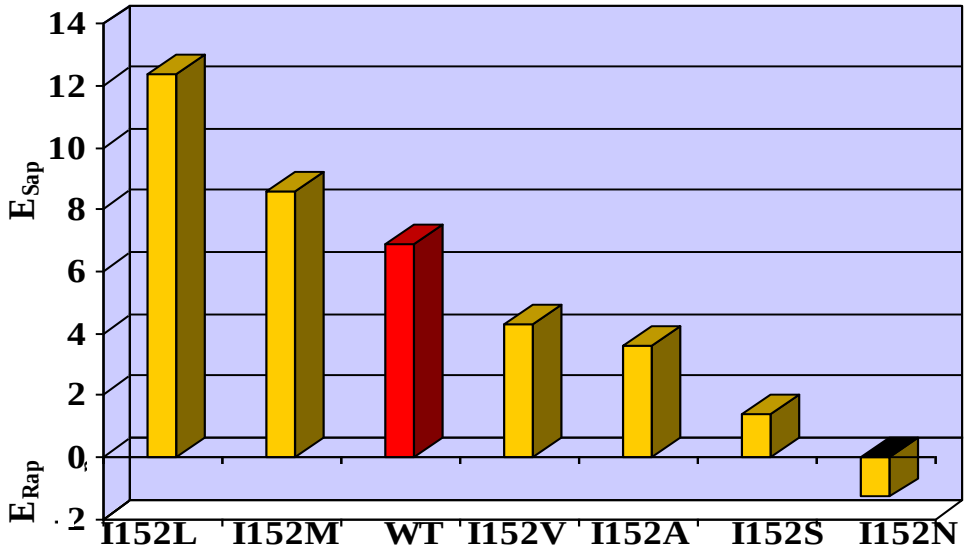
Relative activities towards S (■) and R (■) enantiomer



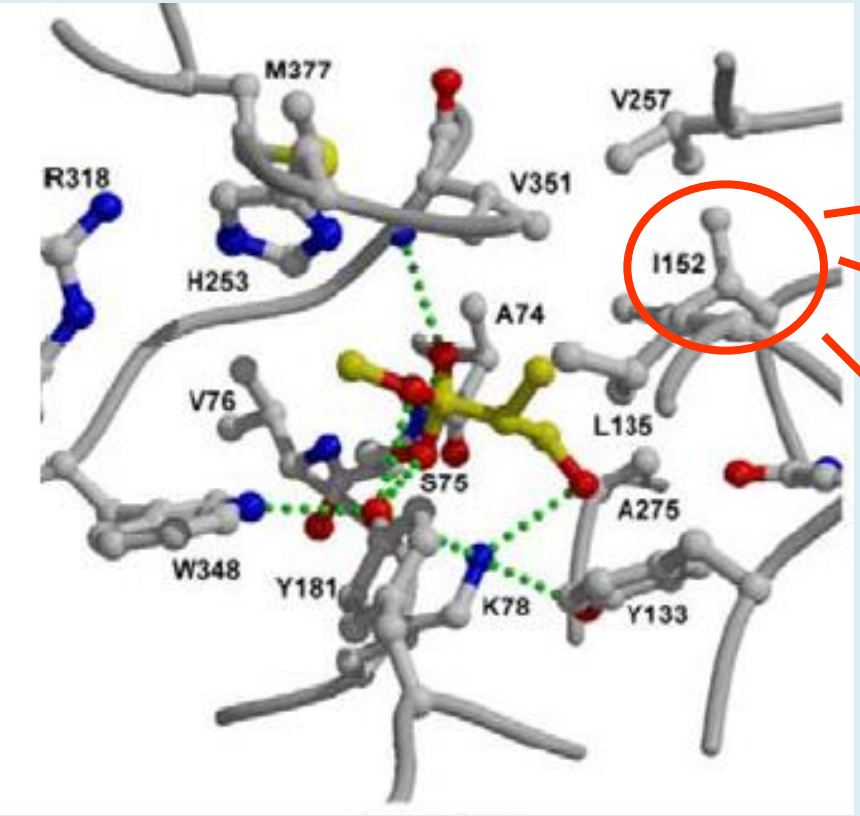
Specific activity towards racemic substrate (■)



Saturation mutagenesis at position 152



Enantioselectivity improvement of esterase EstB



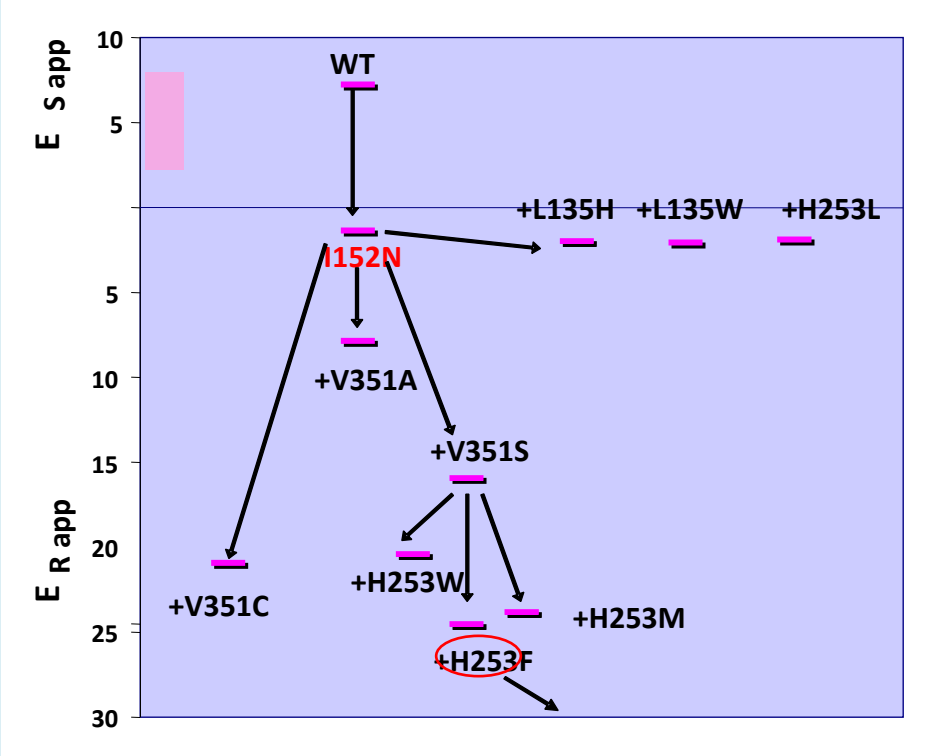
I152L → enhanced S-selectivity

I152V → lowered S-selectivity

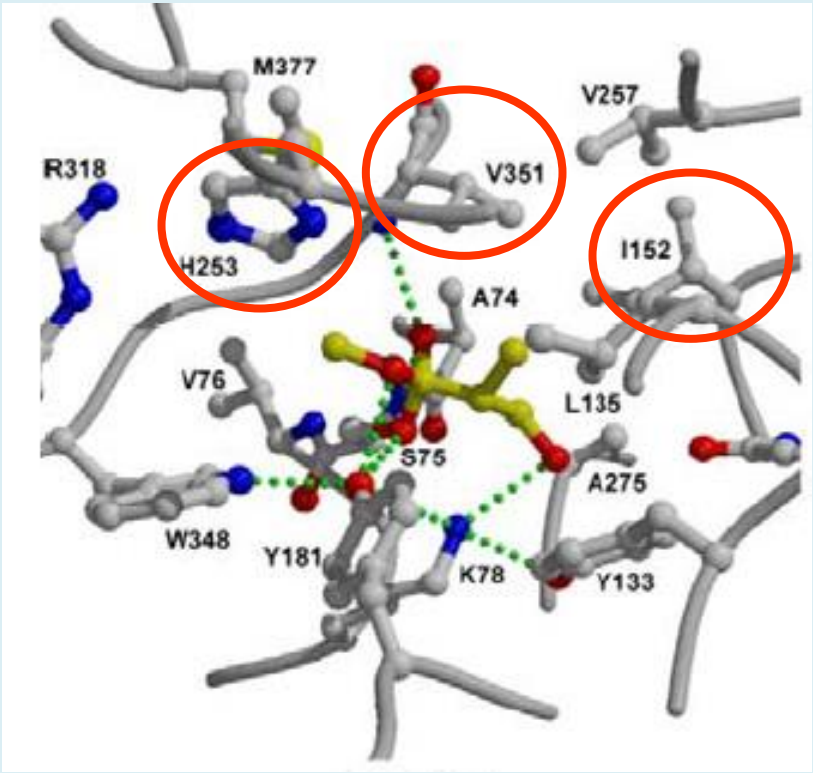
I152N → conversion to R-selectivity

methyl-ß-hydroxyisobutyrate modelled in active site

Improvement of the R-stereoselectivity by semi-random approach



Mutations at positions 152, 253 and 351 increase R-selectivity



Esterase variant showing the highest R-enantioselectivity (E_{Rapp}= 24.6) harbors mutations I152N, H253F and V351S

Activities of R-selective mutations

Mutations	Relative activity a_S	a_R	E_{app}
Wild type	1	1	5.6 S
I152N	0,53	4,5	1.5 R
I152N, V351S	0,08	7,4	15.9 R
SI152N, V351S, H253F	0.09	15.7	28.9 R

Creating a New Enzymatic Functionality

Nature has developed Hnl activity out of different basic protein structures by natural evolution over millions of years

Can we perform this by means of protein engineering within short time??

Hnl Enzymes have evolved from different protein structures

Table 1. Well characterized and sequenced HNLs

	*Me*HNL [22,52]	*Hb*HNL [53,54]	*Pa*HNL [55]	*Lu*HNL [56,57]	*Sb*HNL [58]	*At*HNL [9,28]
Source organism	*Manihot esculenta* (cassava)	*Hevea brasiliensis* (para rubber tree)	*Prunus amygdalus* (bitter almond)	*Linum usitatissimum* (flax)	*Sorghum bicolour* (millet)	*Arabidopsis thaliana* (mouse ear cress)
Stereo-selectivity	*S*	*S*	*R*	*R*	*S*	*R*
Molecular weight/ subunit	29 kDa	29 kDa	61 kDa	46 kDa	33 kDa/23 kDa	29 kDa
Cofactors	–	–	FAD	Zn^{2+}, NAD (?)	–	–
Posttrans-lational modifications	–	–	glycosylation	–	glycosylation	–
Quarternary structure	homo-tetramer (?)	homodimer	monomer	homodimer	hetero-tetramer	homodimer
Hosts for heterologous expression	*E. coli* *P. pastoris* *S. cerevisiae*	*E. coli* *P. pastoris* *S. cerevisiae*	*P. pastoris* (isoenzyme 5)	*E. coli* *P. pastoris*	–	*E. coli*
Natural substrate	acetone cyanhydrin	acetone cyanhydrin	mandelo-nitrile	acetone cyanhydrin	4-hydroxy-mandelo-nitrile	–
Substrate range	aliphatic/ aromatic-aldehydes & methyl ketones	aliphatic/ aromatic-aldehydes & methyl ketones	aliphatic/ aromatic aldehydes &methyl ketones	aliphatic aldehydes & methyl ketones	aromatic aldehydes & methyl ketones	aliphatic/ aromatic aldehydes & methyl ketones
Crystal structure (pdb code)	1EB8 [59,60]	1QJ4 [61]	1JO2 [62]	–	1GXS [63]	3DQZ
Sequence- and structural similarity	α/β-hydrolase	α/β-hydrolase	glucose-methanol-cholin-oxido-reductase	zinc-dependent alcohol dehydro-genase	serine carboxy-peptidase/α/ β-hydrolase	α/β-hydrolase
Technical application (examples)		3-phenoxy-benzalde-hyde cyano-hydrin [64]	2-chloro mandelo-nitrile [64]	-	-	-

Andexer et al., Trends Biotechnol.2009 Oct;27(10):599-607

Novel bacterial Esterases

```
Xv_EstD    GGDPTRVAVMGHSAGAHIAGLLVTDRRWLQAQG
Rrh_EstA   GGDPTRIVLAGDSAGGNLAASVAIAARDGGGPA
Rru_EstA   AAADAPLIVGGDSAGGNLAAVVAQRAVRENGPE
Rrh_EstC   GGDPDRVTIAGESAGAMSVVSLLAMPAARGLFR
Xv_EstA    GIDAQRVGVMGFSAGGHVAASLGTRYAAQVYPA
Xv_EstB    GGDAGNVTVFGQSGGGAKIATLMAMPAARGLFH
```

GxSxG Family

```
Bs_EstA    LHPDRPFVLFGHSMGGMVAFRLAQKLEREGIYP
Bg_EstC    ALGHPRVVLVGHSMGGVAITAAAERAPERIAAL
Bg_EstD    TLGLEKPLLVGHSLGGAIALAVGLDHPDSVSRI
Bg_EstE    QLGAGPVHLVGHSRGGCVAFYMAHRYPELVRSL
```

```
Rrh_EstB   FGIERLALVTGGSMGAQQTYEWAVRFPDKVLRA
Rrh_EstD   ECPLTTYVLTGFSQGAVIVGDVAAQIGAGNGPV
```

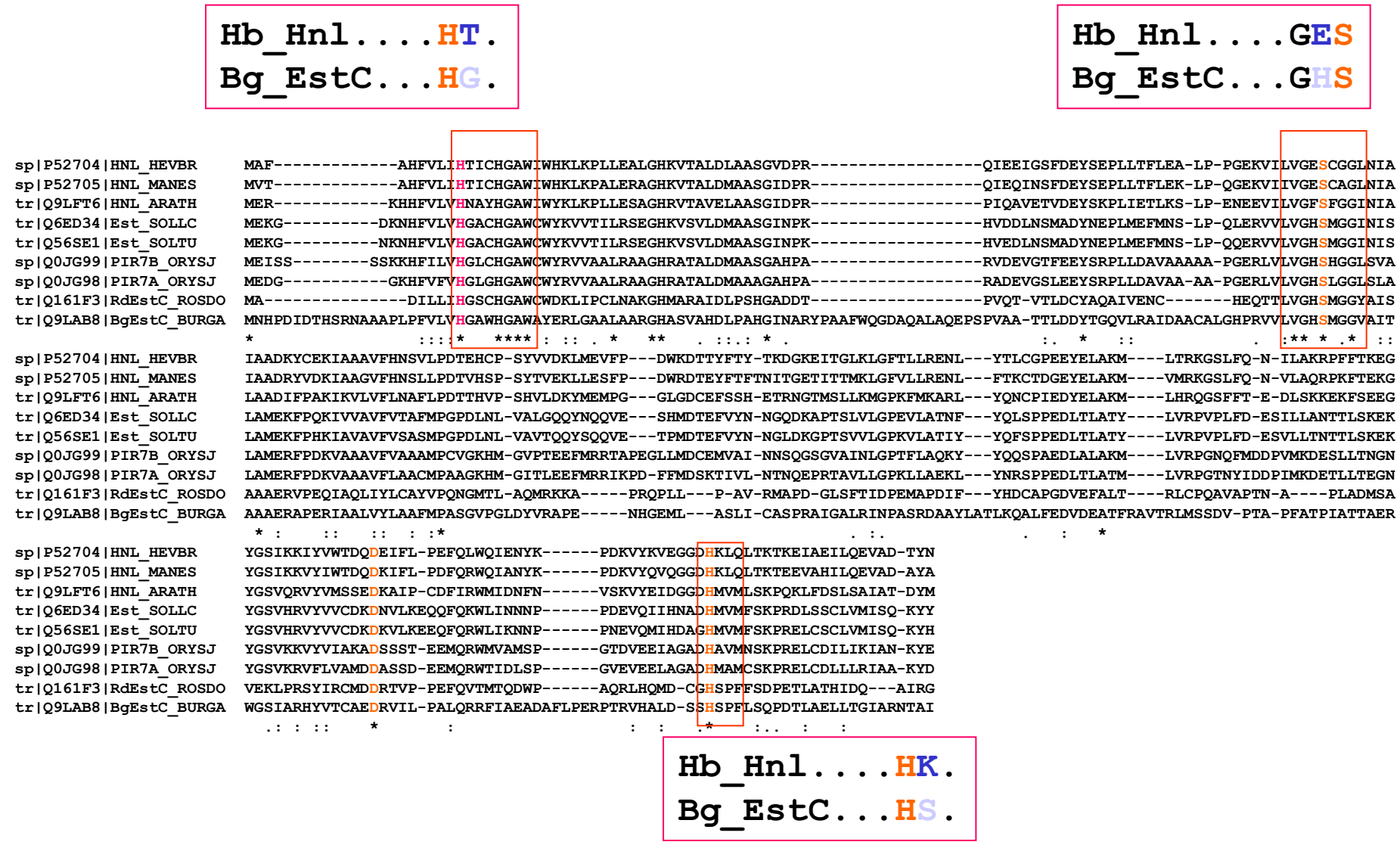
```
Xv_EstE    DSAFDQTVFFGDSLTDSGYYNPLLPAASRAVTG
Bg_EstA    AGVQKQIVSFGDSLSDAGTYSPQILLGFGGGRF
Xv_EstC    PLAASKIVLVGDSTTAVHGGWGPSFCAQHVTSF
```

GDSL Family

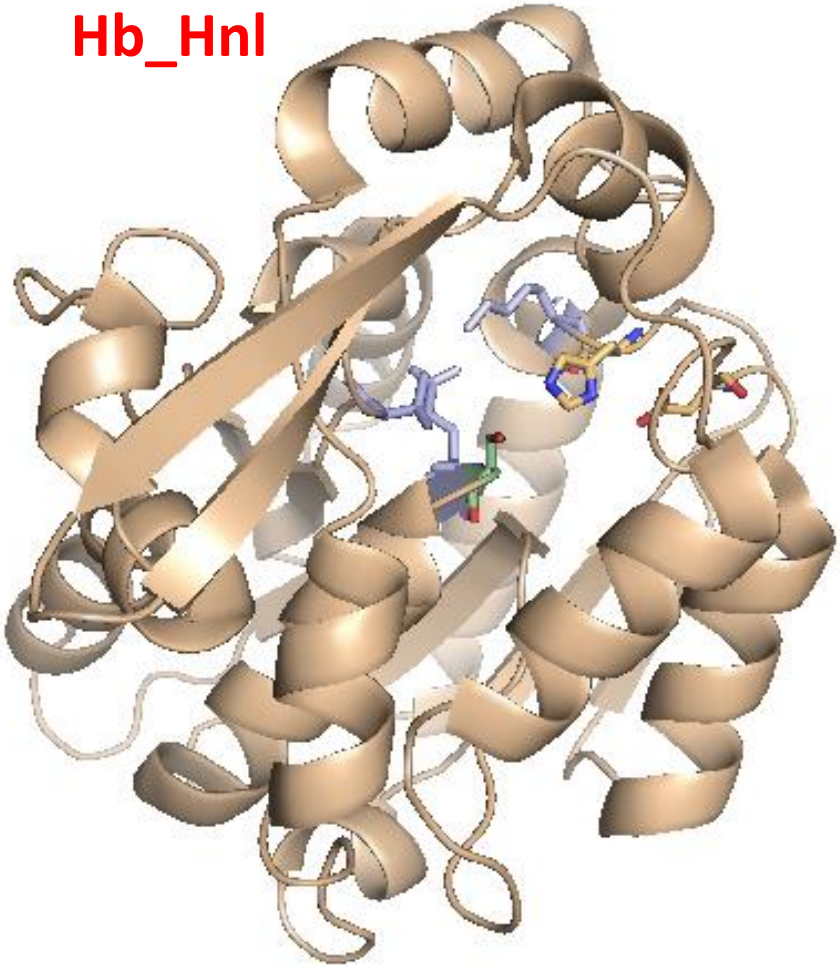
```
Bg_EstB    RPMREDTLFRLASVTKPIVALAVLRLVARGELA
```

SxxK Family

Esterases and Hydroxynitrile Lyases



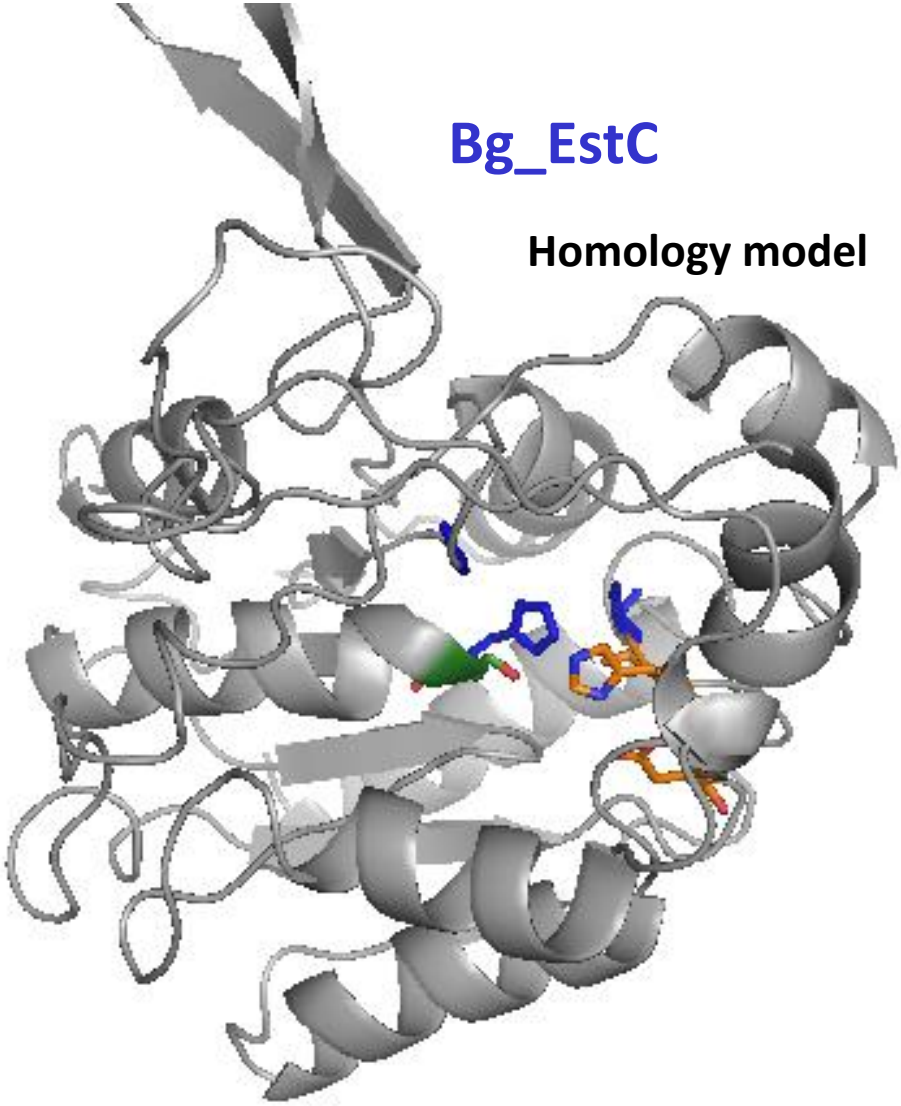
Hb_Hnl



Triade: Ser80, His235, Asp207
Important: Lys236

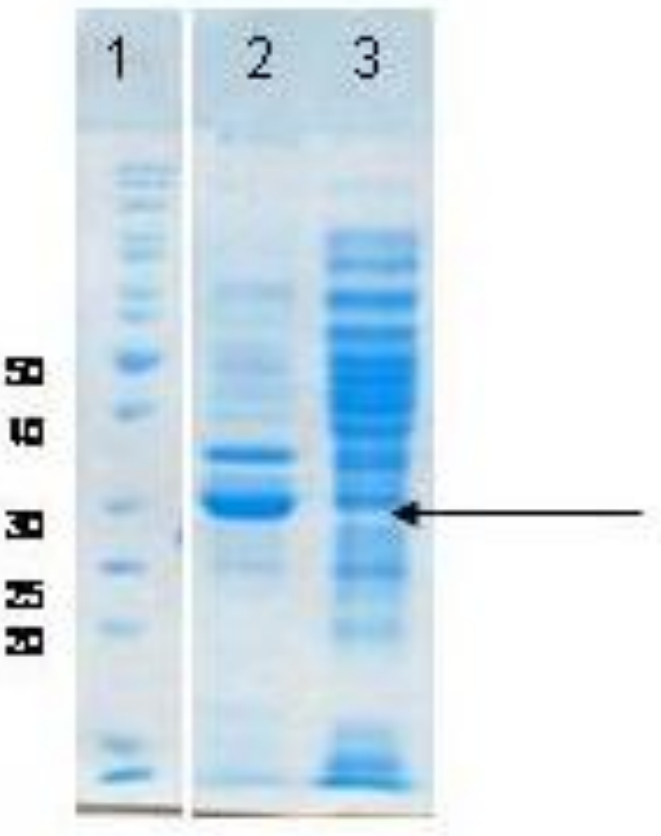
Bg_EstC

Homology model



Triade: Ser112, His275, Asp242

Bg_EstC Muteins designed for Hnl activity



1st Generation:

S276K

2nd Generation:

S276K
G24T
H111E

1: #SM0661 (Fermentas) (5 µl)
2: *Bg*_EstC S276K pellet (2 µl)
3: *Bg*_EstC S276K raw lysate (2 µl)

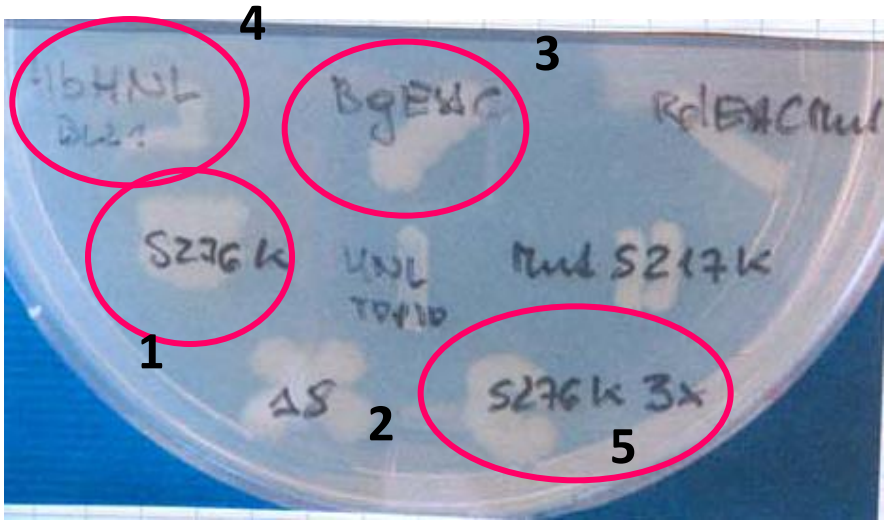
Cyanohydrin cleavage reaction

1: Bg_EstC S276K
2: Vector strain
3: Bg_EstC wt
4: Hb_HNL wt

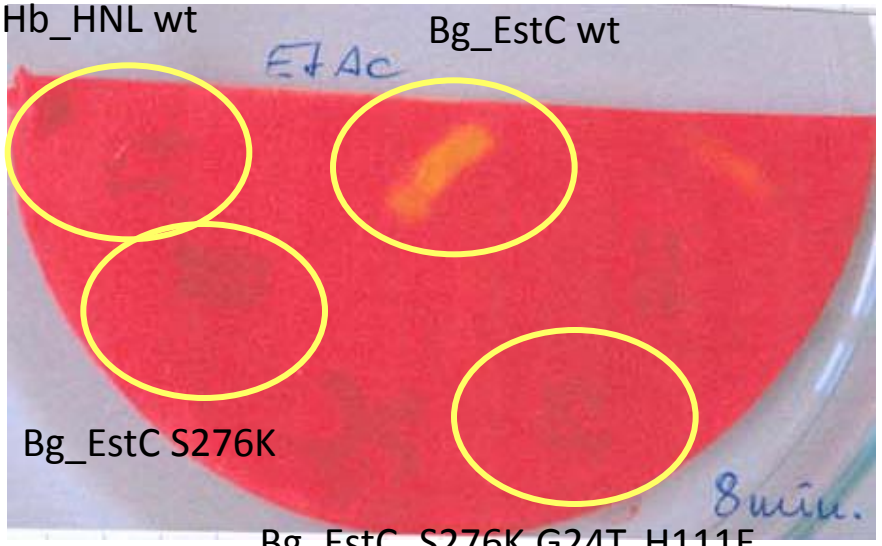
(S)-**m-phenoxy benzaldehyde cyanohydrin, pH 4.5, 56 min**

acetone cyanohydrin, pH 3.5, 3.5 min

Esterase activity



Activity on ethyl acetate



Bg_EstC_S276K,G24T, H111E

1: *Bg*_EstC S276K
2: Vector strain
3: *Bg*_EstC wt
4: *Hb*_HNL wt
5: *Bg*_EstC_S276K,G24T, H111E

Cyanohydrin Synthesis Reaction