A white line-art sketch of a large, multi-story building with many windows and architectural details, serving as a background for the text.

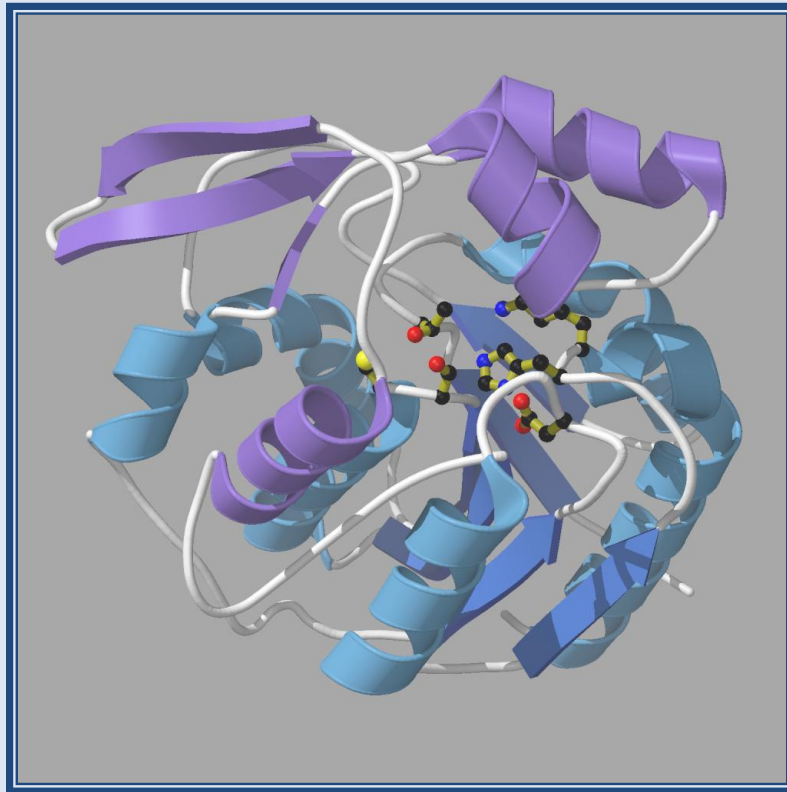
Protein Engineering

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*

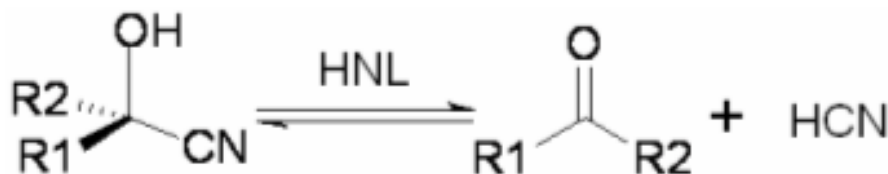
Hb_Hnl

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

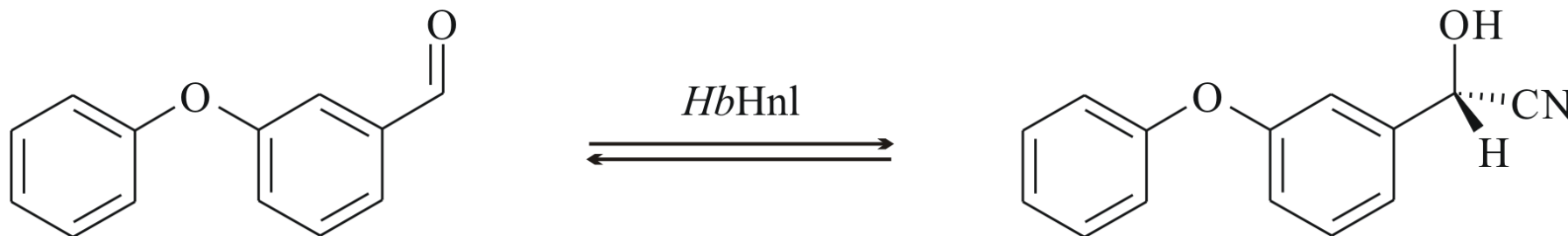
(R)-Hnl of *Prunus amygdalus*

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):

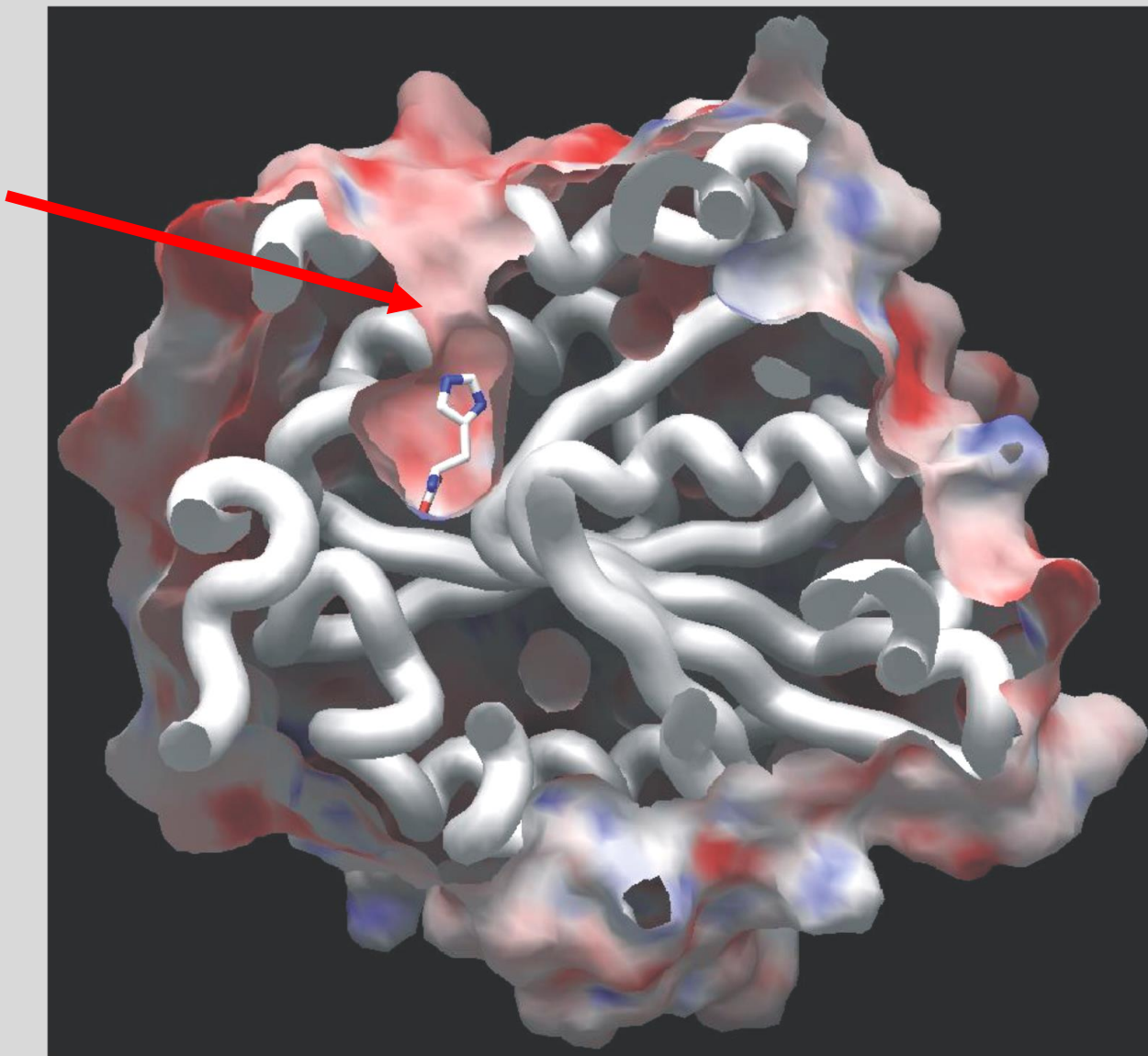


(S)-m-phenoxybenzaldehyde cyanohydrin

- Intermediate in the production of synthetic pyrethroids
- Biocatalytic production employing S-selective *Hb*_HNL provides product in **enantiopure quality**

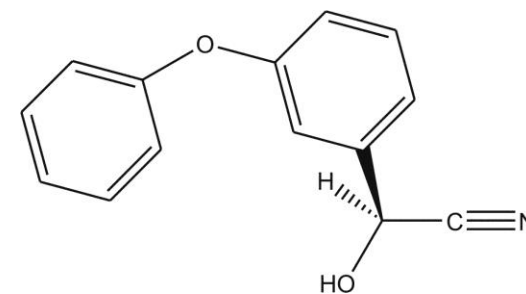
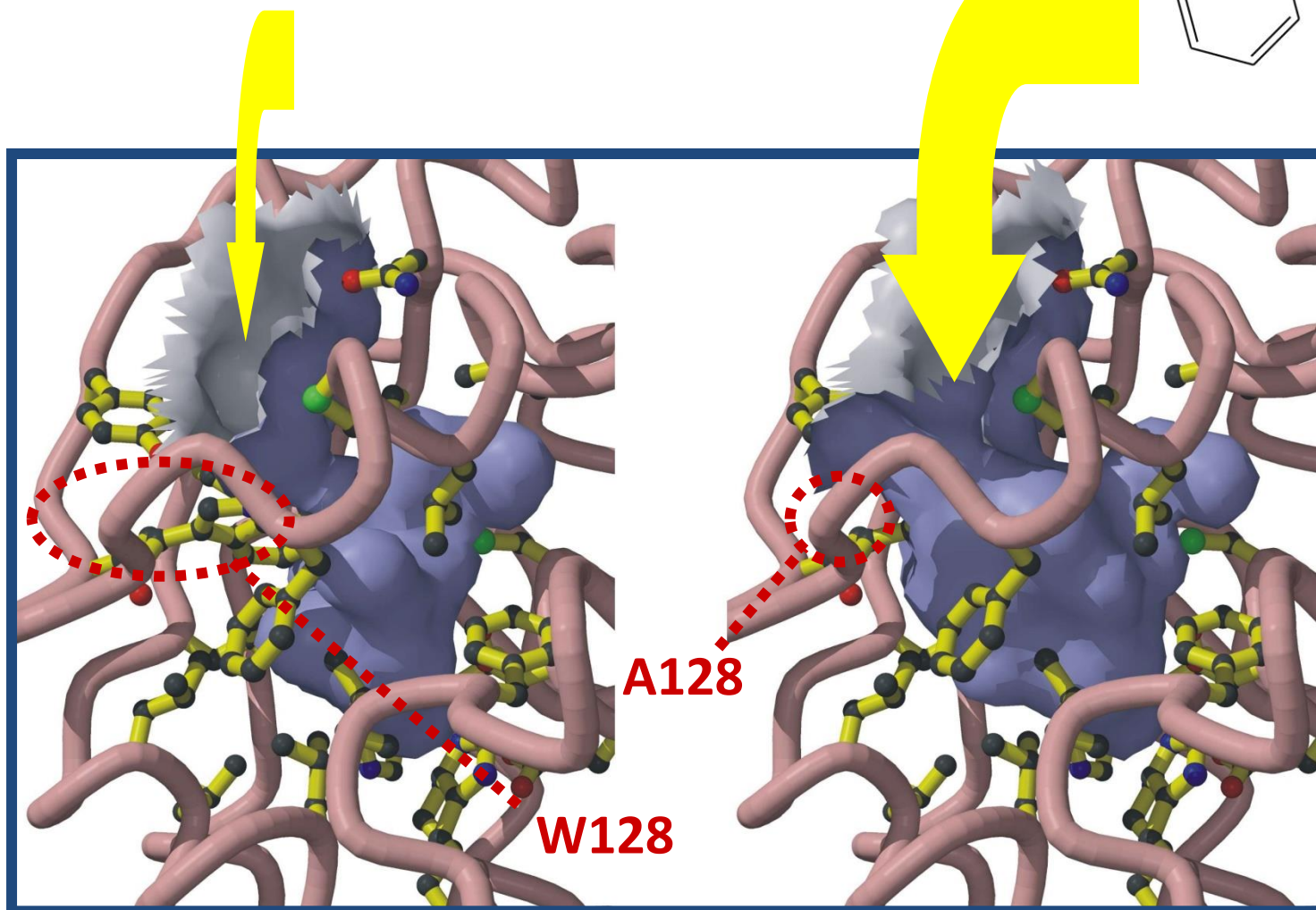
Hb_Hnl – tunnel to active site

Tunnel to
active site



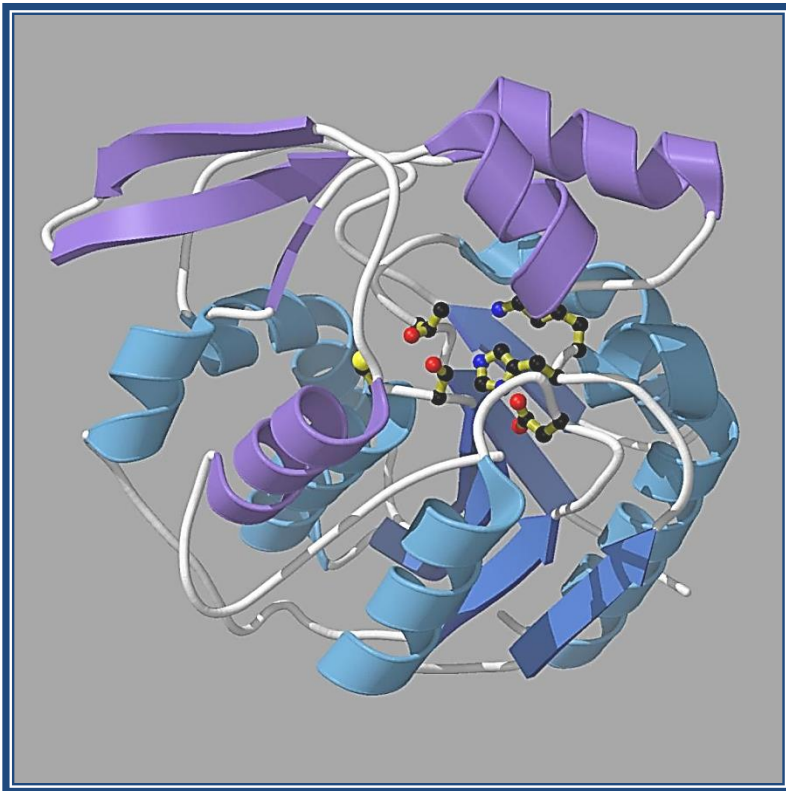
Hb_HNL tunnel mutant

wild-type



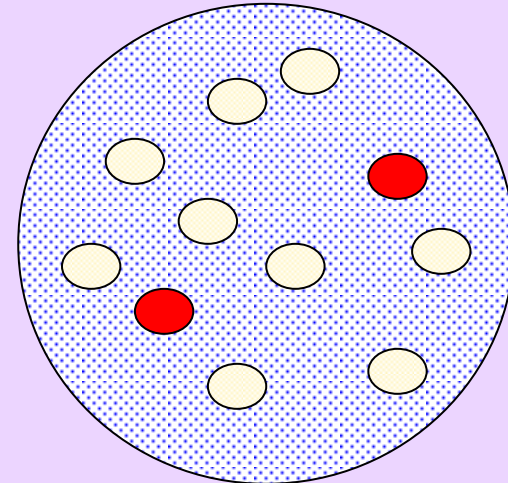
W128A

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

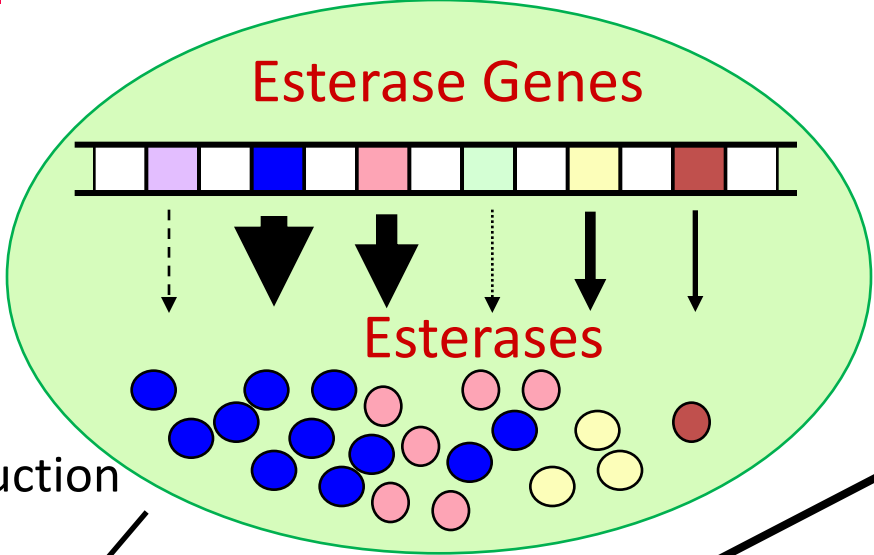
Mutant Library	Mutagenesis conditions	Hits selected/ top / parental
<i>Hb-Hnl-wt</i>	B	0
<i>Hb-Hnl-wt</i>	C	1 / 1 (Evo9) / 0
<i>Hb-Hnl-W128F</i>	C	3 / 2 / 0
<i>Hb-Hnl-W128A</i>	C	13 / 2 / 3
<i>Hb-Hnl-Evo</i>	B	5 [*] / 1 / 2

Evo9: Mutation W128A

* 1 Clone Mutation F128C

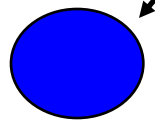
Wild type organism

New Esterases Genome screening

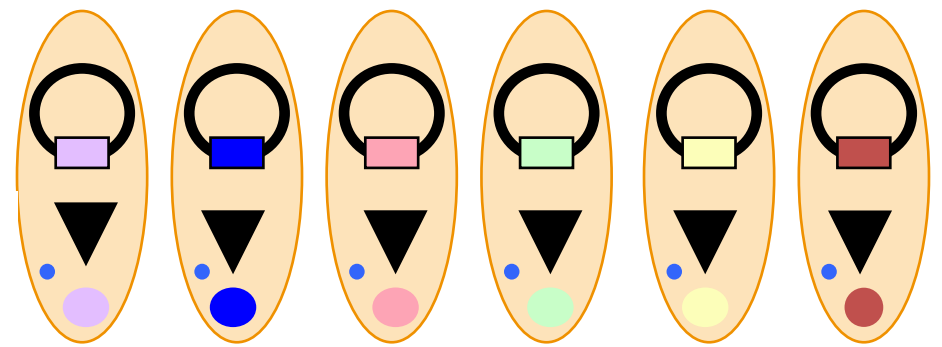


Induction

Screening



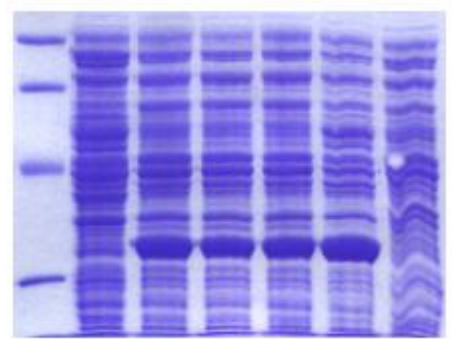
Cloned Esterase Genes heterologous host (*E.coli*)



Screening



Expression



1 2 3 4 5 6 7

Novel bacterial Esterases

Xv_EstD **GGD**PTRVAVM**GH****S**AGAHIAGLLVTD~~RR~~WLQAQ**G**
Rrh_EstA **GGD**PT~~RI~~VLAG**D****S**AG**GN**LAASVAIAARDGGGPA
Rru_EstA AAADAPLIV**G****D****S**AG**GN**LA~~AV~~VAVQRAVRENGPE
Rrh_EstC **GGD**PDRVTI**AG****E****S**AGAMS~~VV~~SLLAMPAARG~~L~~FR
Xv_EstA **G**IDAQRV**G**VM**G****F****S**AG**G**HVAASLGTRYAAQVYPA
Xv_EstB **GGD**AGNVT**V****F****G****Q****S****G****G**AKIATLMAMPAARG~~L~~FH

Bs_EstA LHPDRPFV**L****F****G****H****S****M****G****M**VAFRLAQKLEREGIYP
Bg_EstC ALGHPRV**V****L****V****G****H****S****M****G****G**VAITAAAERAP**E**RIAAL
Bg_EstD TLGLEK**P****L****L****V****G****H****S****L****G****G**AIALAVGLDHPDSVSRI
Bg_EstE QLGAGPV**H****L****V****G****H****S****R****G****G**CVAFYMAHRY**P**ELVRS**L**

Rrh_EstB FGIERLALV**T****G****G****S****M****G**AQQTYEWAVRFPDKVLRA
Rrh_EstD ECPLTTYV**L****T****G****F****S****Q**GAVIVGDVAAQIGAGNGPV

Xv_EstE DSAFDQ**T****V****F****F****G****D****S****L****T****D****S****G**YYNPLLPAASRAV**T****G**
Bg_EstA AGVQKQ**I****V****S****F****G****D****S****L****S****D****A****G****T****Y****S****P****Q****I****L****L****G****F****G****G****R****F**
Xv_EstC PLAASK**I****V****L****V****G****D****S****T**TAVHGGWG**P**SFCAQH**V****T****S****F**

Bg_EstB RPMREDTLFRLA**S****V****T****K****P**IVALAVLRLVARGELA

GxSxG Family

GDSL Family

SxxK Family

11 **Esterase Platform****Established Set of Key Enzymes:**

Basic families:	GXSXG	SXXK	GDSL
	1 Esterase from <i>Bacillus subtilis</i>		
	4 Esterases from <i>Burkholderia gladioli</i>		
	4 Esterases from <i>Xanthomonas vesicatoria</i>		
	5 Esterases from <i>Rhodococcus spp.</i>		
	5 Esterases from plant endophytic bacteria		

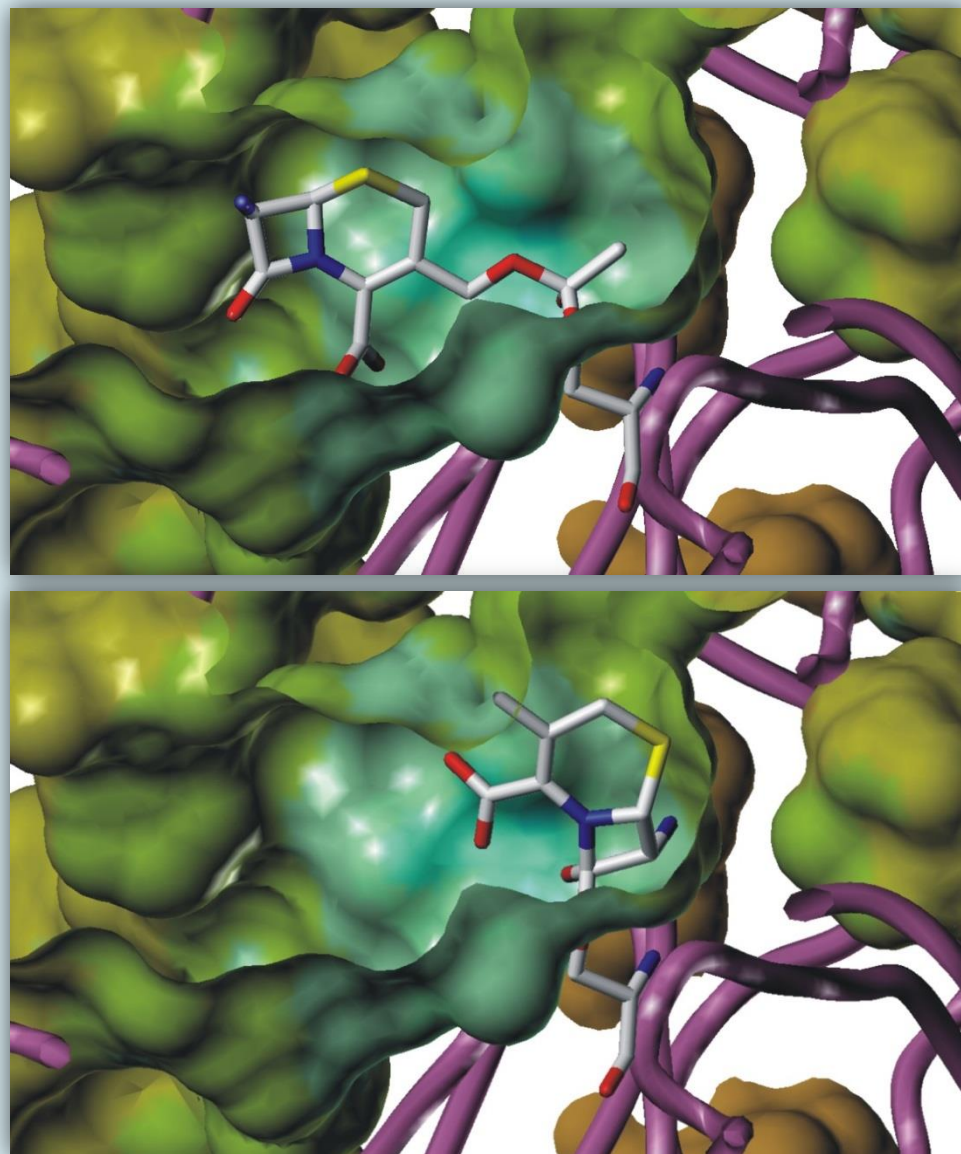
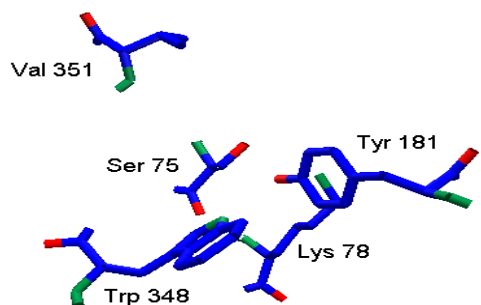
Libraries

- ~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

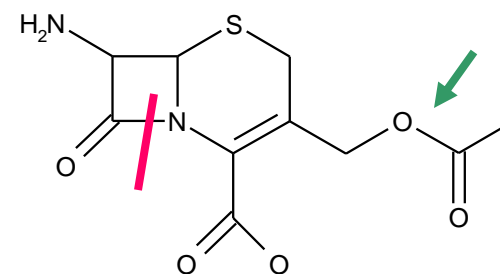
β -Lactamase Activity (U/mg) - Substrates

	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

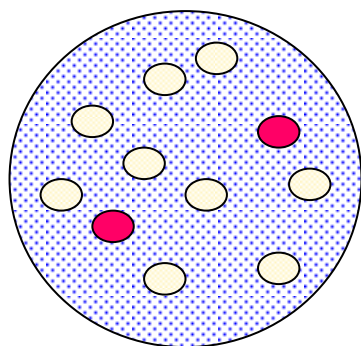
Directed Evolution

Error-Prone PCR

Mutation rate: 0.5%

$5 \cdot 10^4$ Clones screened

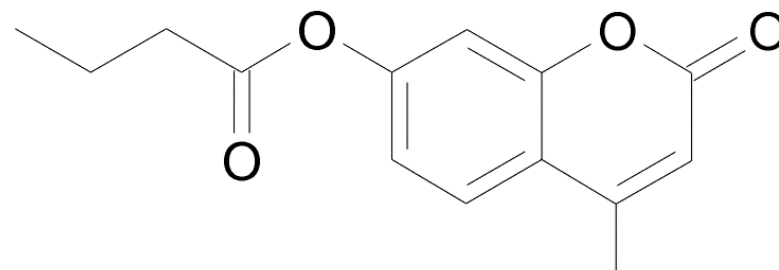
Screening-Assay at Colony-Level



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

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

4-methylumbelliferon-butyrate



Surrogate substrate

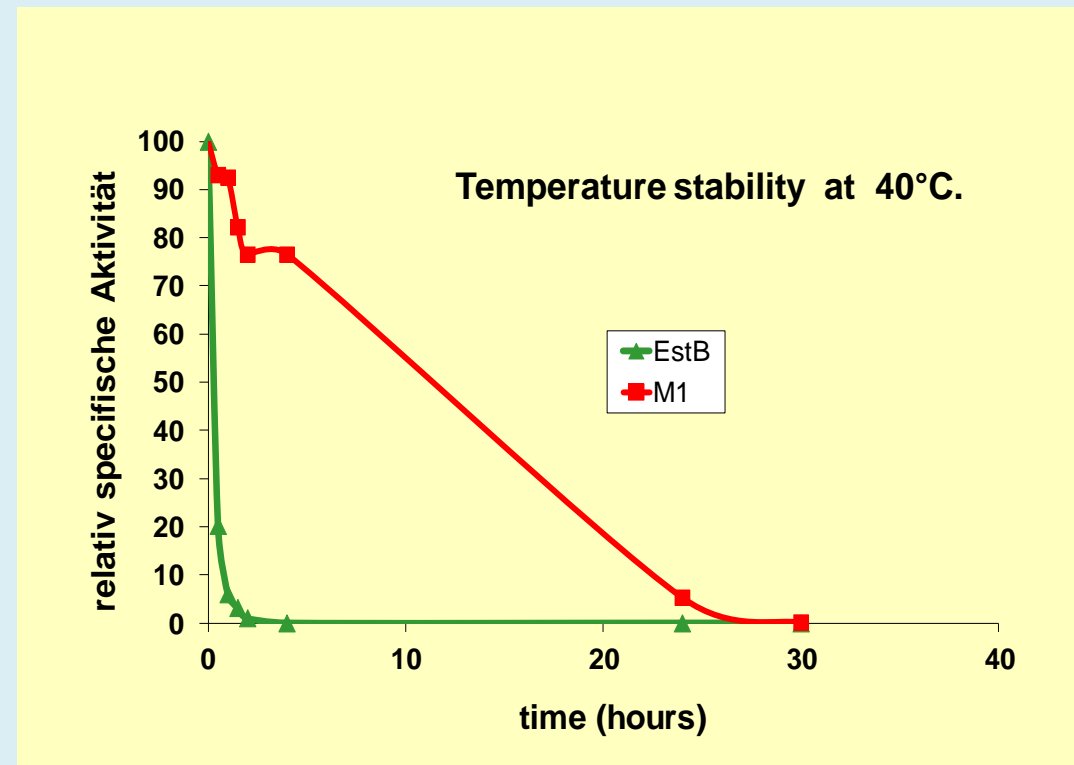
Burkholderia gladioli Esterase EstB

Stability in Vinyl acetate - Mutation W348G

15

Substrate: Cephalosporin C
kinetic data

	Vmax	Km
	$\mu\text{mol}/\text{min}/\text{mg}$	mM
EstB	79	1,3
M1	19	0,8



→ Much lower activity on Cephalosporin

Esterase *Bg_EstB*: Engineering of Stability

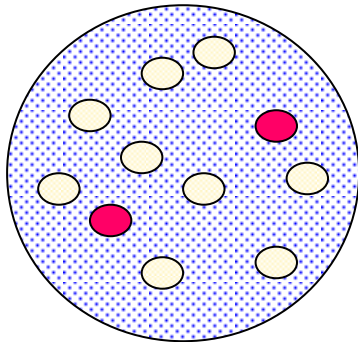
Directed Evolution

Error-Prone PCR

Mutation rate: 0.5%

10^6 Clones screened

Screening-Assay at Colony-Level



Substrate: Cephalosporin

pH-Indicator

Organic solvent (36% DMF)

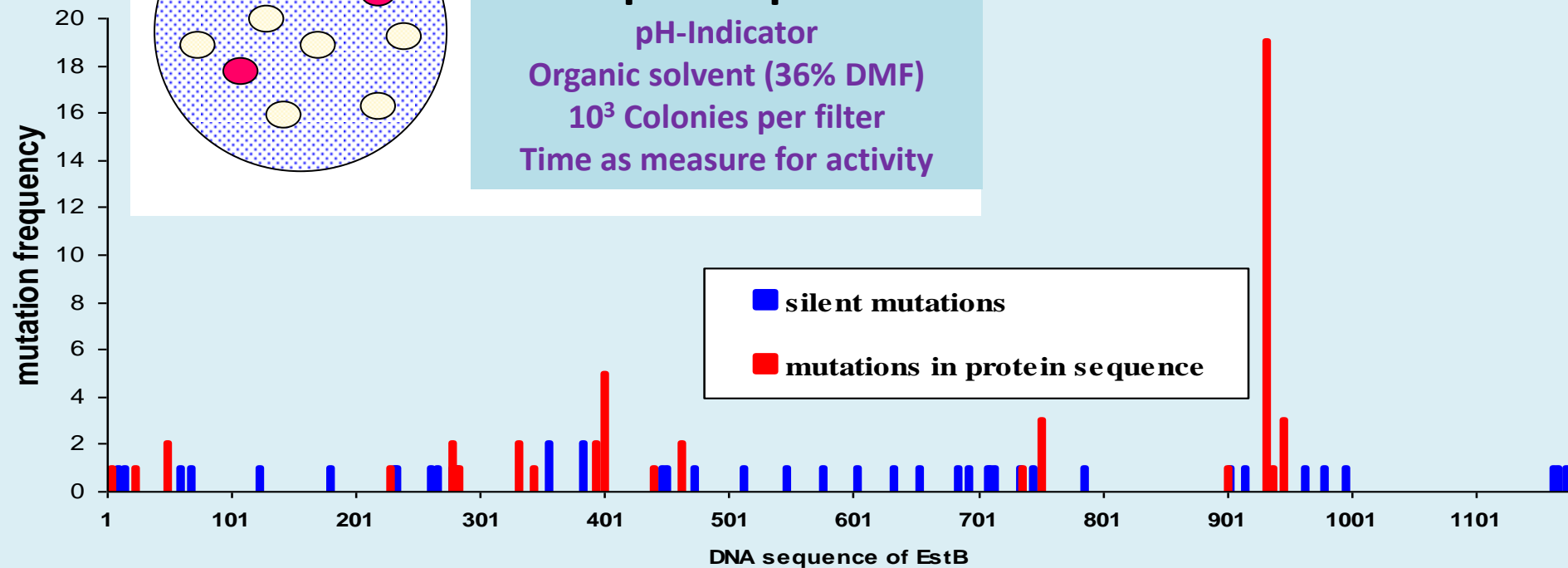
10^3 Colonies per filter

Time as measure for activity

25 Clones isolated and sequenced

19 different clones identified

11 Clones with different aminoacid sequence

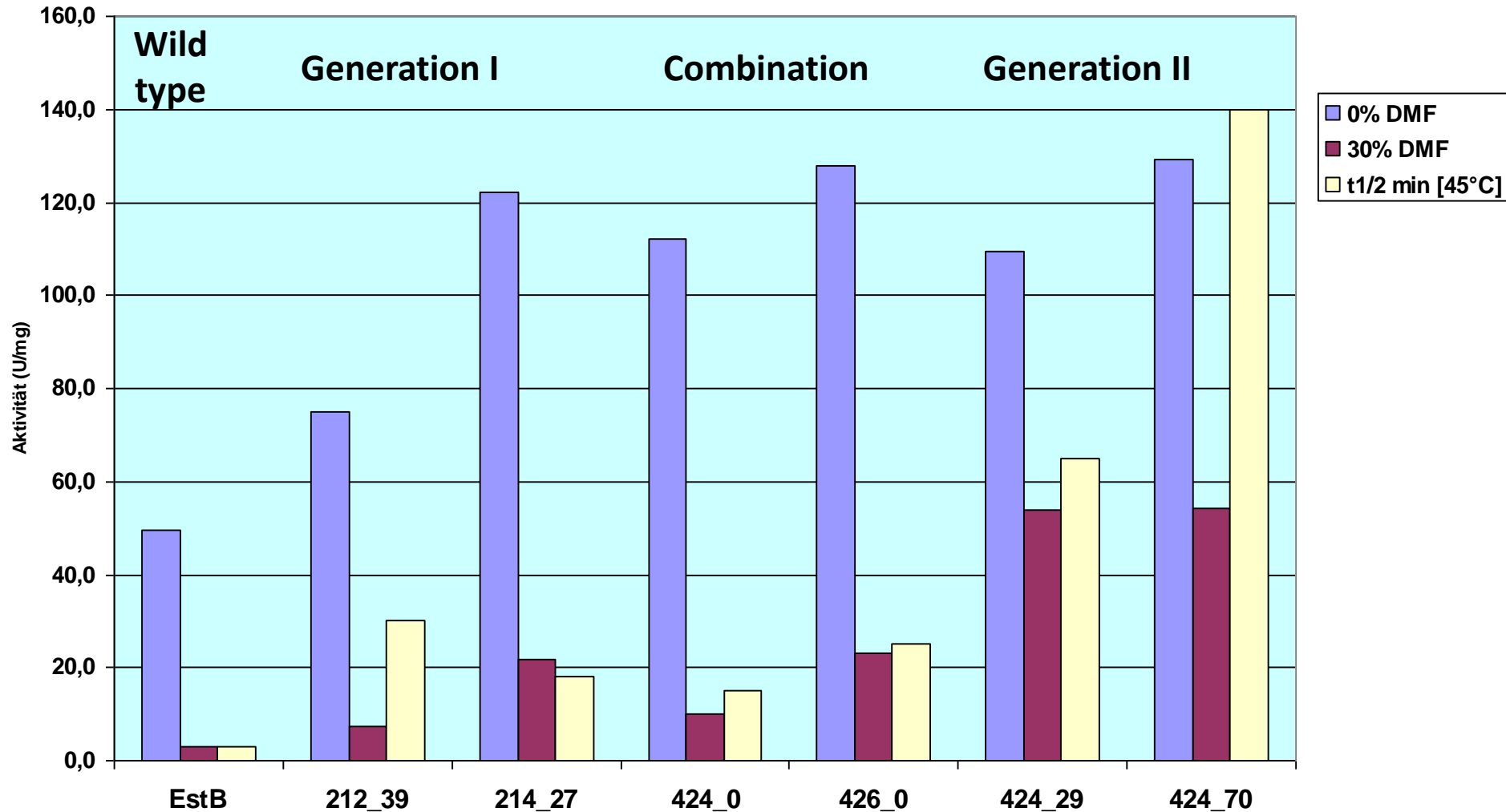


Esterase *Bg_EstB*

Mutants with enhanced Stability (DMF)

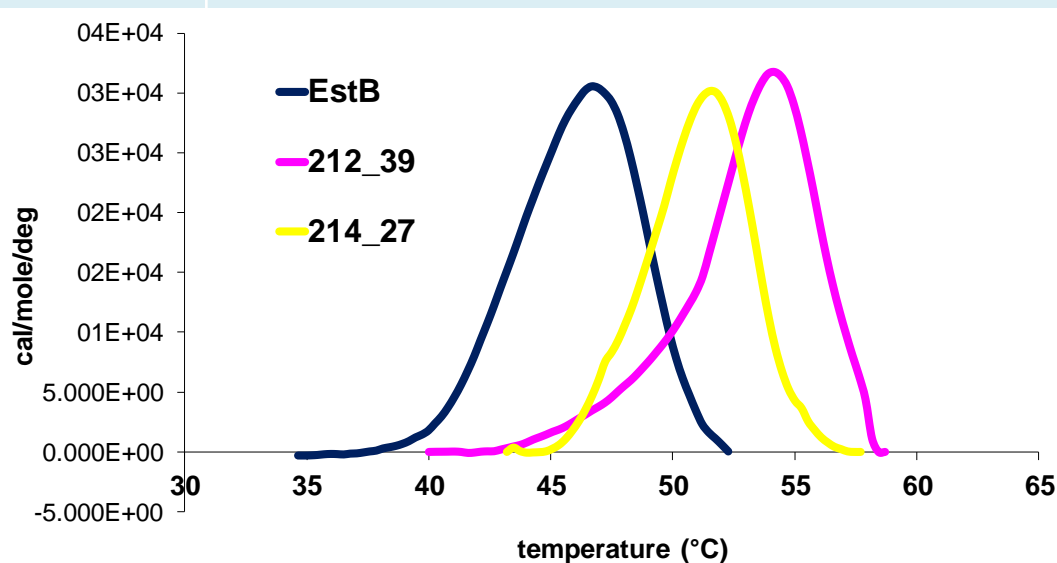
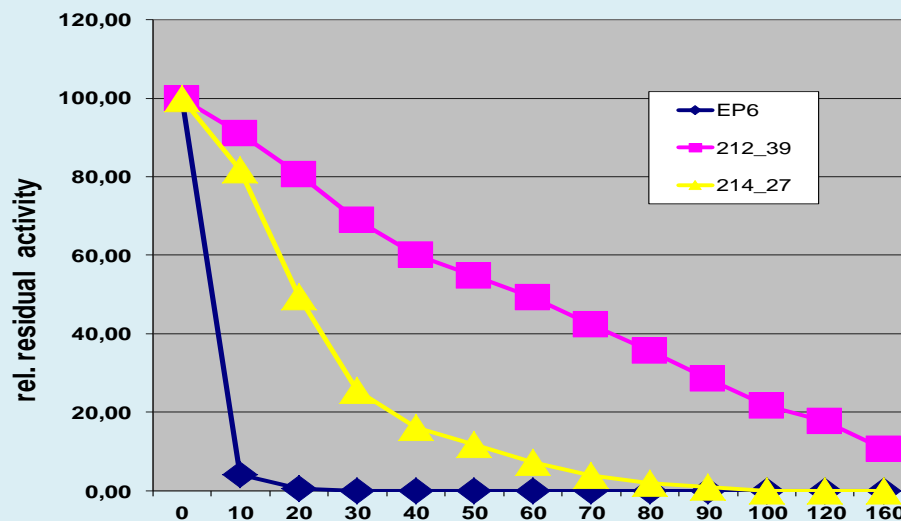
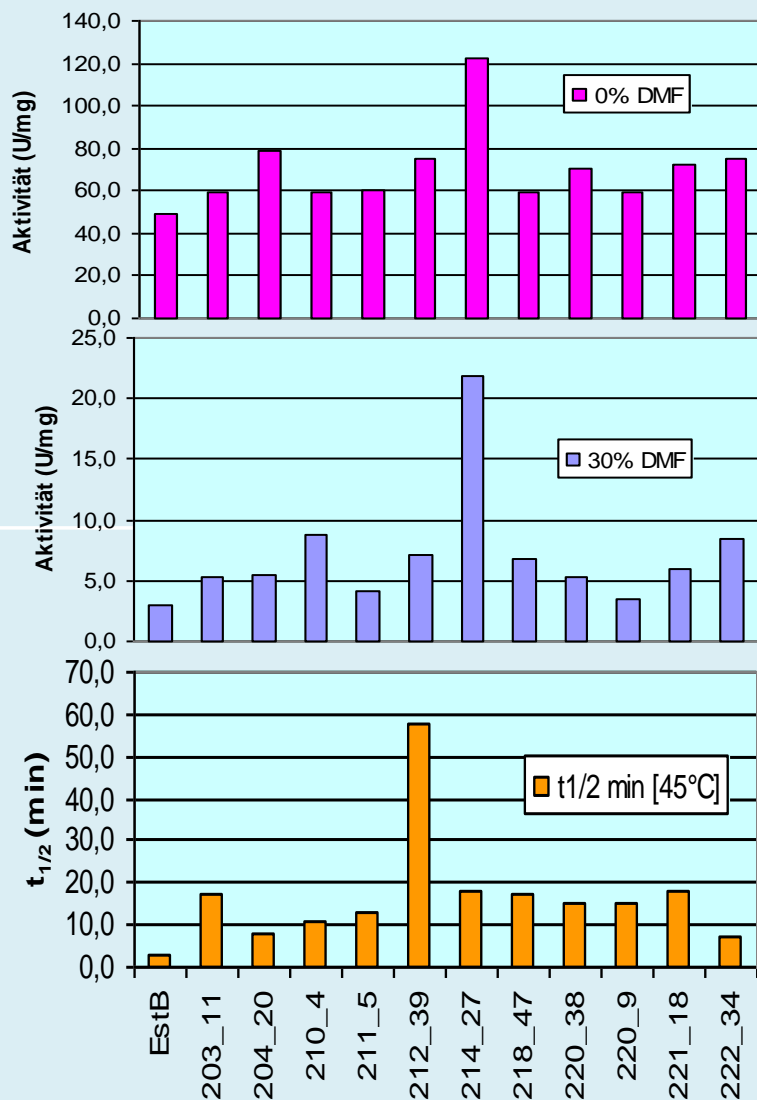
Nr.	Mutations in amino acid sequence															
Generation I																
203_11																A311V
204_20						L111Q				W134R						A311V
210_4		P8L							G132S				R155C			
211_50				E93G												A311V
212_39			S17L										R155C			A311V
214_27			S17L						G132S					E251G	A311V	E313K
218_47	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

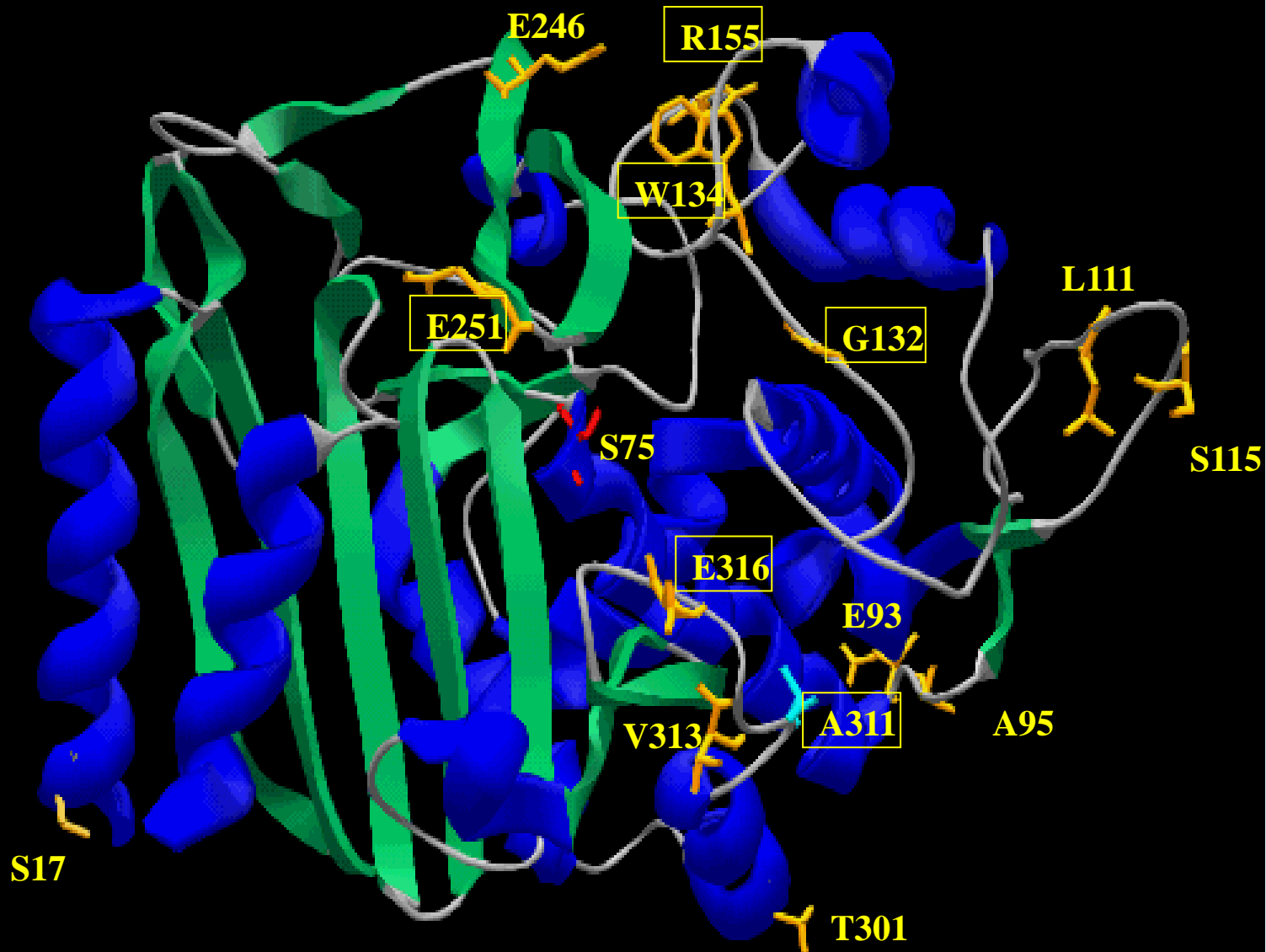
Esterase *Bg_EstB* Mutants with enhanced Stability (DMF)



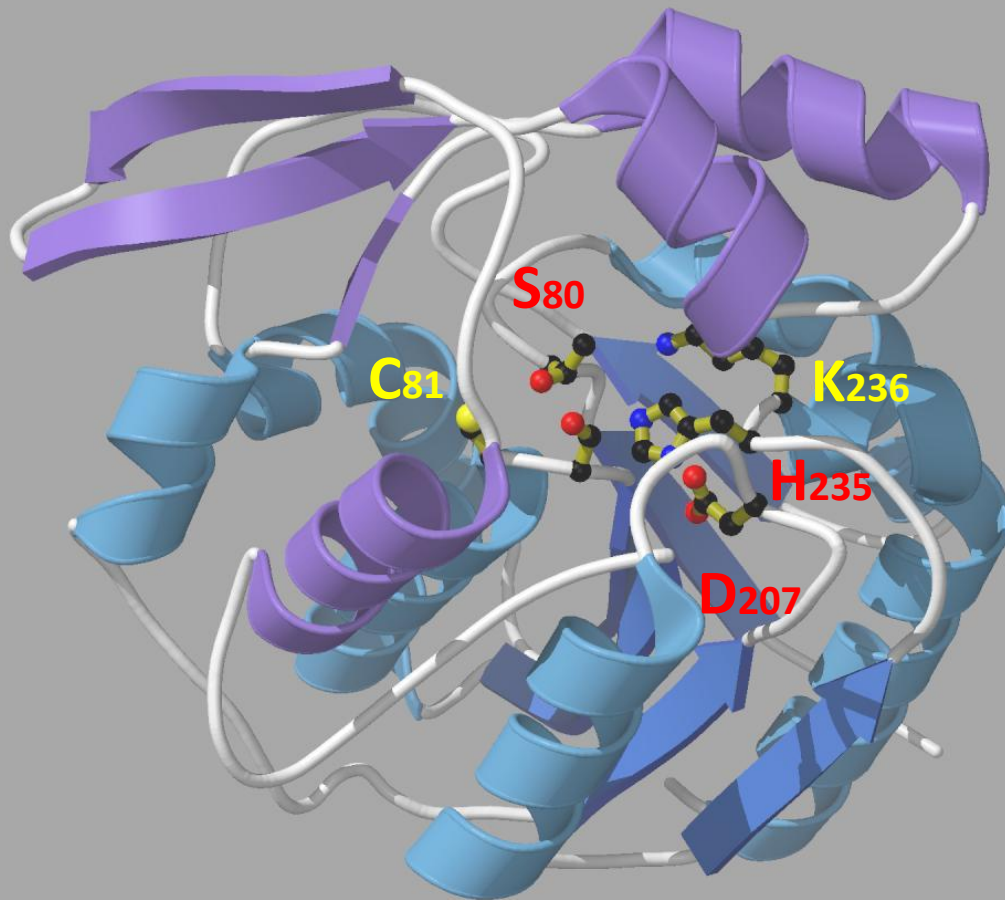
Esterase *Bg_EstB*

Mutants with erhöhter Stabilität (DMF)





Directed Evolution - Engineering



3-D structure of *Hb_HNL*

Example for
Family shuffling
by *in vivo*
Recombination

Hevea brasiliensis
Hb_Hnl

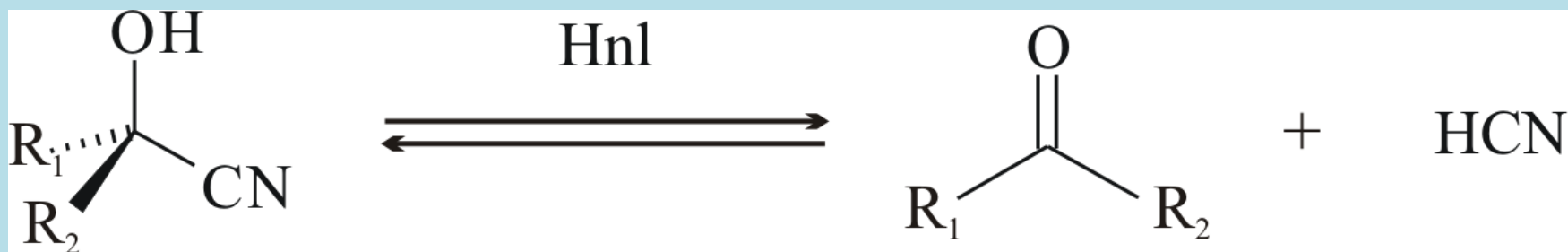
Manihot esculenta
Me_Hnl

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:

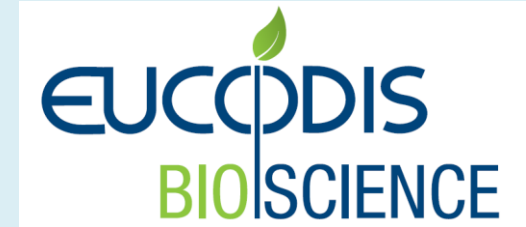
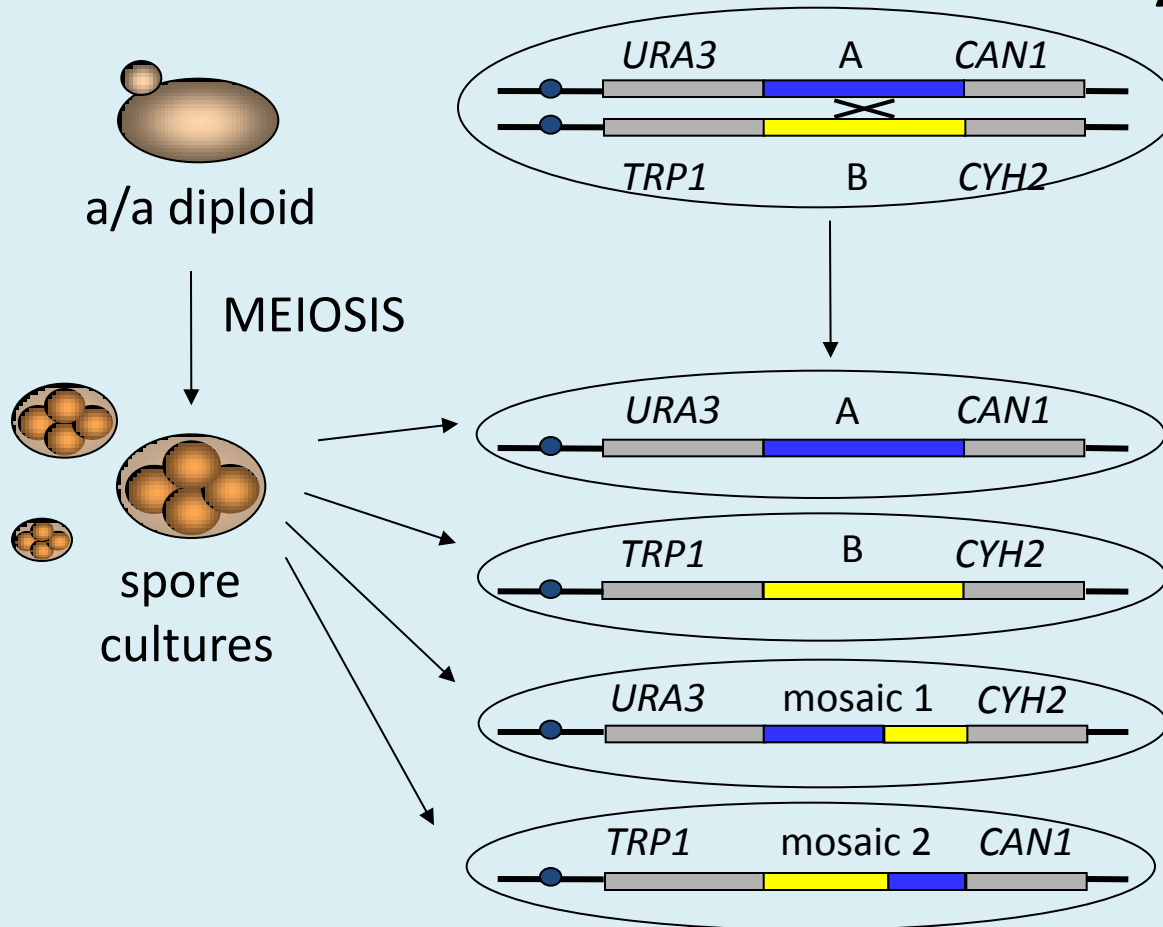
- **Improved stability and activity at low pH**
- **High activity with bulky substrates**

→ **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

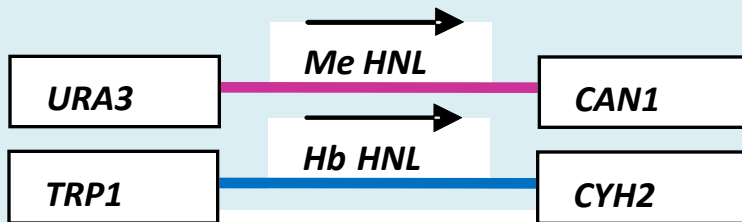
Recombination of MeHNL and HbHNL-W128A

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

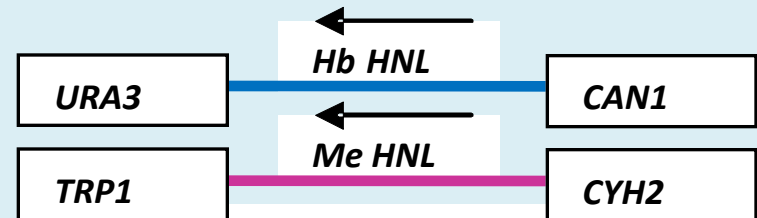
Me_Hnl wt: better stability at low pH

Aims:

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



MXY279
forward orientation

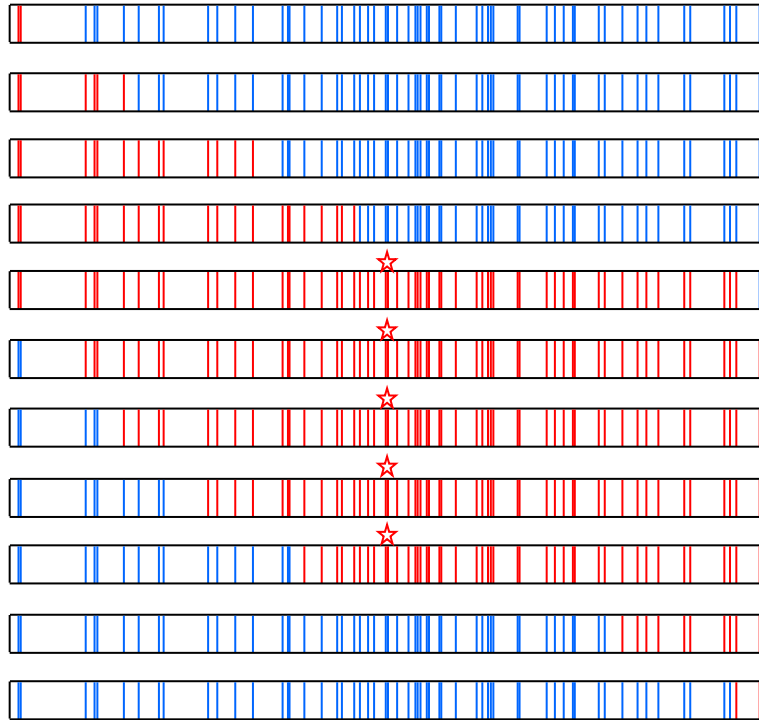


MXY287
reverse orientation

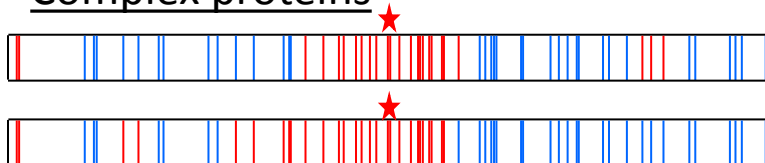
Enzyme Engineering → Recombination

Novel *Hb_HNLW128A* / *Me_Hnl* recombinant proteins

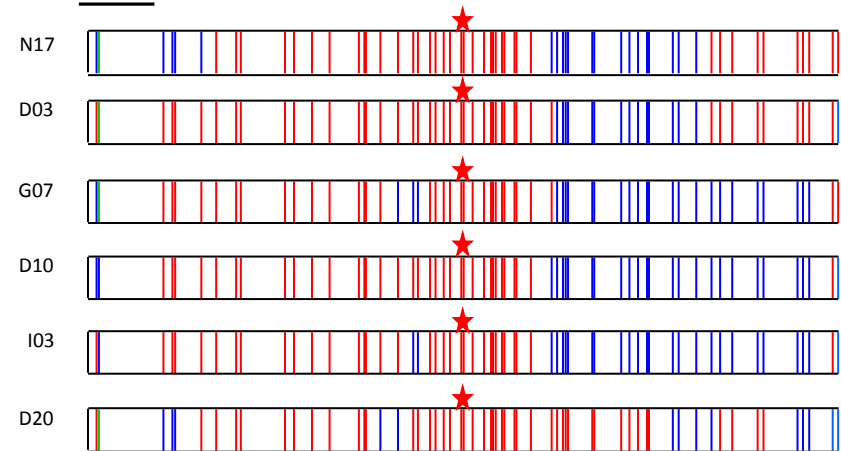
Bipartite proteins



Complex proteins

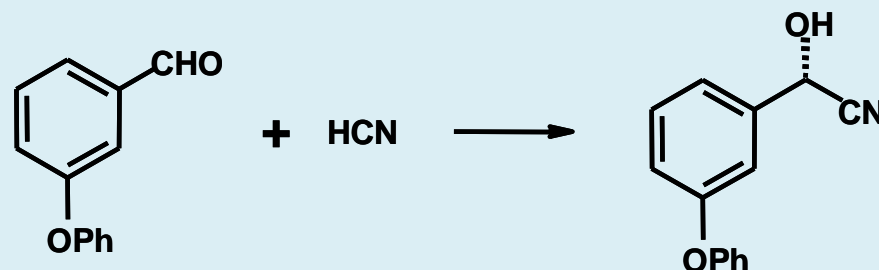


Hits

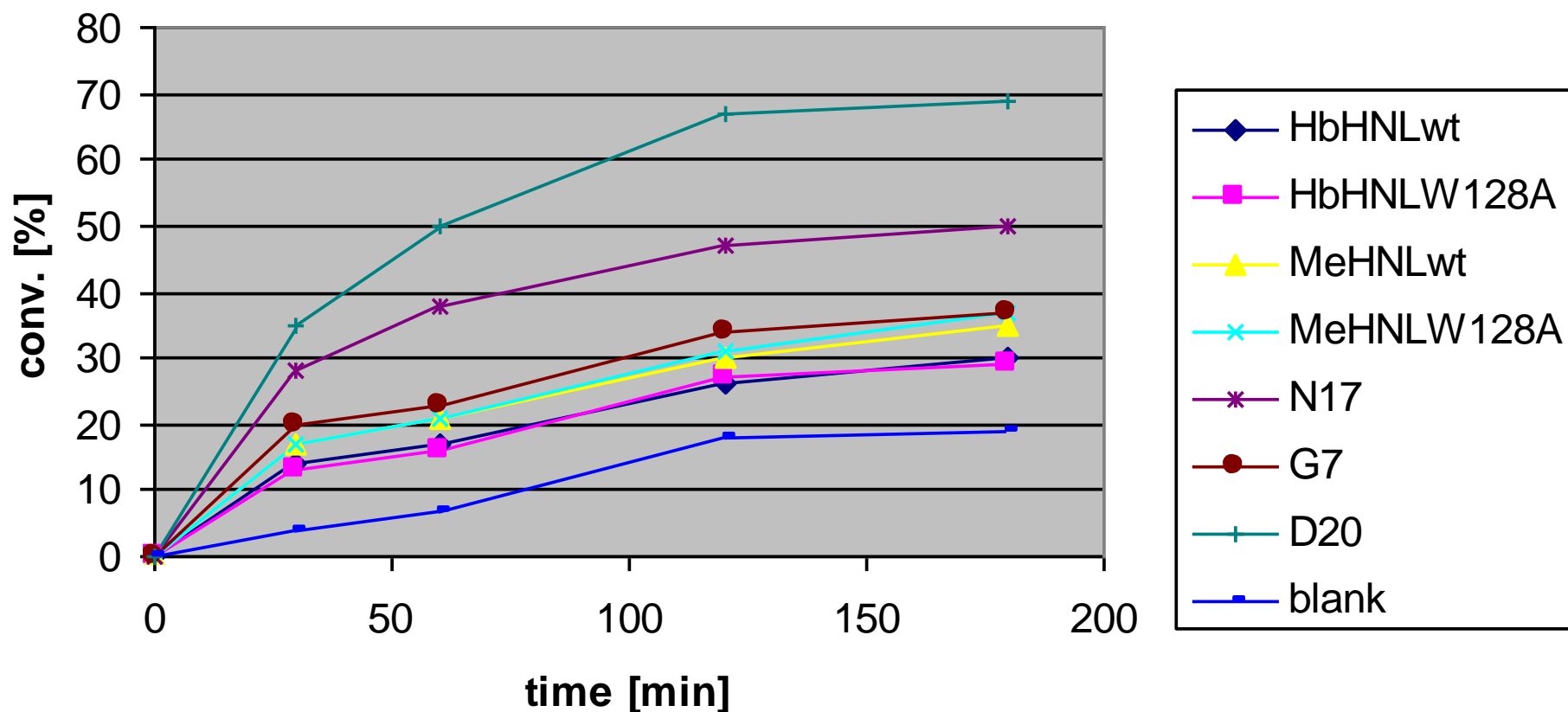


- Hb-specific amino acids
- Me-specific amino acids
- novel amino acids
- ★ Hb W128A alteration

Enzyme Engineering → Recombination



Conversion at pH 5.4



Enzyme Engineering → Recombination

HbHNL_W128A	(1)	MAFAHFVLIHTICHGAWIWHKLKPLLEALGHKVTALDLAAS
Hn1_N17	(1)	MVIAHFVLIHTICHGAWIWHKLKPALE <u>RAG</u> HKVTALDMAAS
HNL_D20	(1)	MAIAHFVLIHTICHGAWIWHKLKPALE <u>RAG</u> HKVTALDLAAS
MeHn1_WT (D7)	(1)	MVTAHFVLIHTICHGAWIWHKLKPALERAGHKVTALDMAAS

HbHNL_W128A	(42)	GVDPRQIEEIGSFDEYSEPLLTFLEALPPGEKVIIVGESC
Hn1_N17	(42)	GVDPRQIE <u>EIG</u> SFDEYSEPLLTFLE <u>ALP</u> GEKVIIVGESC
HNL_D20	(42)	GVDPRQIE <u>EIG</u> SFDEYSEPLLTFLE <u>ALP</u> GEKVIIVGESC
MeHn1_WT	(42)	GIDPRQIEQINSFDEYSEPLLTFLEKLPQGEKVIIVGESCA

HbHNL_W128A	(83)	GLNIAIAADKYCEKIAAAVFHNSVLPDTEHCPSYVVDKLME
Hn1_N17	(83)	GLNIAIAAD <u>KYCEK</u> IAAAVFHNSVLPDTE <u>HCPSYVVD</u> KLME
HNL_D20	(83)	GLNIAIAAD <u>KYCEK</u> IAAGVFHNSLLPDTE <u>HCPSYVVD</u> KLME
MeHn1_WT	(83)	GLNIAIAADRYVDKIAAGVFHNSLLPDTVHSPSYTVEKLLLE

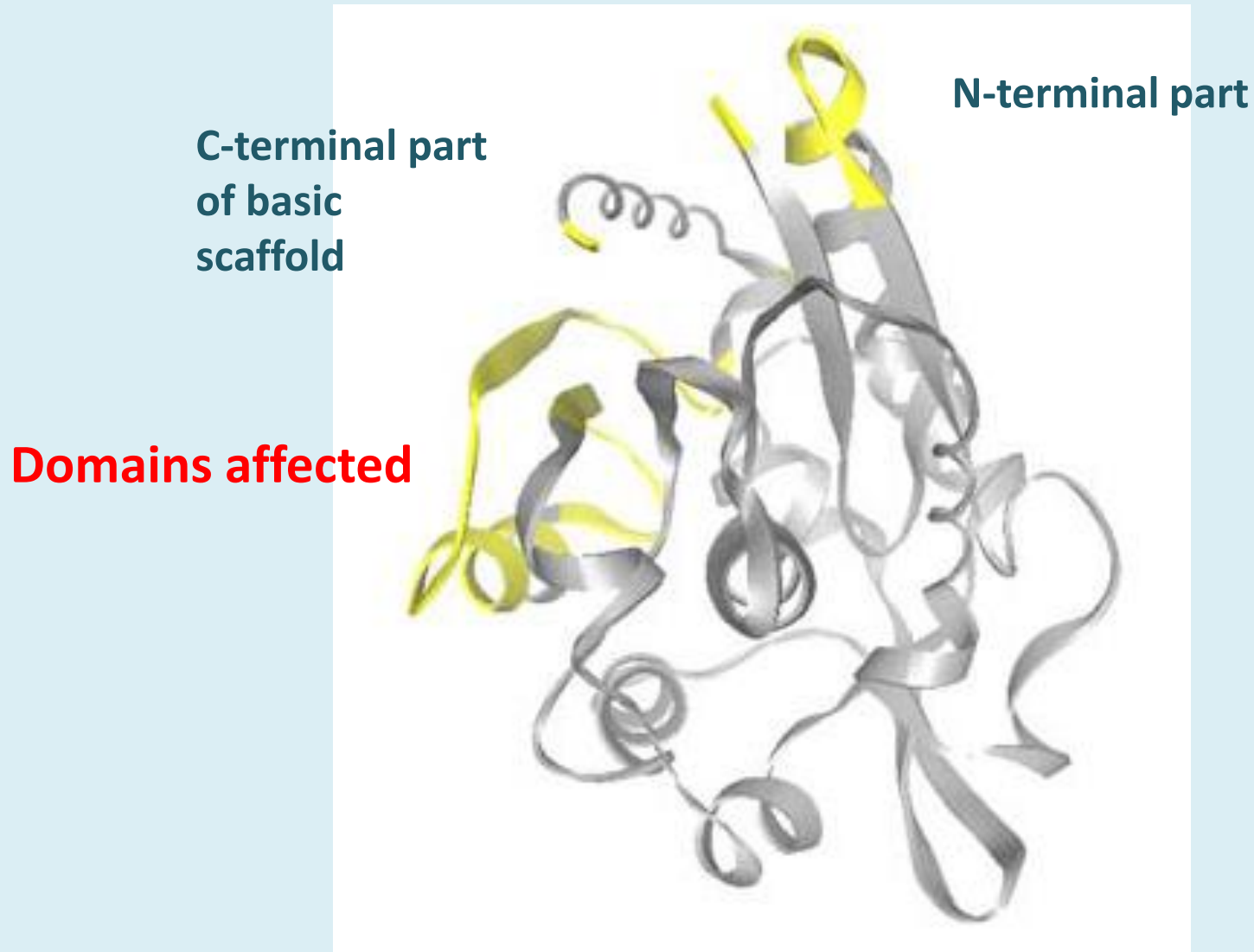
HbHNL_W128A	(124)	VFPDAKDTTYFTYT-KDGKEITGLKLGFTLLRENLYTLCGP
Hn1_N17	(124)	<u>VFPDAKDTTYFTYT-KDGKEITGLKLGFTLLRENLF</u> TKCTD
HNL_D20	(124)	<u>VFPDAKDTTYFTYT-KDGKEITGLKLGFTLLRENLYTLCGP</u>
MeHn1_WT	(124)	SFPDWRDTEYFTFTNITGETITTMKLGFLVLLRENLFTKCTD



Enzyme Engineering → Recombination

HbHNL_W128A	(164)	EEYELAKMLTRKGS LFQ NILAKRPF FT KEGYGSIKKIYVWT
Hn1_N17	(164)	<u>GEYELAKMVMRKGSLFQNVLAQRPKFTEKGYGSIKKVYIWT</u>
HNL_D20	(164)	<u>EEYELAKMLTRKGSLFQILAKRPFFTKEGYGSIKKVYIWT</u>
MeHn1_WT	(165)	GEYELAKMVMRKGS LFQ NVLAQRPKFTEKGYGSIKKVYIWT
HbHNL_W128A	(205)	DQDEIFLPEFQ LWQ IENYKPKDKVYKVEGGDHKLQLTKTKEI
Hn1_N17	(205)	<u>DQDKIFLPEFQLWQIENYKPKDKVYKVEGGDHKLQLTKTKEI</u>
HNL_D20	(205)	<u>DQDKIFLPDFQLWQIENYKPKDKVYKVEGGDHKLQLTKTEEV</u>
MeHn1_WT	(206)	DQDKIFLPDFQ RWQ IANYKPKDKVYQVQGGDHKLQLTKTEEV
HbHNL_W128A	(246)	AEILQEVADTYN
Hn1_N17	(246)	<u>AEILQEVADTYN</u>
HNL_D20	(246)	<u>AHILQEVADAYA</u>
MeHn1_WT	(247)	AHILQEVADAYA

Enzyme Engineering → Recombination



12.5.15

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

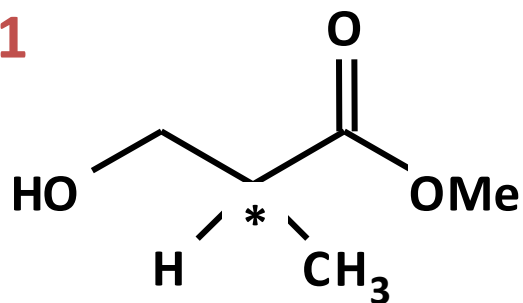
region-specific randomized oligonucleotide mutagenesis

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

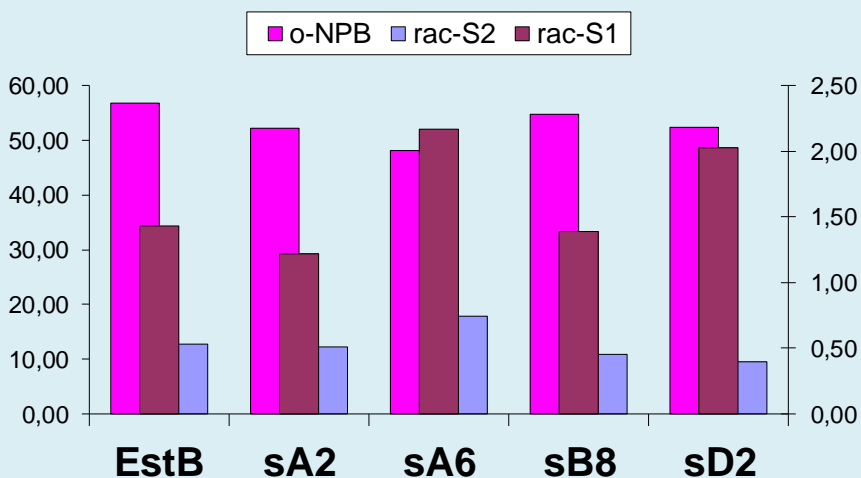
6000 clones screened
Colonie-Filter assay, pH-shift
Differential screen → (R) und (S)-S1
4 clones showing altered selectivity
1 clone showing enhanced (S)-selectivity

Esterase *Bg_EstB*

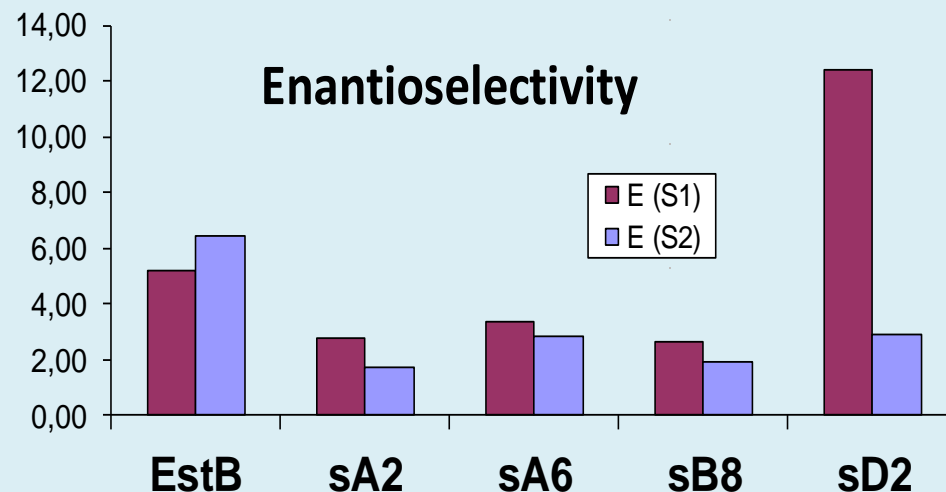
Mutants showing altered selectivity

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

Specific activity

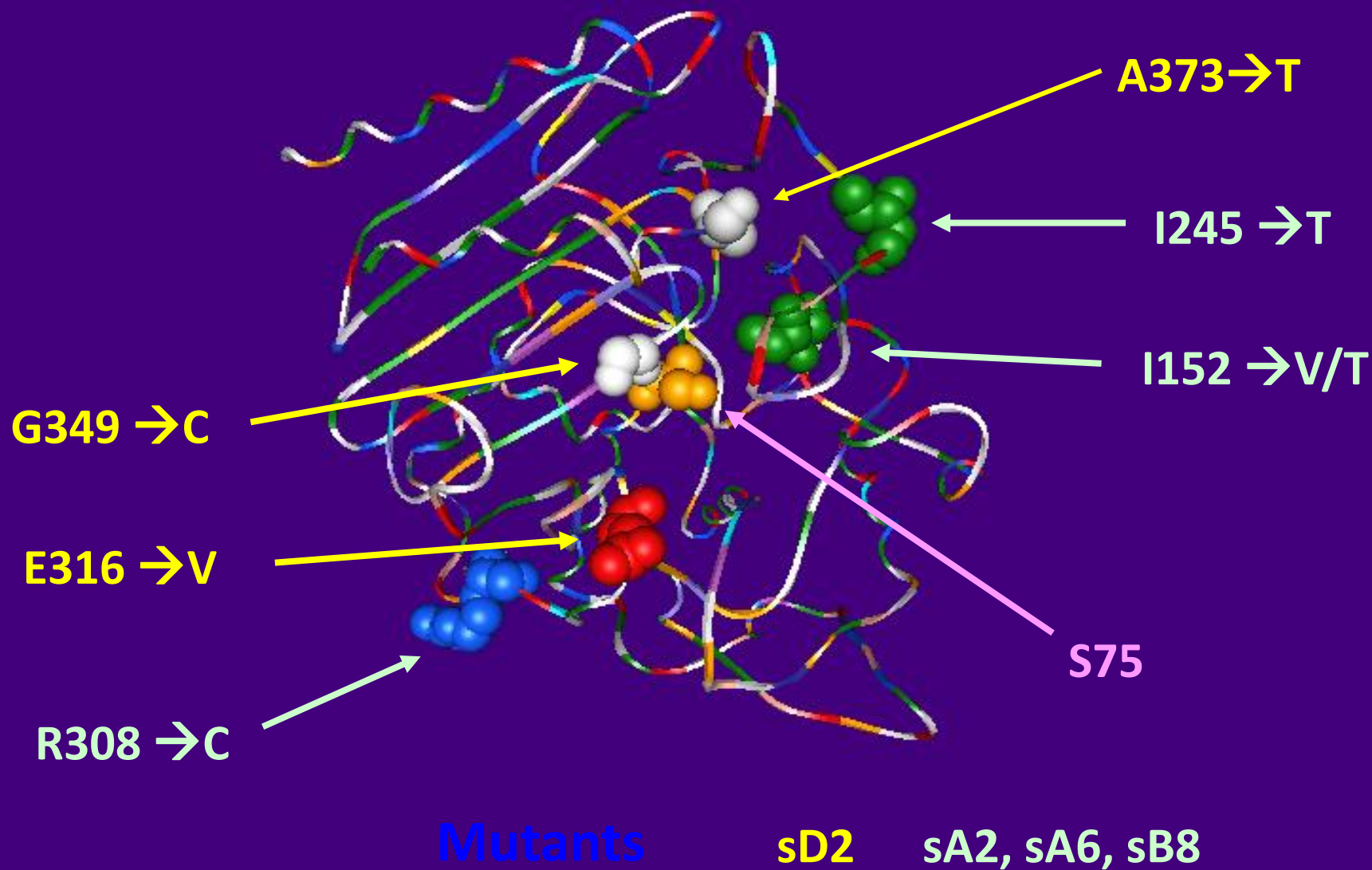


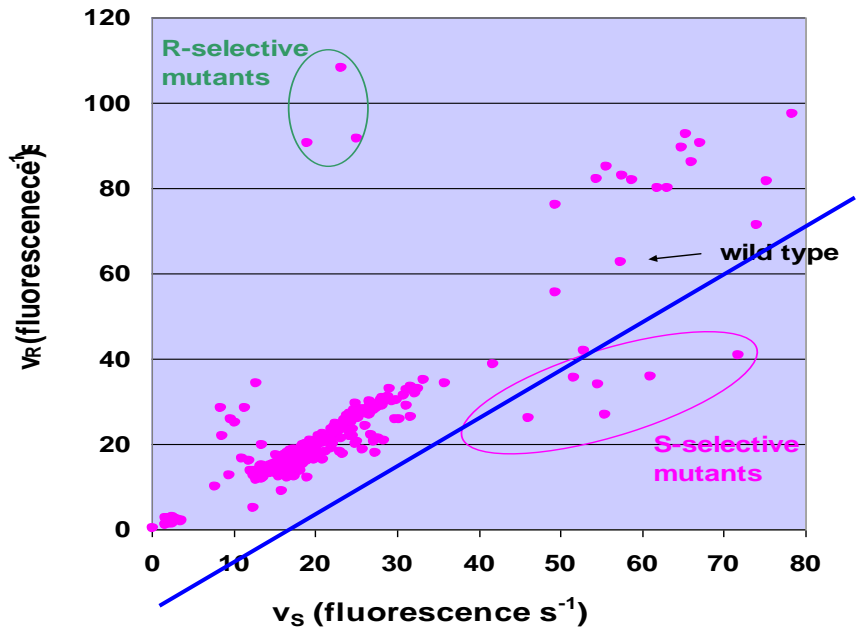
Enantioselectivity



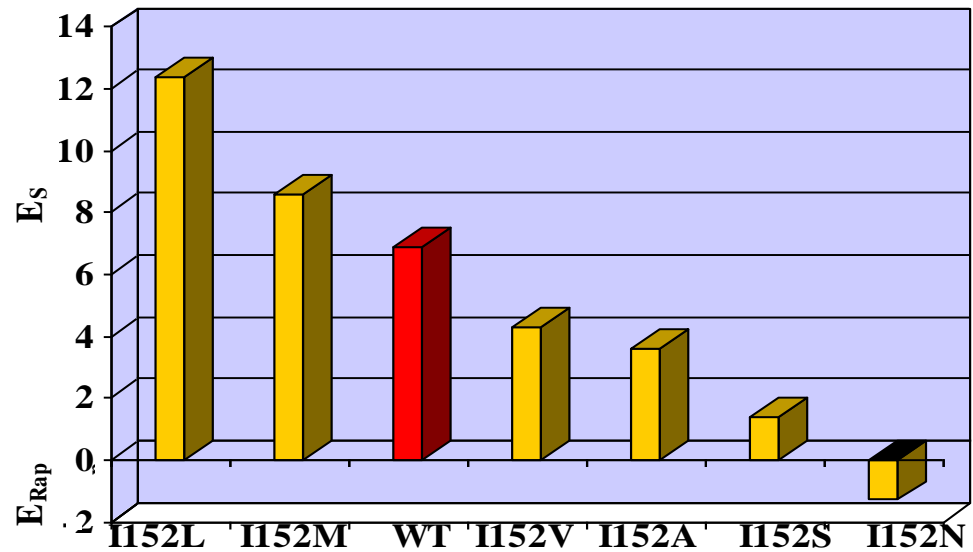
Esterase *Bg_EstB*

Mutants with improved selectivity





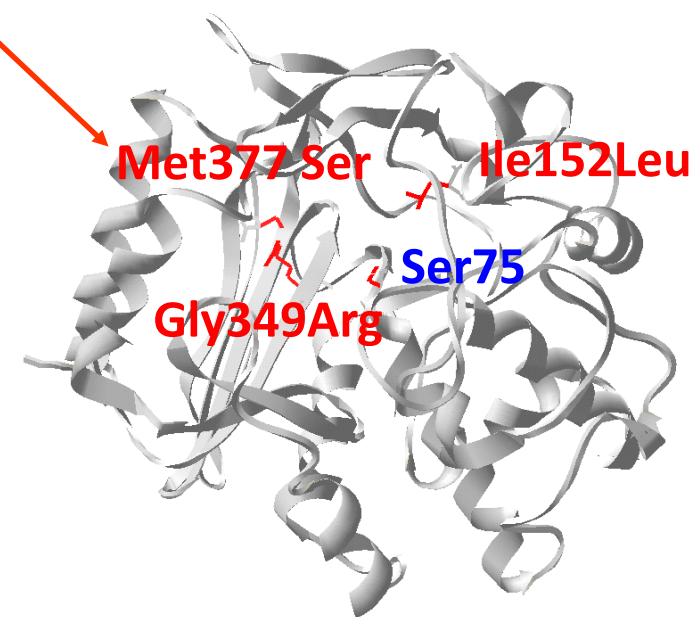
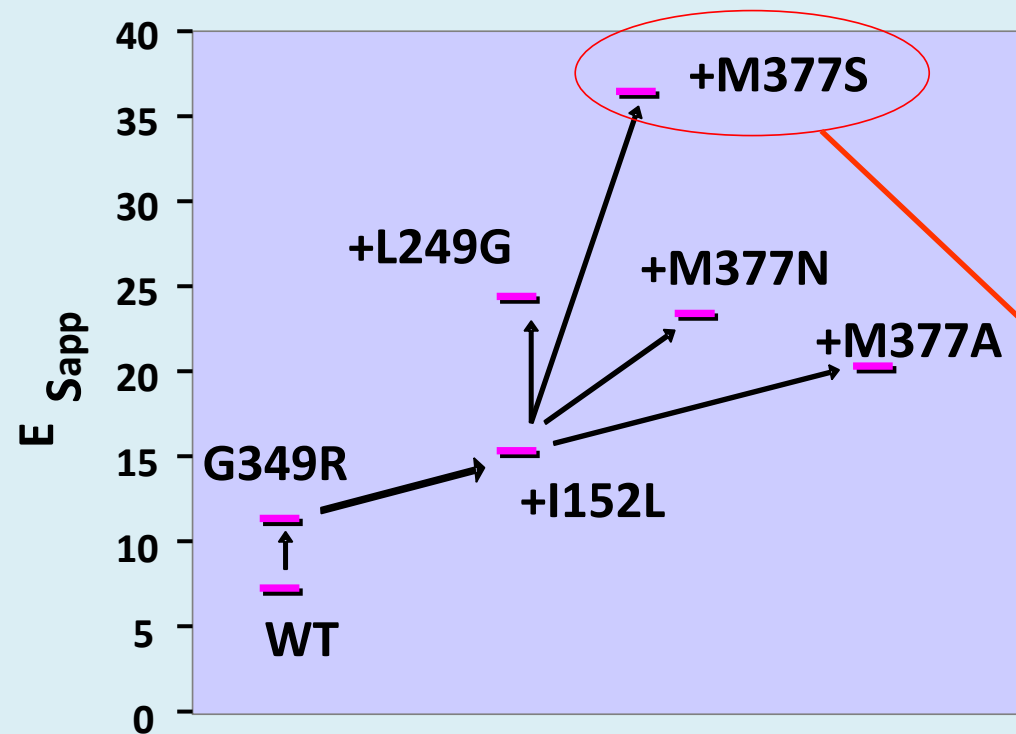
Saturation mutagenesis at position 152



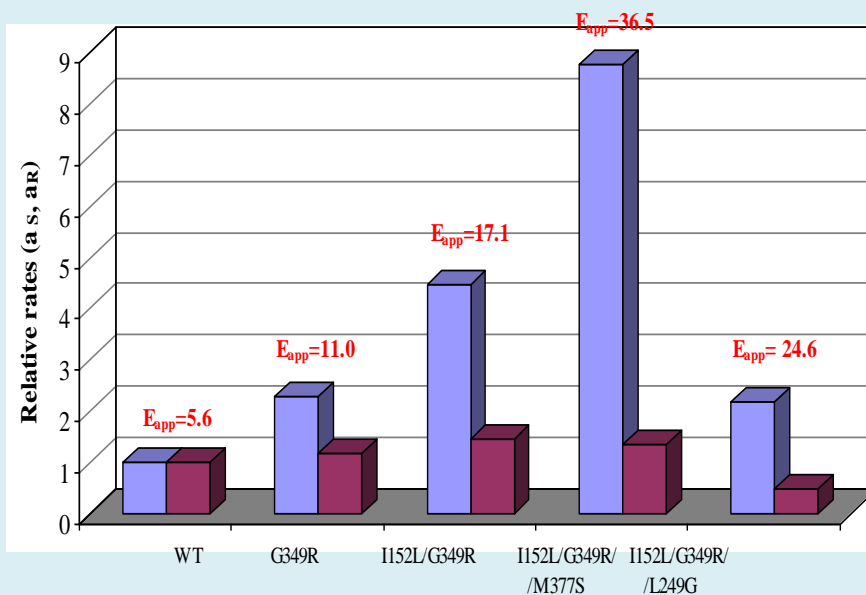
Improvement of S-enantioselectivity by semi-random approach

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

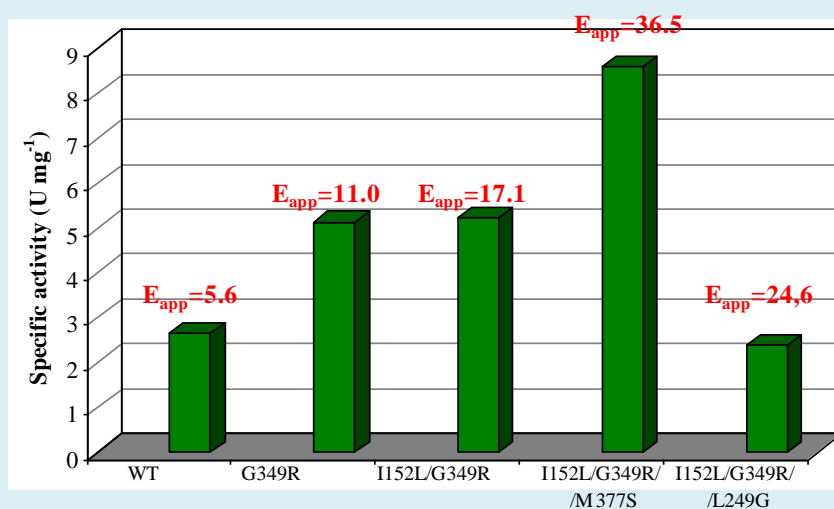
Positions 152, 249, 349 and 377 found to enhance S-selectivity



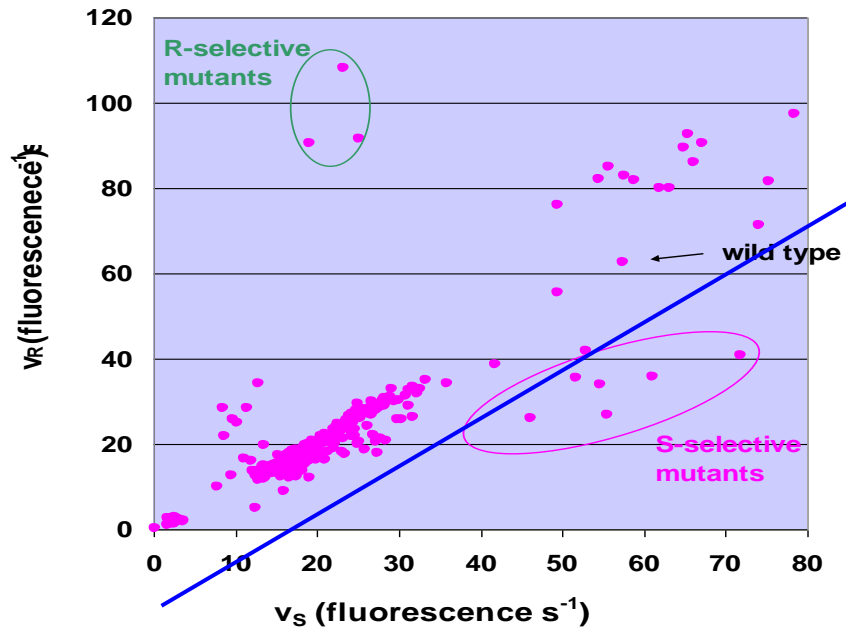
Esterase variant showing the highest S-enantioselectivity ($E_{Sapp} = 36$) harbors mutations I152L, G349R and M377S



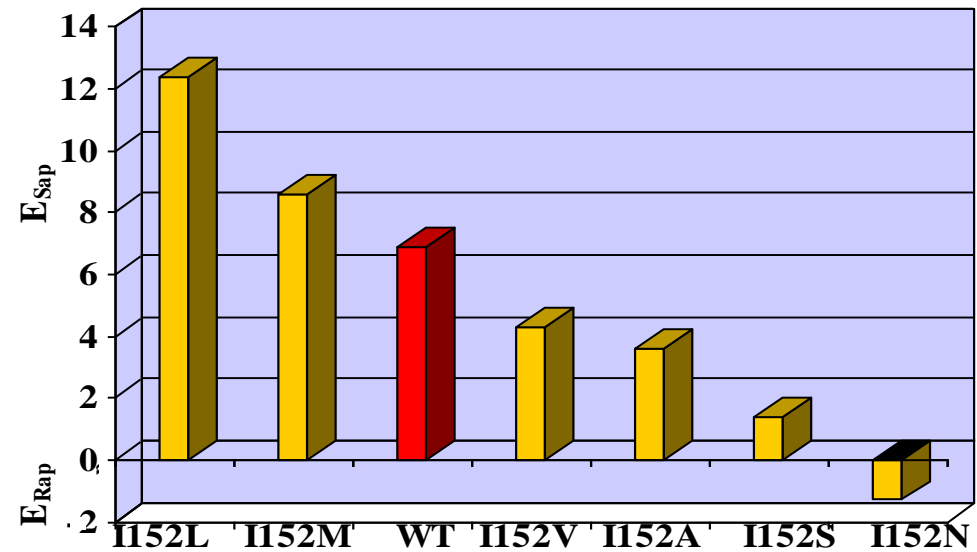
Relative activities towards S (■) and R (■) enantiomer



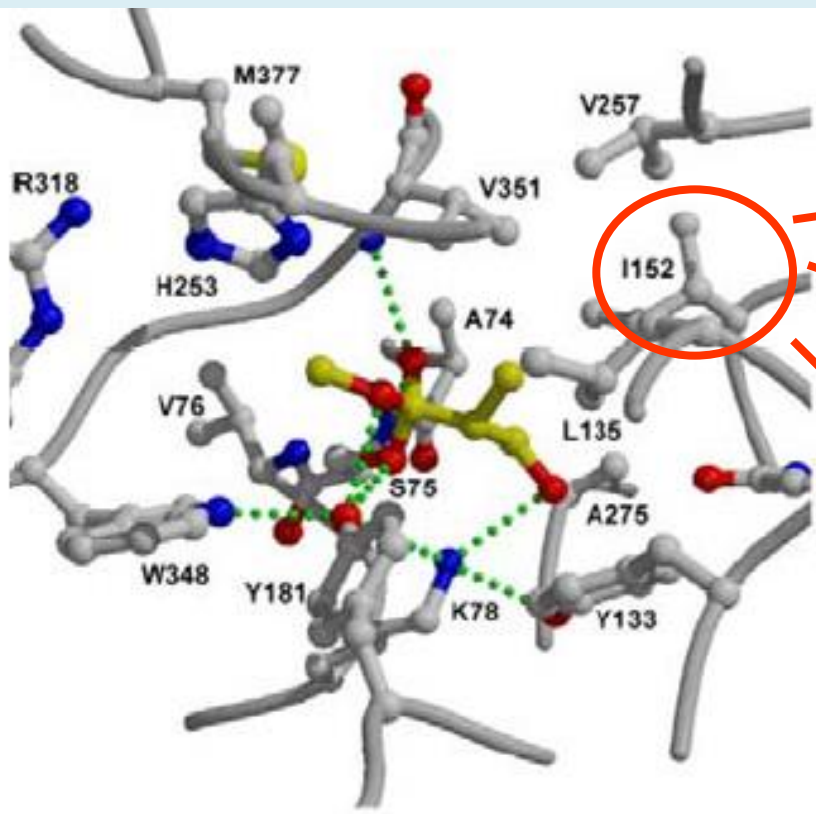
Specific activity towards racemic substrate (■)



Saturation mutagenesis at position 152



Enantioselectivity improvement of esterase EstB



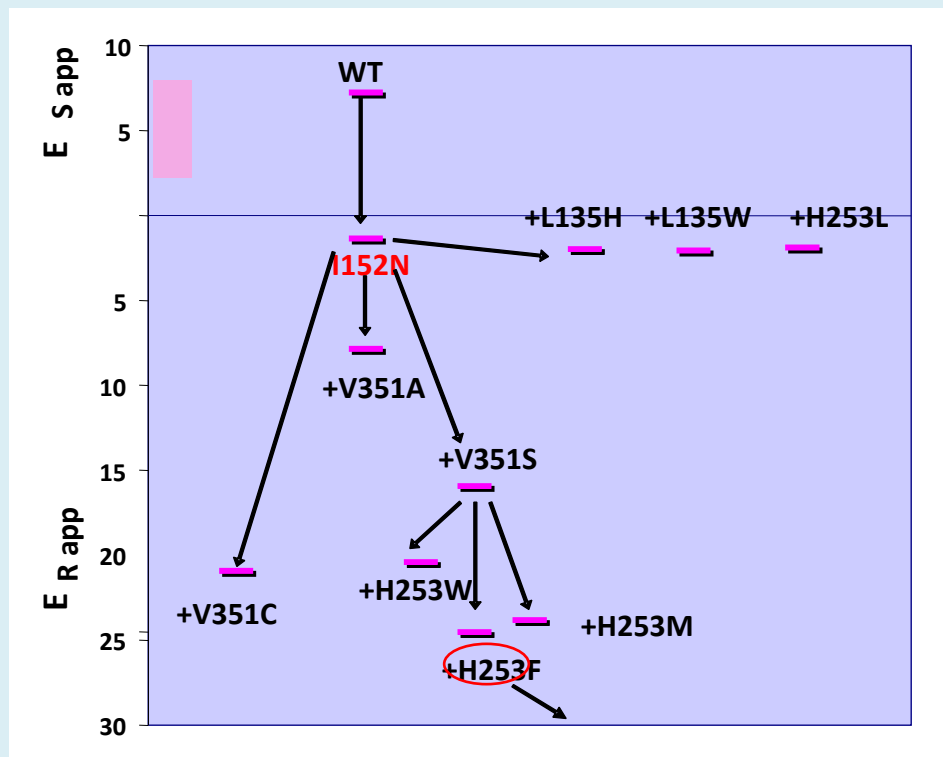
I152L → enhanced S-selectivity

I152V → lowered S-selectivity

I152N → conversion to R-selectivity

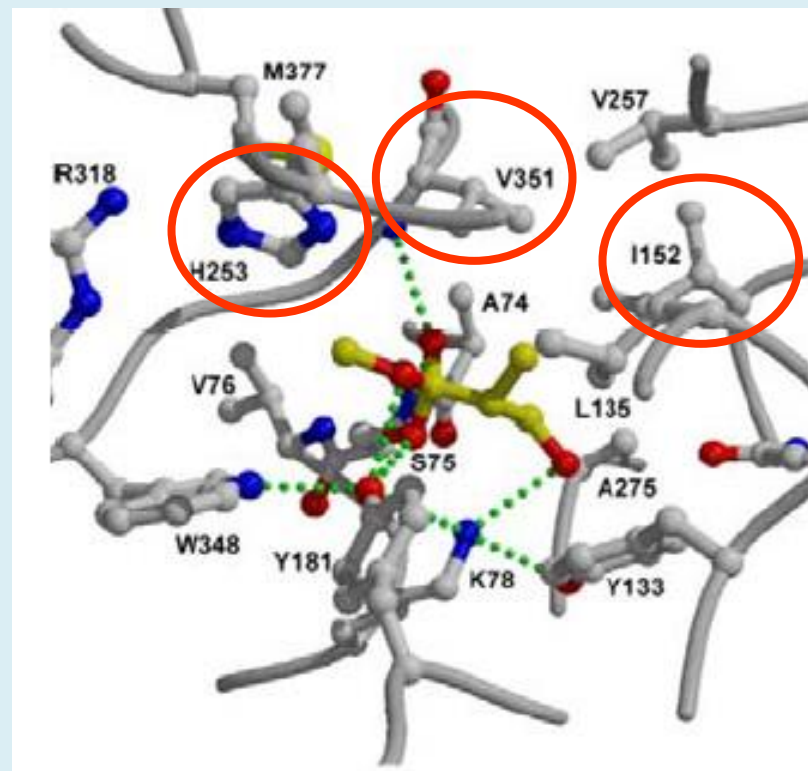
methyl- β -hydroxyisobutyrate modelled in active site

Improvement of the R-stereoselectivity by semi-random approach



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

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



Activities of R-selective mutations

Mutations	Relative a_S	activity 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??**

Table 1. Well characterized and sequenced HNLs

	<i>MeHNL</i> [22,52]	<i>HbHNL</i> [53,54]	<i>PaHNL</i> [55]	<i>LuHNL</i> [56,57]	<i>SbHNL</i> [58]	<i>AtHNL</i> [9,28]
Source organism	<i>Manihot esculenta</i> (cassava)	<i>Hevea brasiliensis</i> (para rubber tree)	<i>Prunus amygdalus</i> (bitter almond)	<i>Linum usitatissimum</i> (flax)	<i>Sorghum bicolor</i> (millet)	<i>Arabidopsis thaliana</i> (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 (?)	-	-
Posttranslational modifications	-	-	glycosylation	-	glycosylation	-
Quarternary structure	homo-tetramer (?)	homodimer	monomer	homodimer	hetero-tetramer	homodimer
Hosts for heterologous expression	<i>E. coli</i> <i>P. pastoris</i> <i>S. cerevisiae</i>	<i>E. coli</i> <i>P. pastoris</i> <i>S. cerevisiae</i>	<i>P. pastoris</i> (isoenzyme 5)	<i>E. coli</i> <i>P. pastoris</i>	-	<i>E. coli</i>
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]	1JU2 [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-benzaldehyde cyano-hydrin [64]	2-chloro mandelo-nitrile [64]	-	-	-

Novel bacterial Esterases

Xv_EstD **GGD**PTRVAVM**GH****S**AGAHIAGLLVTD~~RR~~WLQAQ**G**
Rrh_EstA **GGD**PT~~RI~~VLAG**D****S**AG**GN**LAASVAIAARDGGGPA
Rru_EstA AAADAPLIV**G****D****S**AG**GN**LA~~AV~~VQRAVRENGPE
Rrh_EstC **GGD**PDRVTI**AG****E****S**AGAMS~~VV~~SLLAMPAARG~~L~~FR
Xv_EstA **G**IDAQRV**G**VM**G****F****S**AG**G**HVAASLGTRYAAQVYPA
Xv_EstB **GGD**AGNVT**V****F****G****Q****S****G****G**AKIATLMAMPAARG~~L~~FH

Bs_EstA LHPDRPFV**L****F****G****H****S****M****G****M**VAFRLAQKLEREGIYP
Bg_EstC ALGHPRV**V****L****V****G****H****S****M****G****G**VAITAAAERAP**E**RIAAL
Bg_EstD TLGLEK**P****L****L****V****G****H****S****L****G****G**AIALAVGLDHPDSVSRI
Bg_EstE QLGAGPV**H****L****V****G****H****S****R****G****G**CVAFYMAHRY**P**ELVRS**L**

Rrh_EstB FGIERLALV**T****G****G****S****M****G**AQQTYEWAVRFPDKVLRA
Rrh_EstD ECPLTTYV**L****T****G****F****S****Q**GAVIVGDVAAQIGAGNGPV

Xv_EstE DSAFDQ**T****V****F****F****G****D****S****L****T****D****S****G**YYNPLLPAASRAV**T****G**
Bg_EstA AGVQKQ**I****V****S****F****G****D****S****L****S****D****A****G****T****Y****S****P****Q****I****L****L****G****F****G****G****R****F**
Xv_EstC PLAASK**I****V****L****V****G****D****S****T**TAVHGGWG**P**SFCAQH**V****T****S****F**

Bg_EstB RPMREDTLFRLA**S****V****T****K****P**I**V**A**L**A**V**L**R**L**V**A**R**G**E**L**A**

GxSxG Family

GDSL Family

SxxK Family

Esterases and Hydroxynitrile Lyases

Hb_Hnl...HT.
Bg_EstC...HG.

Hb_Hnl...GES
Bg_EstC...GHS

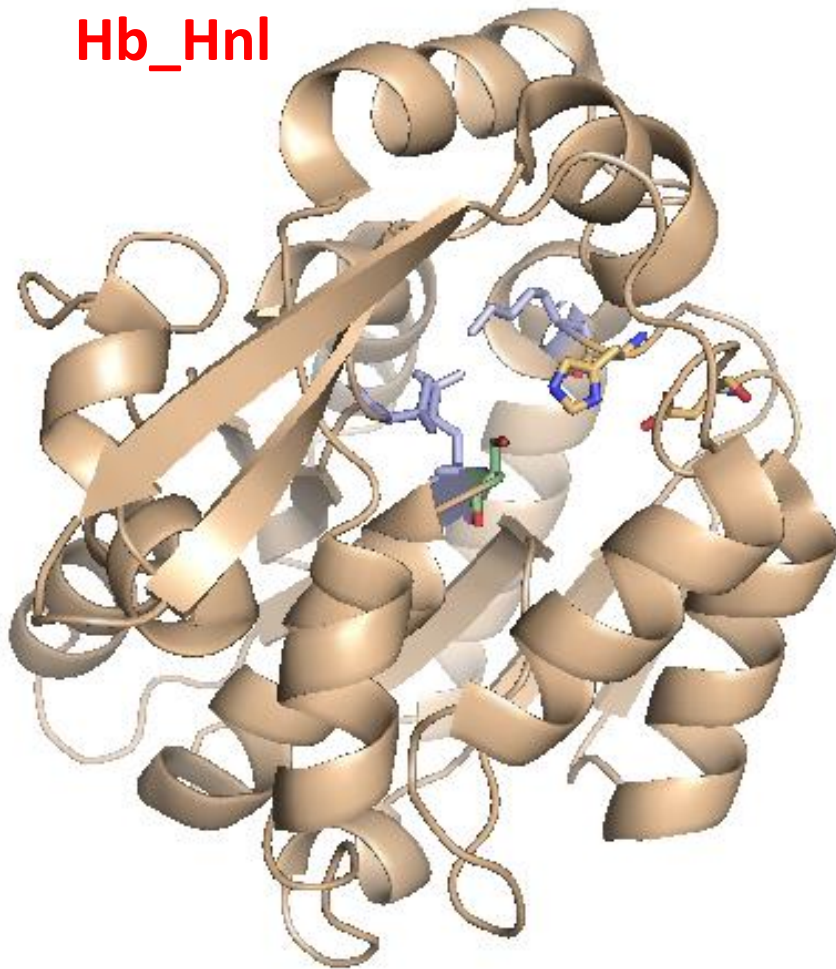
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sp|P52705|HNL_MANES     MVT-----AHFVLIHTICHGAWIWHKLPALERAGHKVTALDMAASGIDPR-----QIEQINSFDEYSEPLLTFLFK-LP-QGEKVIIVGESCAGLNIA
tr|Q9LFT6|HNL_ARATH     MER-----KHFFVLIHAYHGAWIWKLPPLLESAGHRVTAVELAASGIDPR-----PIQAVETVDEYSKPLIETLKS-LP-ENEVILVGFSGGINIA
tr|Q6ED34|Est_SOLLC     MEKG-----DKNHFVLIHGACHGAWCWYKVVVILRSEGHKVSVDMAASGINPK-----HVDDLNSMADYNEPLMEFMNS-LP-QLERVVLVGHSMGGINIS
tr|Q56SE1|Est_SOLTU     MEKG-----NKNHFVLIHGACHGAWCWYKVVVILRSEGHKVSVDMAASGINPK-----HVEDLNSMADYNEPLMEFMNS-LP-QQERVVLVGHSMGGINIS
sp|Q0JG99|PIR7B_ORYSJ  MEISS-----SSKKHFILVHGLCHGAWCYRVVAALRAAGHRATALDMAASGAHPA-----RVDEVGTFEEYSRPLLDAAVAAAA-PGERLVLVGHSHGGLSVA
tr|Q0JG98|PIR7A_ORYSJ  MEDG-----GKHFFVLIHGCHGAWCYRVVAALRAAGHRATALDMAAGAHPA-----RADEVGSLEEYSRPLLDAAVAA-AA-PGERLVLVGHSLGGLSLA
tr|Q161F3|RdEstC_ROSDO MA-----DILLIHGSCGAWCWKLIPLCLNAKGHMARAIPLPSHGADT-----PVQT-VTLDCYAQAIVENC-----HEQTVLVGHSMGGYAIS
tr|Q9LAB8|BgEstC_BURGA MNHPDIDTHSRNAAAPLPEVLIHGAWHGAWAYERLGAALAAARGHASVAHDLPAGHINARYPAAFWQGDAQALAQEPPSVAA-TTLLDDYTQQVLRIDAACALGHPRVVLVGHSMGGVAIT
* : : : * * * * : : . * ** . : : : * . : : * : : * * * * . : :
IAADKYCEKIAAAVFHNSVLPDTEHCP-SYVVDKLMEVFP---DWKDTTYFTY-TKDGEITGLKLGFTLLRENL---YTLCGPEEYELAKM---LTRKGSFLQ-N-ILAKRPFFTEKG
sp|P52705|HNL_MANES     IAADRYVDKIAAGVFHNSLLPDTVHSP-SYTVKELLESP---DWRDTEYFTFTNITGETITTMKLGFTLLRENL---FTKCTDGEYELAKM---VMRKGSLFQ-N-VLAQRPKFTEKG
tr|Q9LFT6|HNL_ARATH     LAADIFPAKIKVLVFLNAFLPDTTHVP-SHVLDKYMEMP---GLGDCEFSSH-ETRNGTMSLLKMGPKFMKARL---YQNCPIEDYELAKM---LHRQGSFFT-E-DLSKKEKFSEEG
tr|Q6ED34|Est_SOLLC     LAMEKFPQKIVVAVFVTAEMPGPDLNL-VALGQQYNQVQVE---SHMDTEFVYN-NGQDKAPTSLVLGPEVLATNF---YQLSPPEDLTLATY---LVRPVPLFD-ESILLANTLSKEK
tr|Q56SE1|Est_SOLTU     LAMEKFPKHIAVAVFVSASMPGPDNL-VAVTQQYSQQVE---TPMDTEFVYN-NGLDKGPSTSVVLGPKVLATYIY---YQFSPPEDLTLATY---LVRPVPLFD-ESVLLTNTLSKEK
sp|Q0JG99|PIR7B_ORYSJ  LAMERFPDKVAAAVFLAACMPAAGKHM-GVTTEEFMRRTAPEGLLMDCEMVAI-NNSQSGVAINLGPFLAQKY---YQSPAEIDLALAKM---LVRPGNQFMDDPVMKDESLLTNGN
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tr|Q161F3|RdEstC_ROSDO AAAERVPEQIAQLIYLCAVYPQNGMTL-AQMRKKA---PRQPLL---P-AV-RMAPD-GLSFTIDPEMAPDIF---YHDCAPGDVEFALT---RLCPQAVAPTN-A---PLADMSA
tr|Q9LAB8|BgEstC_BURGA AAAERAPERIAALVYLAAMPASGVPGLDYVRAPE-----NHGEML---ASLI-CASPRAIGALRINPASRDAAYLATLQALFEDVDDEATFRAVTRLMSSDV-PTA-PFATPIATTAER
* : : : * * * * : : . * ** . : : : * . : : * : : * * * * . : :
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sp|P52705|HNL_MANES     YGSIKKVIWTDQDKIFL-PDFQRWQIANYK-----PDKVYQVGGDKHKLQTKTEEVAHILQEVAD-AYA
tr|Q9LFT6|HNL_ARATH     YGSVQRVYVMSSEDKAIP-CDFIRWMIDNFN-----VSKVYEIDGGHMVMLSKPKLFDLSAIAT-DYM
tr|Q6ED34|Est_SOLLC     YGSVHRVYVVCDDKNVLEKQQFQKWLINNNP-----PDEVQIIHNADHMVMFSKPRDLSSCLVMISQ-KYY
tr|Q56SE1|Est_SOLTU     YGSVHRVYVVCDDKVLKEEQFORWLKNNP-----PNEVQMIHDAGHMVMFSKPRELCSCLVMISQ-KYH
sp|Q0JG99|PIR7B_ORYSJ  YGSVKVYVIAKADSSST-EEMQRWLVWAMP-----GTDVEEIIAGADHAVMNSKPRELCLLIKIAN-KYE
sp|Q0JG98|PIR7A_ORYSJ  YGSVVRVFLVAMDASSD-EEMQRWTTDLSP-----GVEVEELAGADHMAMCSKPRELCLLLRIA-KYD
tr|Q161F3|RdEstC_ROSDO VEKLPERSYIRCMDRTVP-PEFQVTMTQDWP-----AORLHQMDCGHSPPFSDPETLATHIDQ---AIRG
tr|Q9LAB8|BgEstC_BURGA WGSIAHVVTCAEDRVIL-PALQRRFIAEADAFLPERPTRVHALD---SSHSPPFSQPDTLAELLTGIAARNTAI
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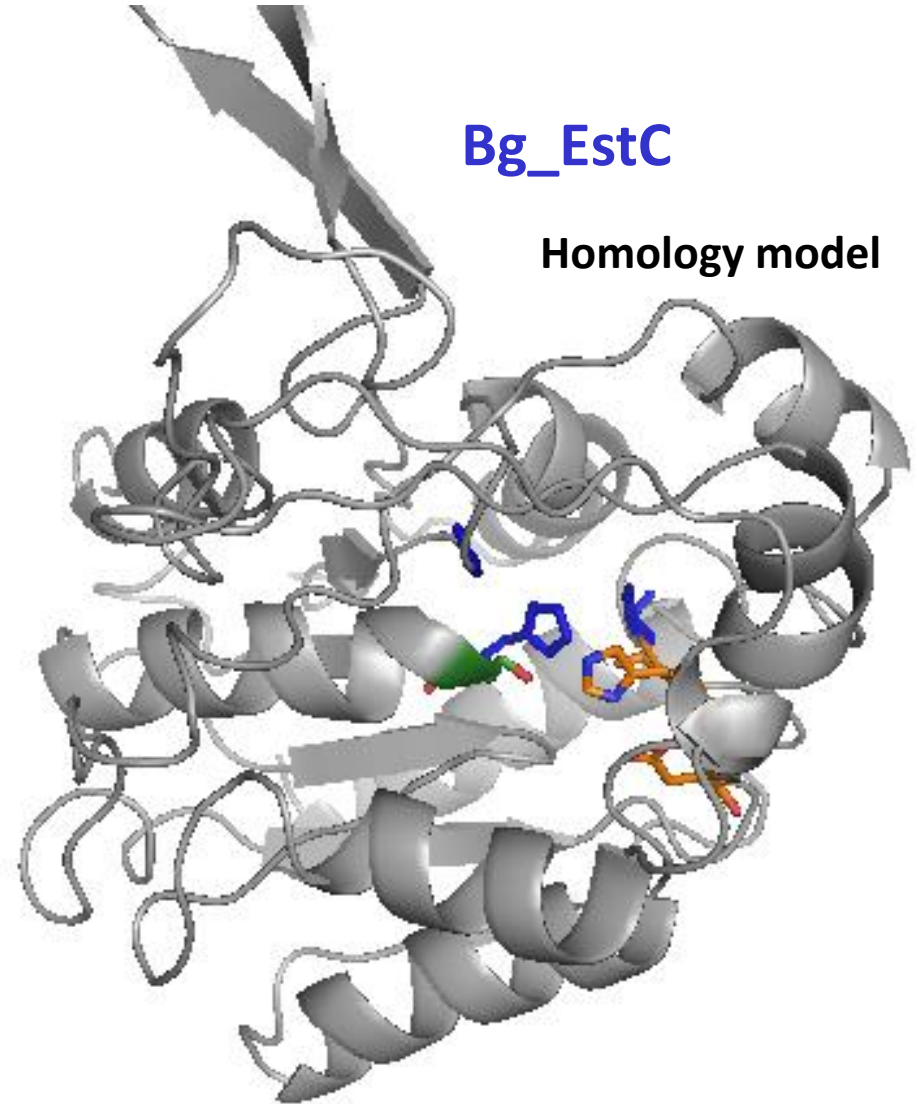
Hb_Hnl...HK.
Bg_EstC...HS.

Hb_Hnl



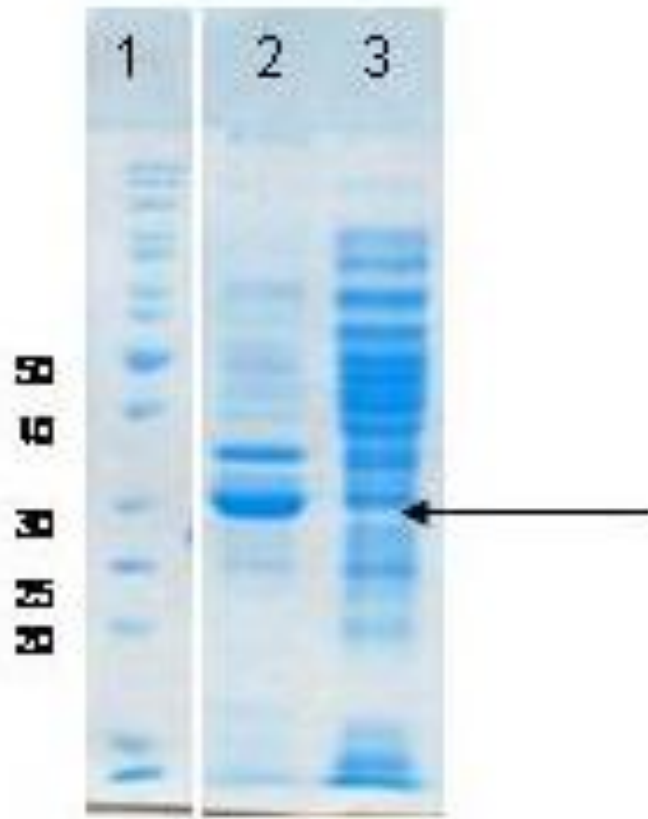
Triade: Ser80, His235, Asp207
Important: Lys236

Bg_EstC



Triade: Ser112, His275, Asp242

Bg_EstC Muteins designed for Hnl activity



1st Generation:

S276K

2nd Generation:

S276K

G24T

H111E

1: #SM0661 (Fermentas) (5 μ l)

2: *Bg_EstC* S276K pellet (2 μ l)

3: *Bg_EstC* S276K raw lysate (2 μ l)

Cyanohydrin cleavage reaction



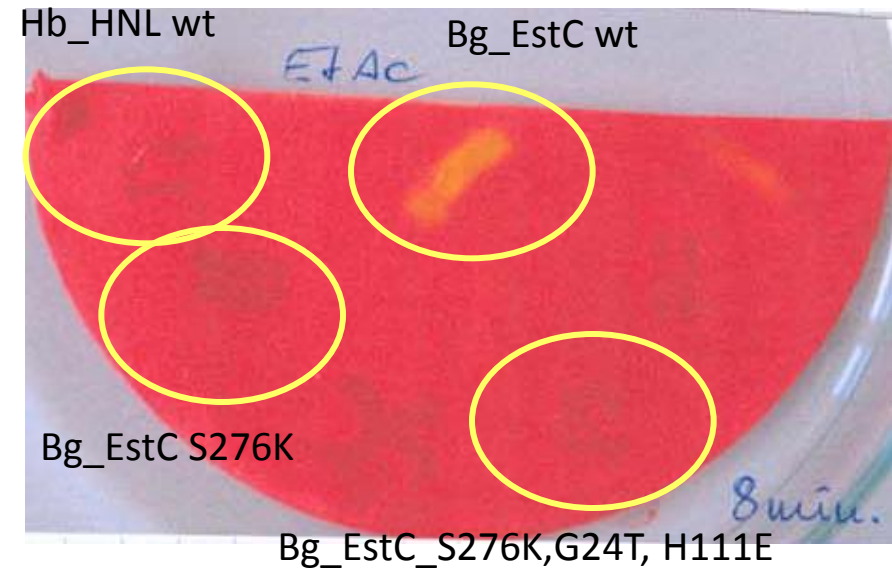
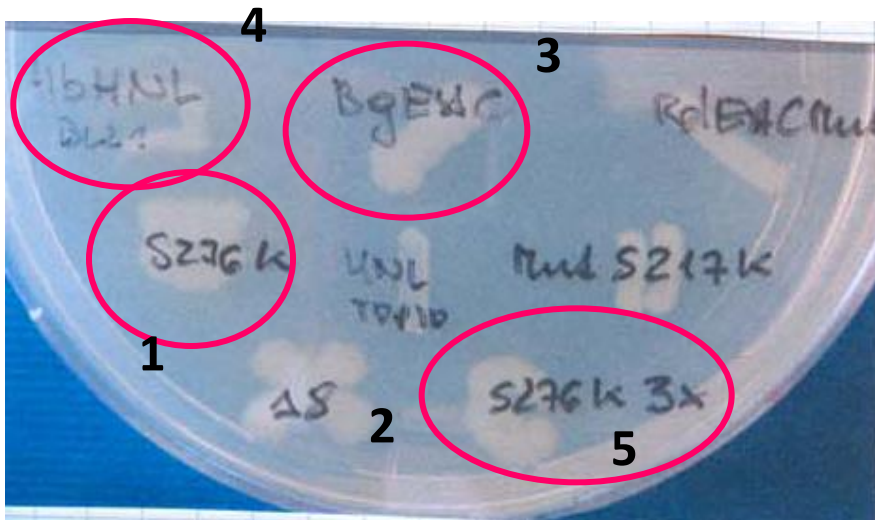
- 1: Bg_EstC S276K
- 2: Vector strain
- 3: Bg_EstC wt
- 4: Hb_HNL wt

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

acetone cyanohydrin,
pH 3.5, 3.5 min

Esterase activity

Activity on ethyl acetate



1: *Bg_EstC* S276K

2: Vector strain

3: *Bg_EstC* wt

4: *Hb_HNL* wt

5: *Bg_EstC_S276K,G24T, H111E*

Cyanohydrin Synthesis Reaction

