Enzymes in Lipid Modification

From Classical Biocatalysis with Commercial Enzymes to Advanced Protein Engineering Tools

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Outline

- Introduction
- Lipase-Catalyzed Synthesis of Structured Triglycerides
- Lipase-Catalyzed Synthesis of Sugar Esters
- Characterization of Lipases from Metagenome
- Removal of 3-MCPD with Dehalogenase/Epoxide Hydrolase
- Phospholipase C in Degumming
- Protein Engineering to Alter Fatty Acid Selectivity of Lipase
- Summary

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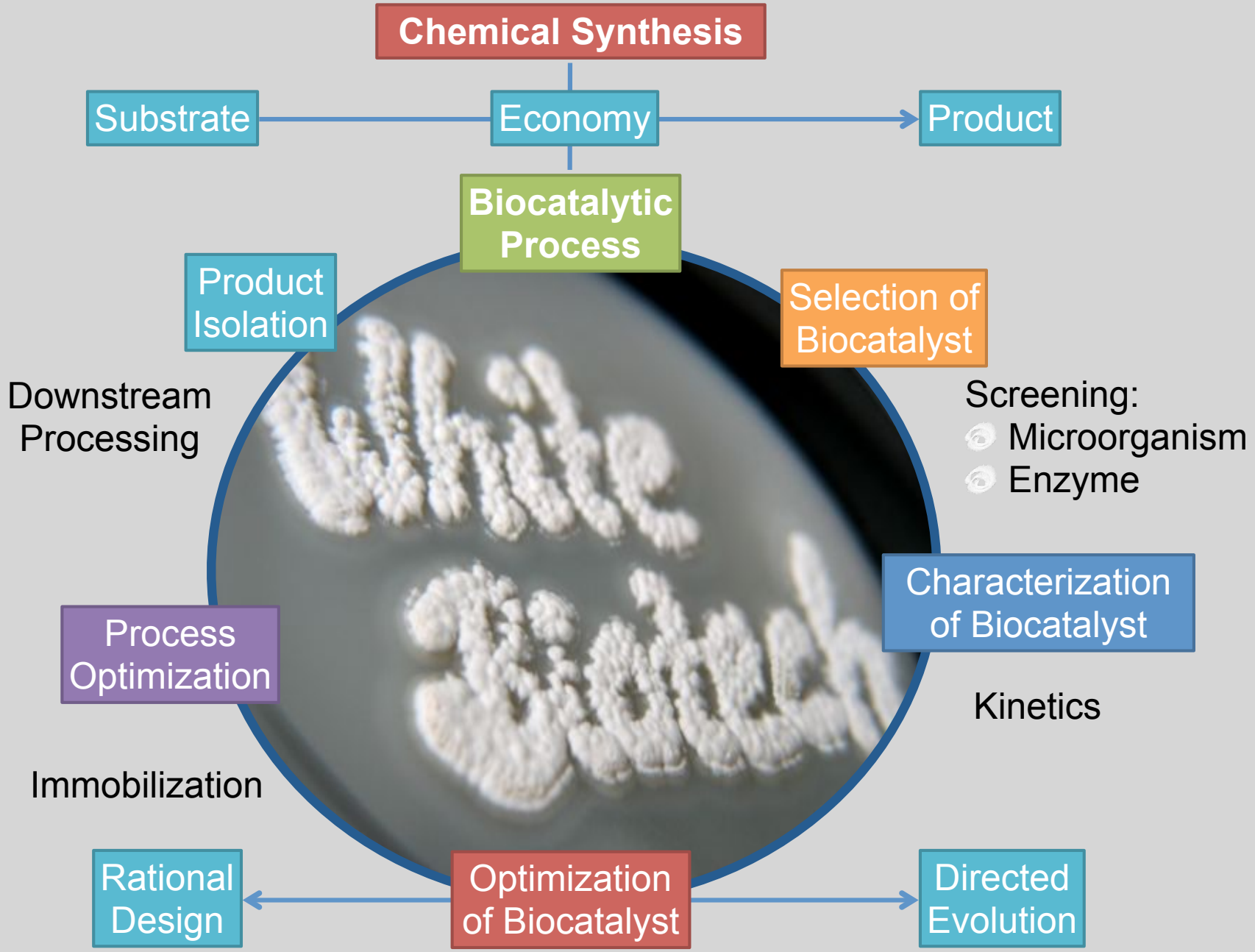
Why Biocatalysis in Lipid Modification?

Advantages of enzymatic reactions

- Mild reactions conditions / low temperatures
- Conversion of sensitive compounds (e.g. polyunsaturated fatty acids)
- Often simplified downstream processing
- Products often biodegradable
- High chemo-, regio- and stereoselectivity
  - no / less by-products
  - no need for protecting group chemistry
  - no need for solvents

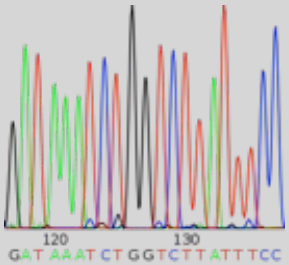
"White Biotechnology"

The Biocatalysis Cycle



Enzyme Sources

- Commercial suppliers (screening in catalogs)
- Classical screening by enrichment culture
- *In silico* screening in data bases
- Metagenome-screening:
  - >99% of all MO are 'non-cultivable'
  - broad diversity accessible, already recombinant
- Protein Engineering by
  - Rational protein design
  - Directed (molecular) evolution
  - Combined approaches



Looking Back to the 1990ies

- Until the early 90ies, the biocatalysis was hampered because only a very limited number of enzymes was available, such as:
  - Lipozyme RMIM (immob. lipase from *Rhizomucor miehei*, Novozymes)
  - Novozyme Sp435/NZ435 (immob. lipase B from *Candida antarctica*)
  - Lipase from *Humicola lanuginosa* (now renamed to *Thermomyces lanuginosis* TLIM), Novoz.
  - Lipase from *Rhizopus oryzae* / delemar (e.g., Amano, Japan)
  - Lipase from *Pseudomonas* sp. (e.g., Amano, Japan)
  - Lipase from *Candida rugosa* (e.g., Meito Sangyo, Amano, Japan)
  - Lipase from *Geotrichum candidum* (e.g., Amano, Japan)
- Protein Engineering was difficult because of lack of protein structures, software
- Standard solution was optimization of reaction conditions
  - changing solvents (medium engineering)
  - investigate water content / water activity
  - change acyl donor or alcohol / enzyme amount / pH / temperature etc.
- If all of this did not solve the problem, then you had a problem !

Which (Isolated) Enzymes are Useful?

Enzyme	Applications	Examples
Lipase	Synthesis of structured triglycerides Enrichment of specific fatty acids Incorporation of specific fatty acids Synthesis of fatty acid derived products Fatty acid methyl ester synthesis	Cocoa-butter equivalent, Betapol PUFA from fish oils PUFA into plant oils Emollient esters, sugar esters Biodiesel
Phospholipase	Removal of fatty acids in *sn*1- or *sn*2-position (PLA_1 or PLA_2) Removal of phosphate head group (PLC) Head group exchange (PLD)	Degumming of oils Lyso-phospholipids Chiral diglycerides Phosphatidylserine
Monooxygenase	Hydroxylation of fatty acids	Precursor for polyesters / lactones
Epoxidase	Epoxidation of double bonds	?
Lipoxygenase	Synthesis of FA-hydroperoxides	?

Metzger, J.O., Bornscheuer, U.T. *Appl. Microb. Biotechnol.*, **71**, 13-22 (2006)
Kourist, R., Brundiek, H., Bornscheuer, U.T. (2010), *Eur. J. Lipid Sci. Technol.*, **112**, 64-74
Biermann, U., Bornscheuer, U.T., Meier, M.A.R., Metzger, J., Schäfer, H.J. *Angew. Chem. Int. Ed* (2011), **50**, 3854-3871

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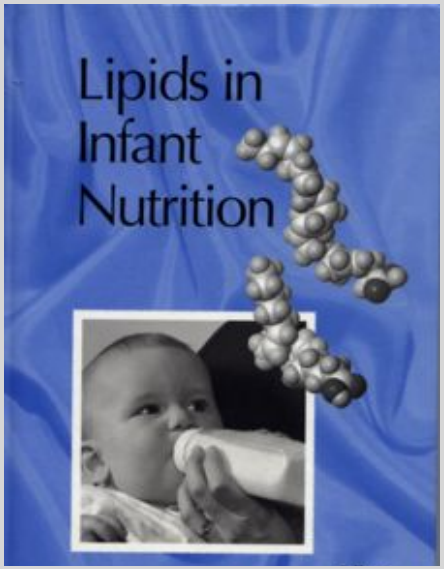
Lipase-Catalyzed Synthesis of Structured Triglycerides

- Structured triglycerides of the ABA-type such as 1-Oleyl-2-Palmitoyl-3-Oleoyl-glycerol (OPO) are important for infant nutrition (e.g., Betapol)
- Others like CyOCy are useful for low energy nutrition or in sports

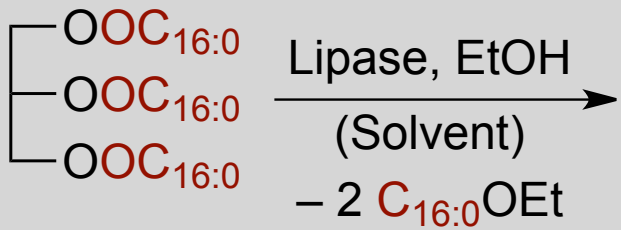


Reviews: U.T. Bornscheuer, *Lipid Technol.*, **7**, 105-107 (2001). Bornscheuer, U.T., Adamczak, M., Soumanou, M.M. In: Lipids as constituents of functional foods (Gunstone, FD, ed.) PJ Barnes & Associates, Bridgwater, pp. 149-182 (2002).

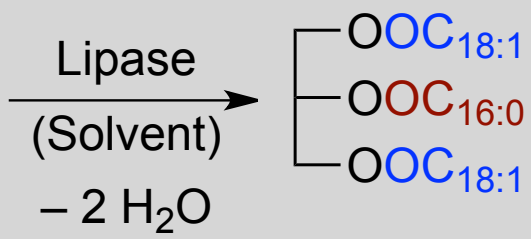
Lipase-Catalyzed Synthesis of Structured Triglycerides

Two step process for the production of Betapol (OPO):
(instead of simple acidolysis encountered with low yield / product purity)

Alcoholysis



Esterification



Advantages:

- ✓ higher yield
- ✓ higher purity
- ✓ only ethanol or acetone used
- ✓ flexible choice of lipases
- ✓ reaction conditions variable
- ✓ up-scaled to multi-kg stage
- ✓ successfully transferred also to PUFA incorporation from fish oil

Lipase	Solvent	Yield [%]	Purity [%]
RML	hexane/MS	70 (5 h)	92
RML	vacuum	89 (24 h)	82
RDL-Celite	hexane/MS	72 (5 h)	94
RDL-EP100	vacuum	78 (16 h)	96

Soumanou, M.M., Bornscheuer, U.T., Schmid, R.D., *J. Am. Oil Chem. Soc.* **75**, 703-710 (1998).
Soumanou, M.M., Bornscheuer, U.T., Menge, U., Schmid, R.D., *J. Am. Oil Chem. Soc.* **74**, 427-433 (1997).
Wongsakul, S., H-Kittikun, A., Bornscheuer, U.T. *J. Am. Oil Chem. Soc.*, **81**, 151-155 (2004)
Wongsakul, S., Prasertsan, P., Bornscheuer, U.T., H-Kittikun, A. *Eur. J. Lipid Sci. Technol.*, **105**, 68–73 (2003).

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Lipase-Catalyzed Synthesis of Sugar Esters

Background:

Sugar fatty acid esters are useful as biodegradable detergents / emulsifiers

Starting materials are renewable resources (sugar and fatty acids from plant oils)

Problem:

Sugar and fatty acid are not miscible; do not dissolve enough in organic solvents

Published 'solutions':

- 'Hydrophobization' w/ acetone or phenylboronic acid requires protection/deprotection
- Use of alkyl-sugars: works, but yields other product properties
- Solid-phase synthesis: high yield, but difficult to up-scale

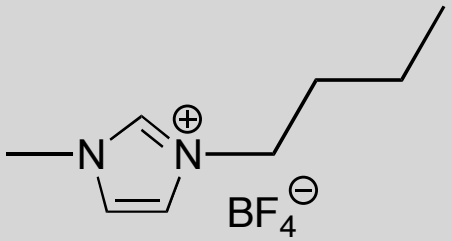
Alternative:

Use of ionic liquids (ILs) as solvent

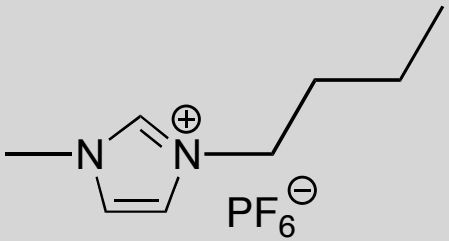
Lipase-Catalyzed Synthesis of Sugar Esters

Ionic Liquids:

- Defined as salts with melting temperatures <100°C (molten salts)
- Organic cation combined w/ inorganic anion
- No measurable vapor pressure, therefore regarded as 'green solvent'
- Properties can be modulated by variation of cation / anion:
- Miscible or immiscible with water / organic solvents



BMIM·BF_4



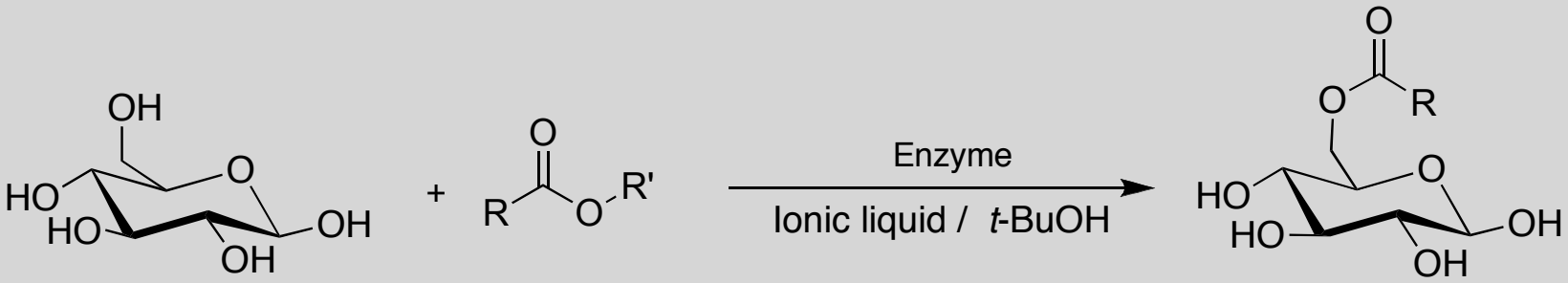
BMIM·PF_6

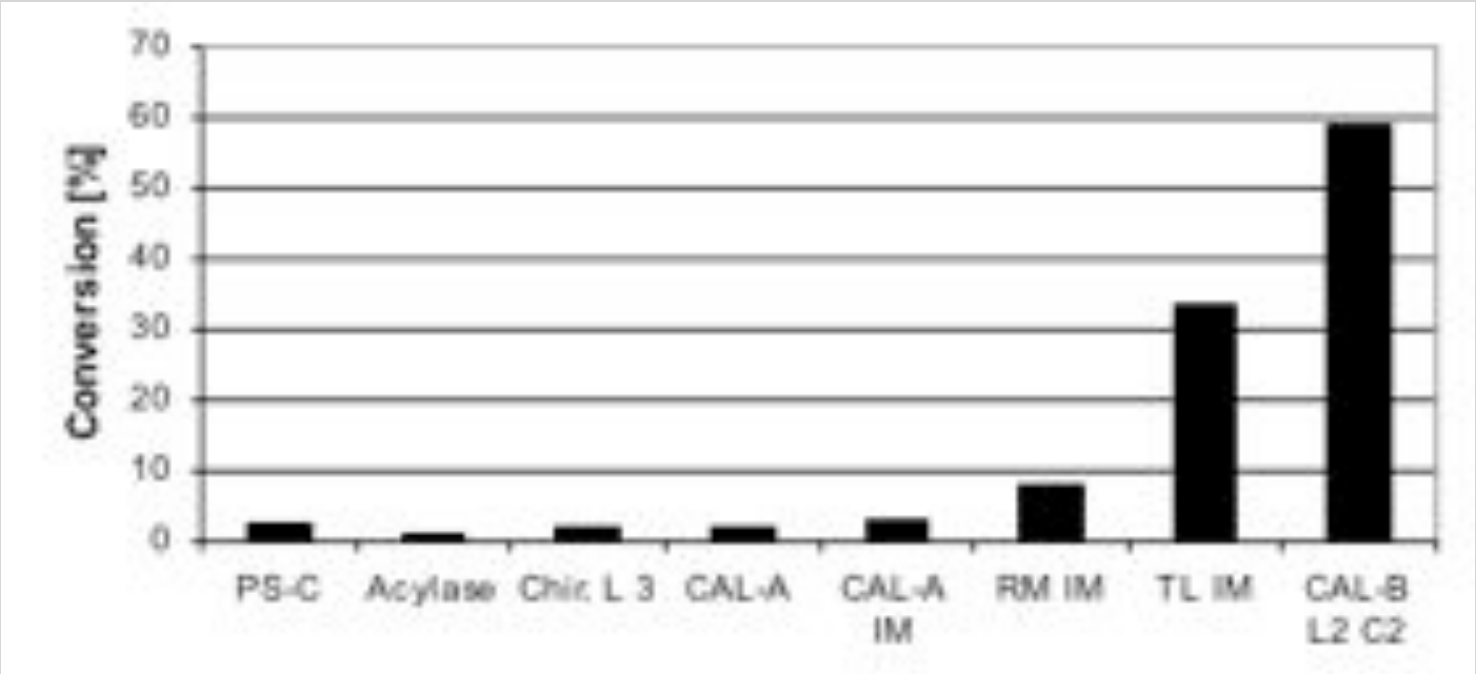
BMIM: 1-butyl-3-methyl-imidazolium

Lipase-Catalyzed Synthesis of Sugar Esters

Reaction I: first steps

- Glucose & fatty acid (vinyl ester) with lipase in IL & *t*-BuOH



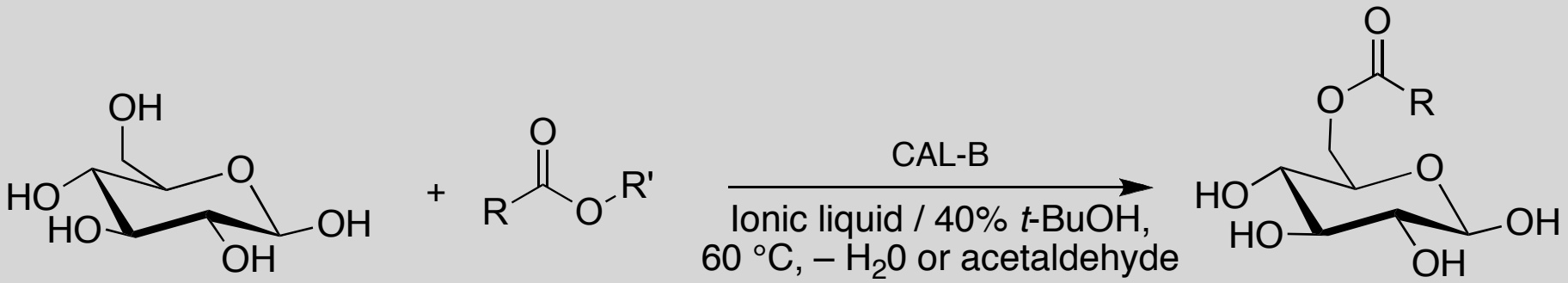


- Lipase B from *Candida antarctica* works best

Lipase-Catalyzed Synthesis of Sugar Esters

Reaction I: Optimization

- Use of fatty acid / vinyl ester, various ILs, concentration of *t*-BuOH, preparative scale:



R = $C_{11}H_{23}$ or $C_{13}H_{27}$ or $C_{15}H_{31}$
R' = H or $CH{=}CH_2$

Glukose &	Conv. [%]	Yield [%]	Purity [%]
C12-Vinylester	90	75	99.8
C14-Vinylester	89	89	95.1
C16-Fettsäure	64	48	99.8

Ganske, F.; Bornscheuer, U.T. Org. Lett., **7**, 3097-3098 (2005)
Ganske, F.; Bornscheuer, U.T. J. Mol. Catal. B: Enzym., **36**, 40-42 (2005)
Ganske, F.; Bornscheuer, U.T. Biotechnol. Lett., **28**, 465-469 (2006)

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Characterization of Lipases from Metagenome

Metagenome

- refers to all genome information present in an environmental sample (i.e. soil sample)
- allows access to 'non-cultivatable' biodiversity (~99 % of all organisms!)
- all enzymes identified are already recombinant and functionally expressed
- numerous and highly diverse biocatalysts accessible
  (i.e. 200 nitrilase found compared to ~15 found by classical screening/cultivation)

Task: Identification of suitable lipases

- 350 lipase/esterase samples provided by Verenium Inc. (formerly Diversa Inc.)
- profiling of fatty acid selectivity using milk fat as model lipid using GC analysis
- further characterization of interesting hits

Handelsman, J. (2005), *Nature Biotechnol* **23**, 38-39; Short, J.M. (1997), *Nature Biotechnol.*, **15**, 1322-1323

Characterization of Lipases from Metagenome

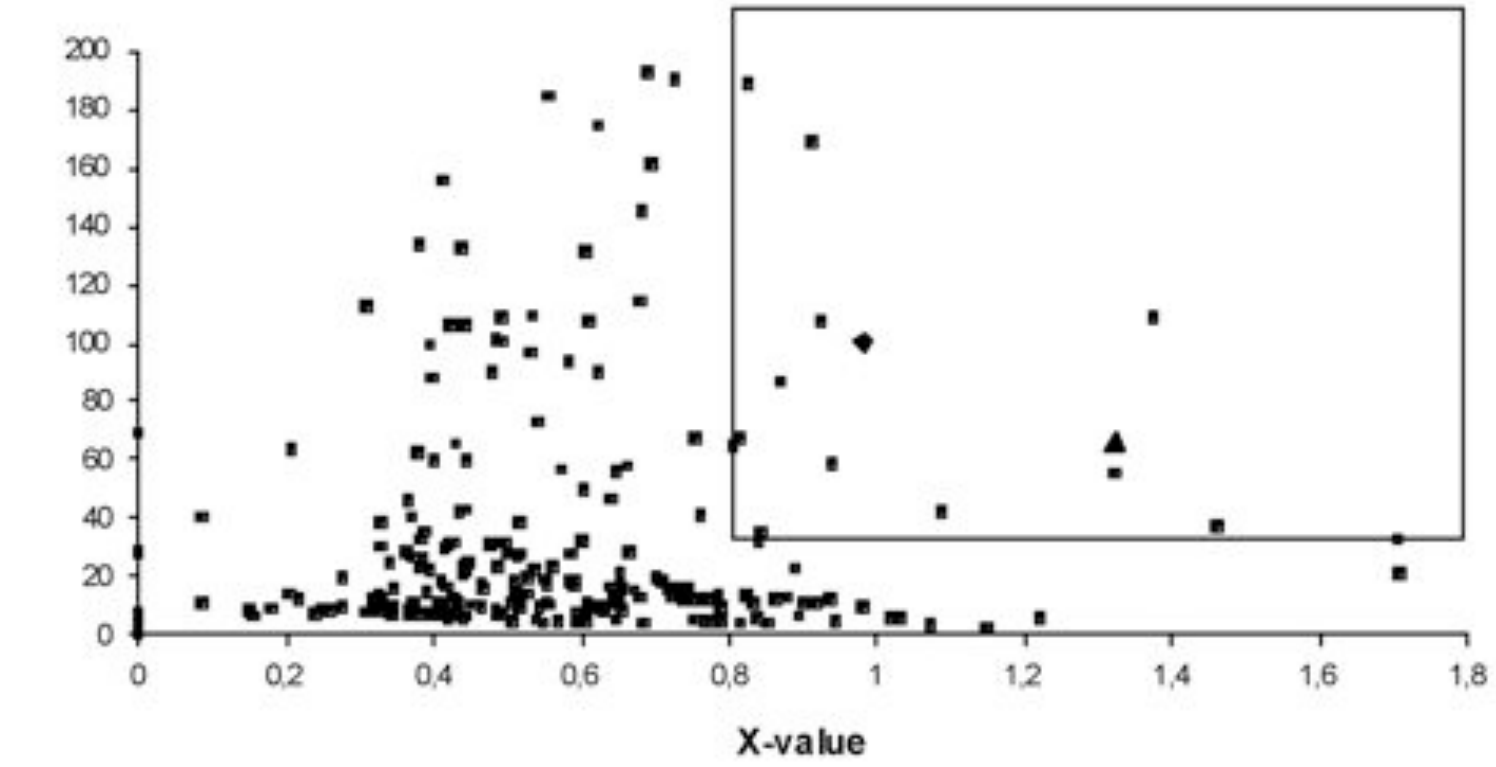
How to deal with so much data?

- Enzymes searched w/ preference for short chain fatty acids
- Selected by suitable X- and Y-values using equation

$$X = \frac{\left(\frac{C4:0+C6:0+C8:0}{3}\right)}{\left(\frac{C10:0+C12:0+C14:0+C16:0+C18:1}{5}\right)}$$

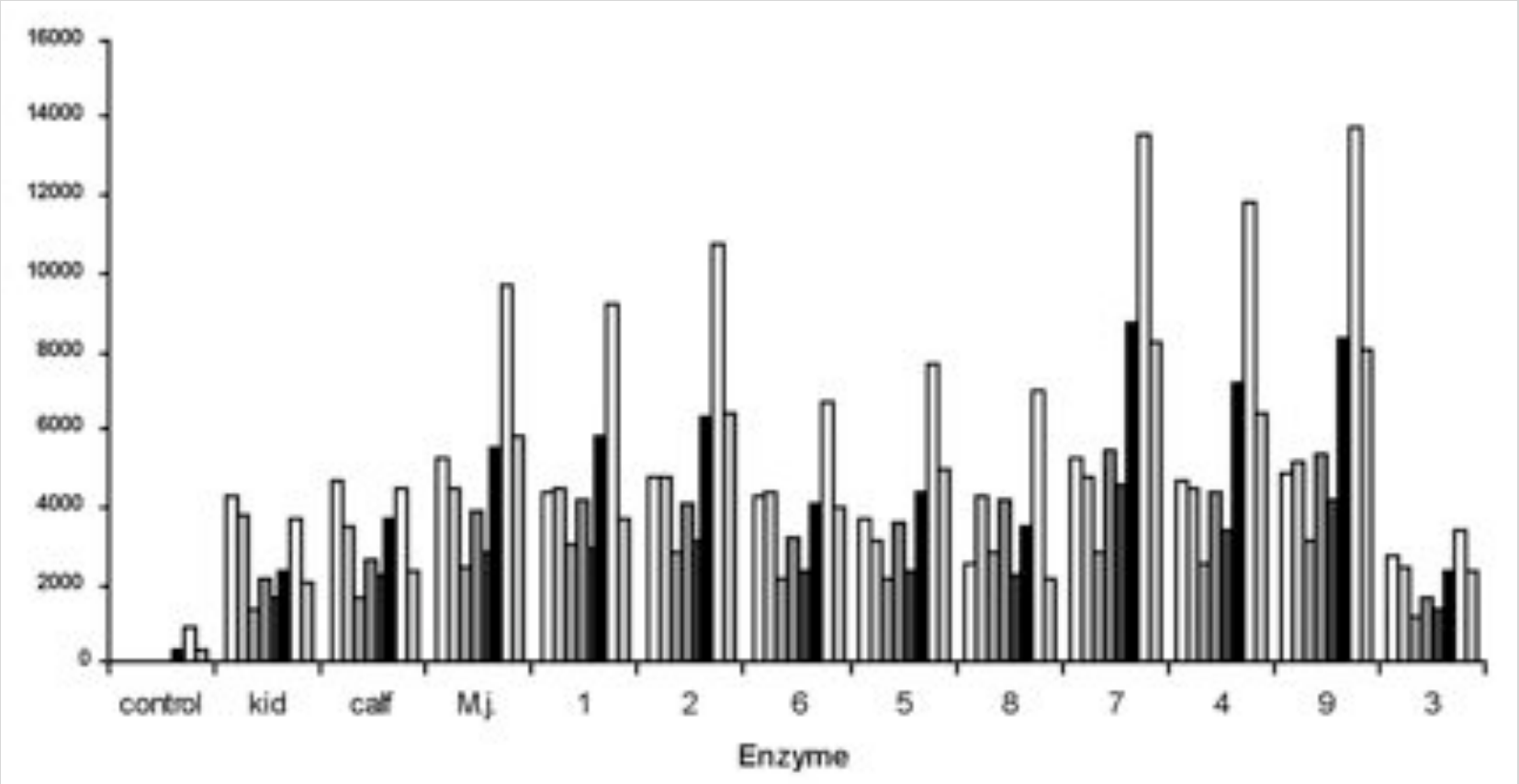
$$Y = C4:0+C6:0+C8:0+C10:0+C12:0+C14:0+C16:0+C18:1$$

Desired enzymes



Characterization of Lipases from Metagenome

- Profile compared to kid and calf lipases used in cheese production
- identified several enzymes with similar fatty acid profile



Bertram, M., Hildebrandt, P., Weiner, D.P., Patel, S.J., Bartnek, F., Hitchman, T.S., Bornscheuer, U.T., (2008) *J. Am. Oil Chem. Soc.,* **85**, 47-53.

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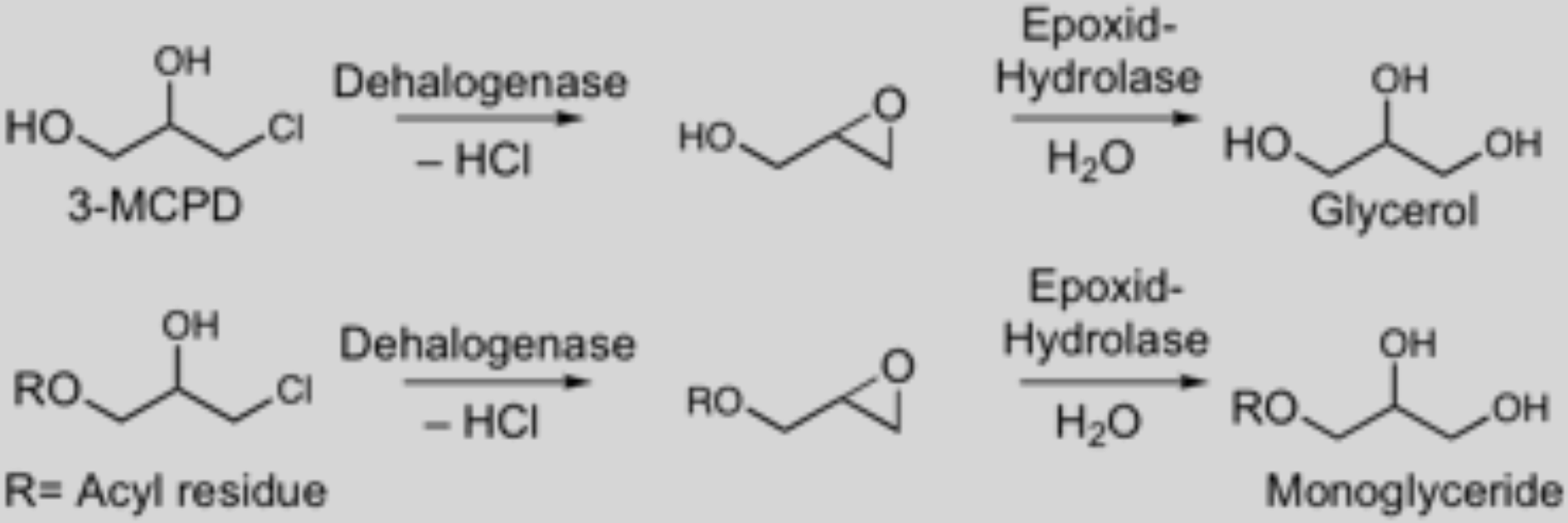
Challenge

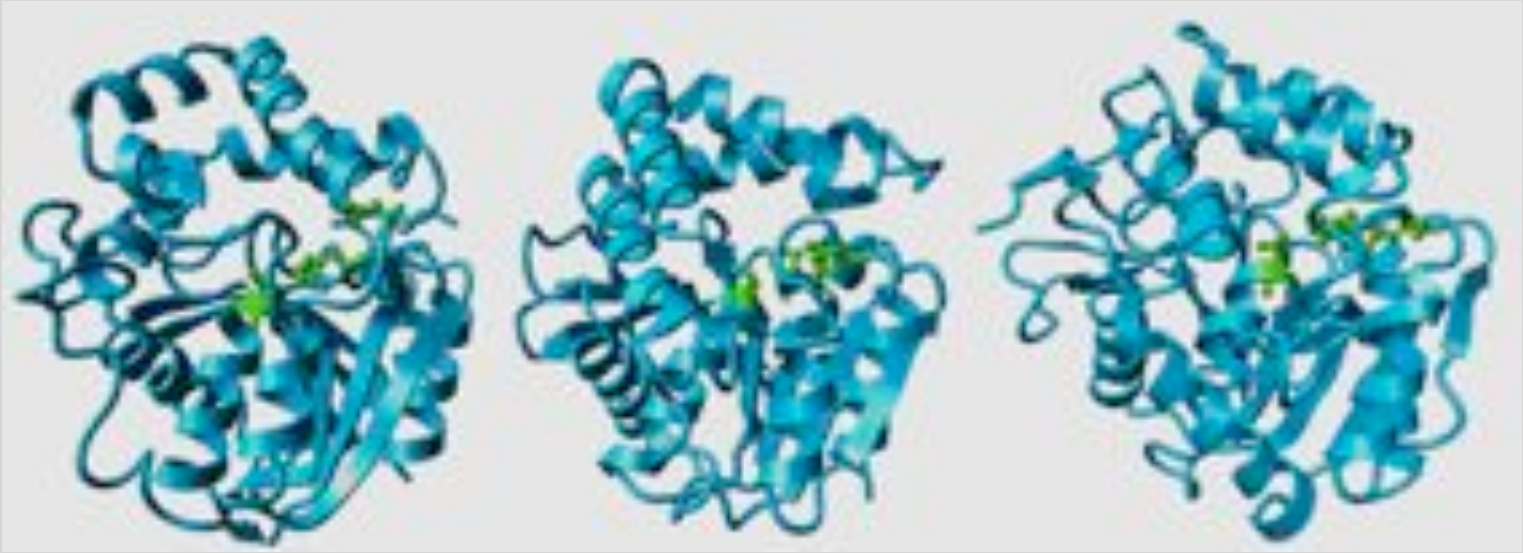
- 3-Monochloro-1,2-propanediol (3-MCPD) is a food processing contaminant
- In heat processed fat-containing foodstuffs it is formed from glycerol or acylglycerols (partial glycerides) and chloride ions
- Only a small percentage is present as free 3-MCPD
- The major part is ester linked with fatty acids
- These compounds are toxic and need to be removed

Enzymatic Concept

A combination of dehalogenase and epoxide hydrolase should allow removal of 3-MCPD



Properties of Epoxide Hydrolases & Dehalogenases



Epoxide hydrolase **Esterase** **Dehalogenase**

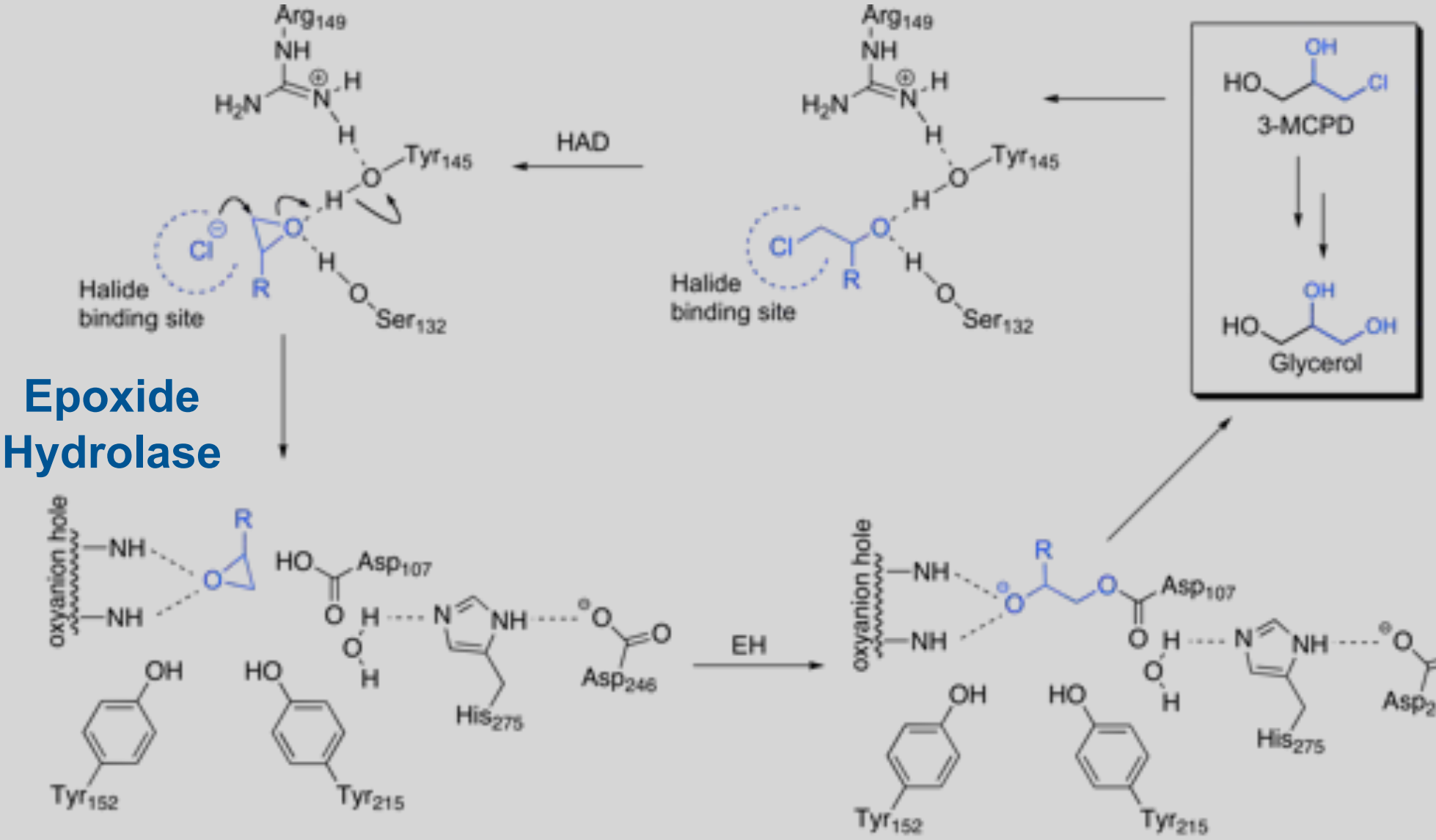
All are α/β-hydrolase fold enzymes:

- Esterase/Lipase/Protease (Ser-His-Asp)
- Hydroxynitrile Lyase (Ser-His-Asp)
- Epoxide Hydrolase (Asp-His-Asp)
- Haloalkane / Halohydrine Dehalogenase (Asp-His-Asp)

Kourist, R., Jochens, H., Bartsch, S., Kuipers, R., Padhi, S.K., Gall, M., Böttcher, D., Joosten, H.-J., Bornscheuer, U.T., *ChemBioChem*, **11**, 1635-1643 (2010)

Enzymatic Mechanisms

Dehalogenase:



Epoxide Hydrolase

Experimental Verification

- Establishment of analytical methods for 3-MCPD analysis
- Investigation of several dehalogenases and epoxide hydrolases for activity on these compounds
- Verification of 3-MCPD conversion in aqueous system
- Transfer to oil samples containing an aqueous phase
- Synthesis of 3-MCPD-monoester of oleic acid
- Verification of enzymatic activity on 3-MCPD-monoester

Conversion of 3-MCPD-Ester in a Two-Phase System

Water [%]	3-MCPD[a] [mM]	Glycidol [mM]	Glycidol[b] [mM]
50	2.6	6.3	0.7
25	1.4	7.2	0
5	0	7.7	0

[a]start conc. 10 mM; [b]after addition of EH

- Dehalogenase and epoxide hydrolase are indeed able to convert 3-MCPD into the harmless glycerol
- For 3-MCPD fatty acid esters, a lipase can be added
- Unexpectedly, all enzymes were active in the highly hydrophobic reaction system

Bornscheuer, U.T., Hesseler, M. (2010) *Eur. J. Lipid Sci. Technol.*, **112**, 552-556

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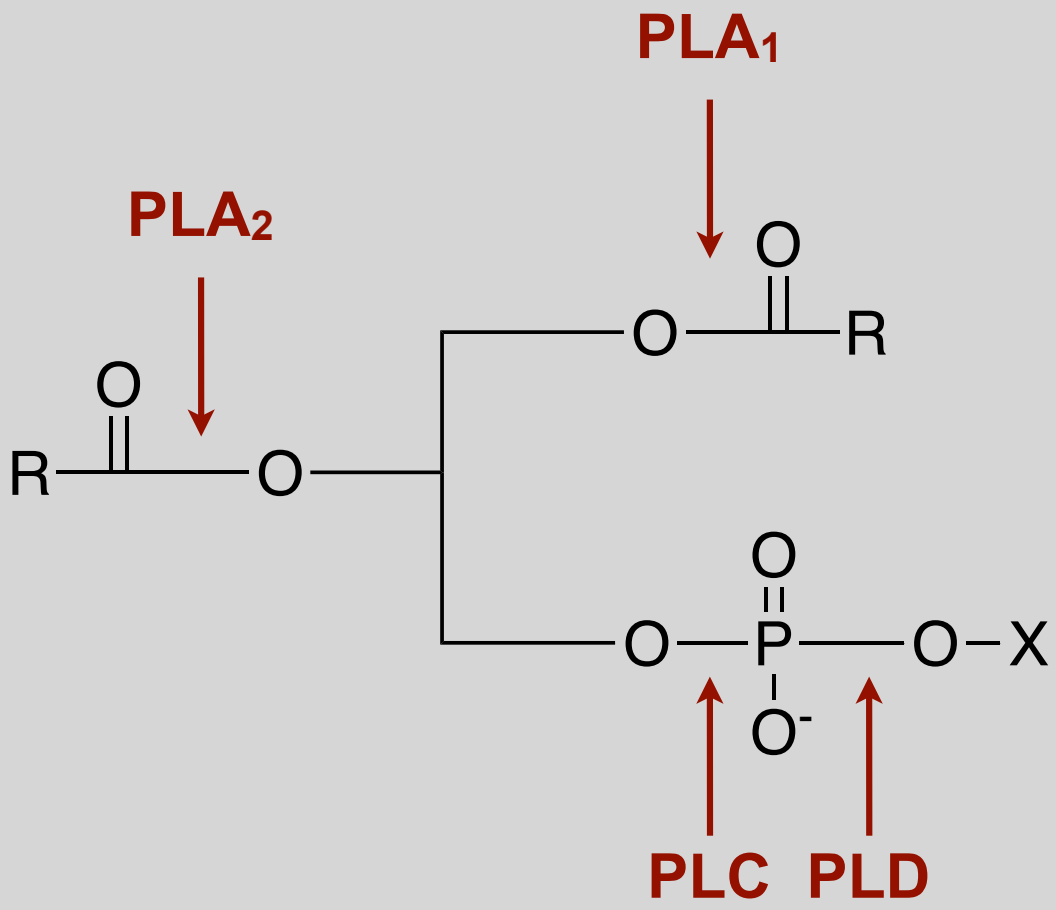
Phospholipase C



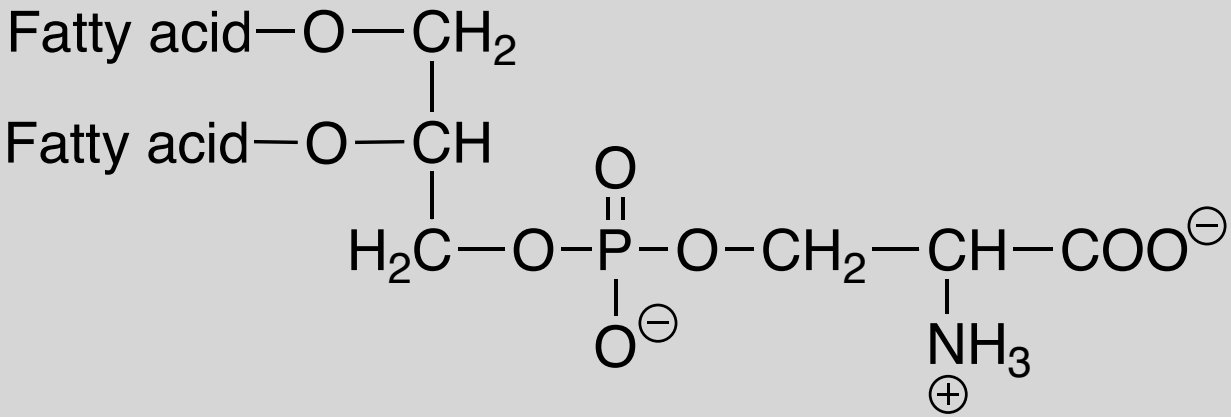
Some Recent Applications

PLA_1 / PLA_2

- Degumming of edible oils (EnzyMax Process)
- Synthesis of specific (lyso-)phospholipids

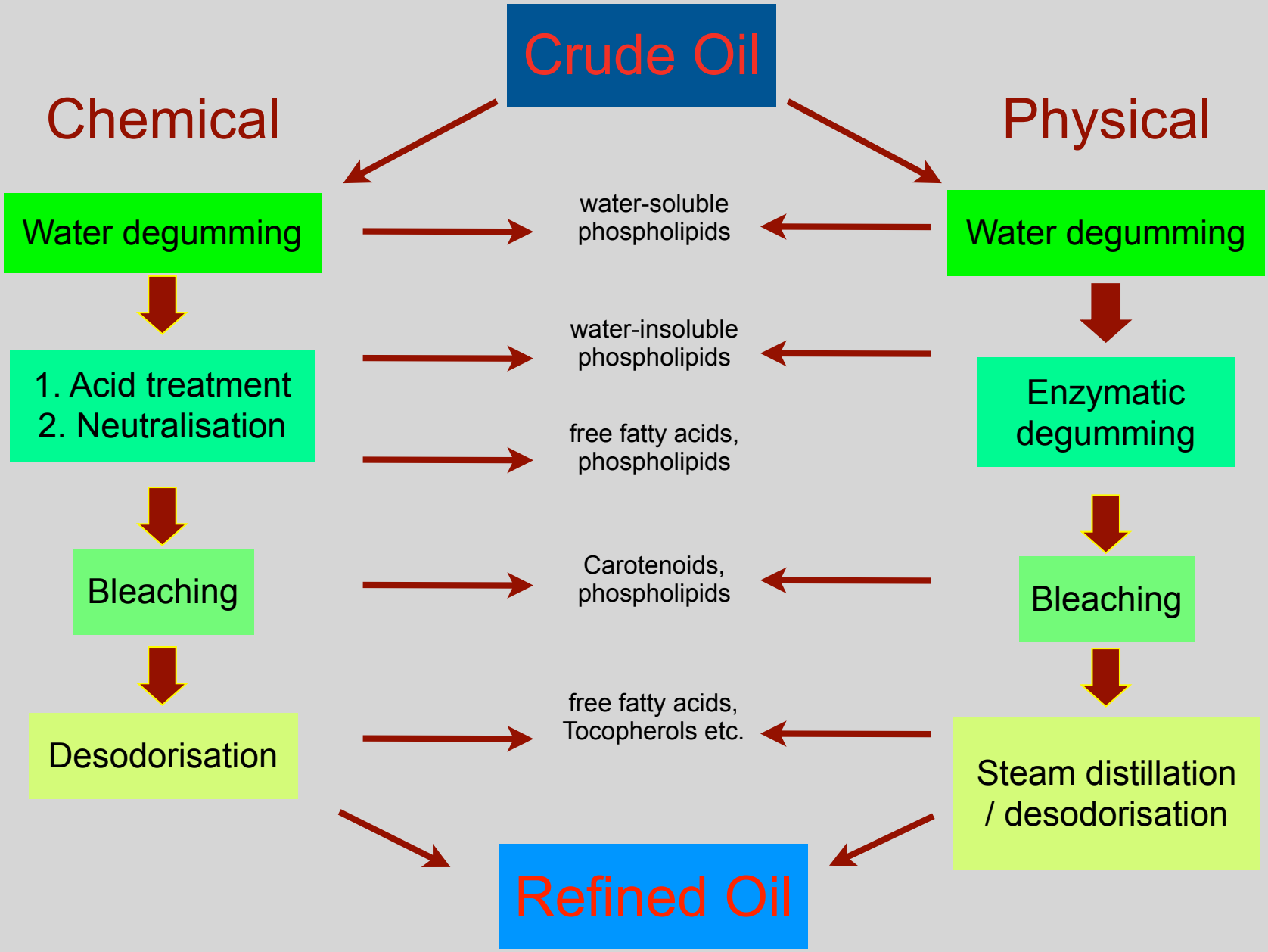
PLD

- Headgroup exchange
- Synthesis of specific phospholipids, e.g. Serines



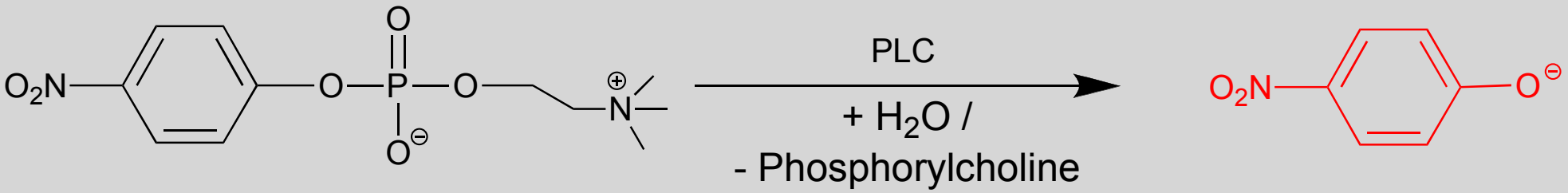
http://www.leci-ps.com/bioactives/html/e/products/brands/lecips/phosphat.htm

Degumming of Edible Oils



Screening of Bacillus sp. Wild-Type Strains

- 25 strains from *B. cereus* and *B. subtilis* were screened for PLC-activity
- All strains were cultivated at 37°C in LB-media in shake flasks
- Cell were harvested by centrifugation, the media was concentrated using
- Centricons and/or lyophilized and used for characterization of PLCs
- Seven *B. cereus* strains show high PLC-activity towards pNPPC



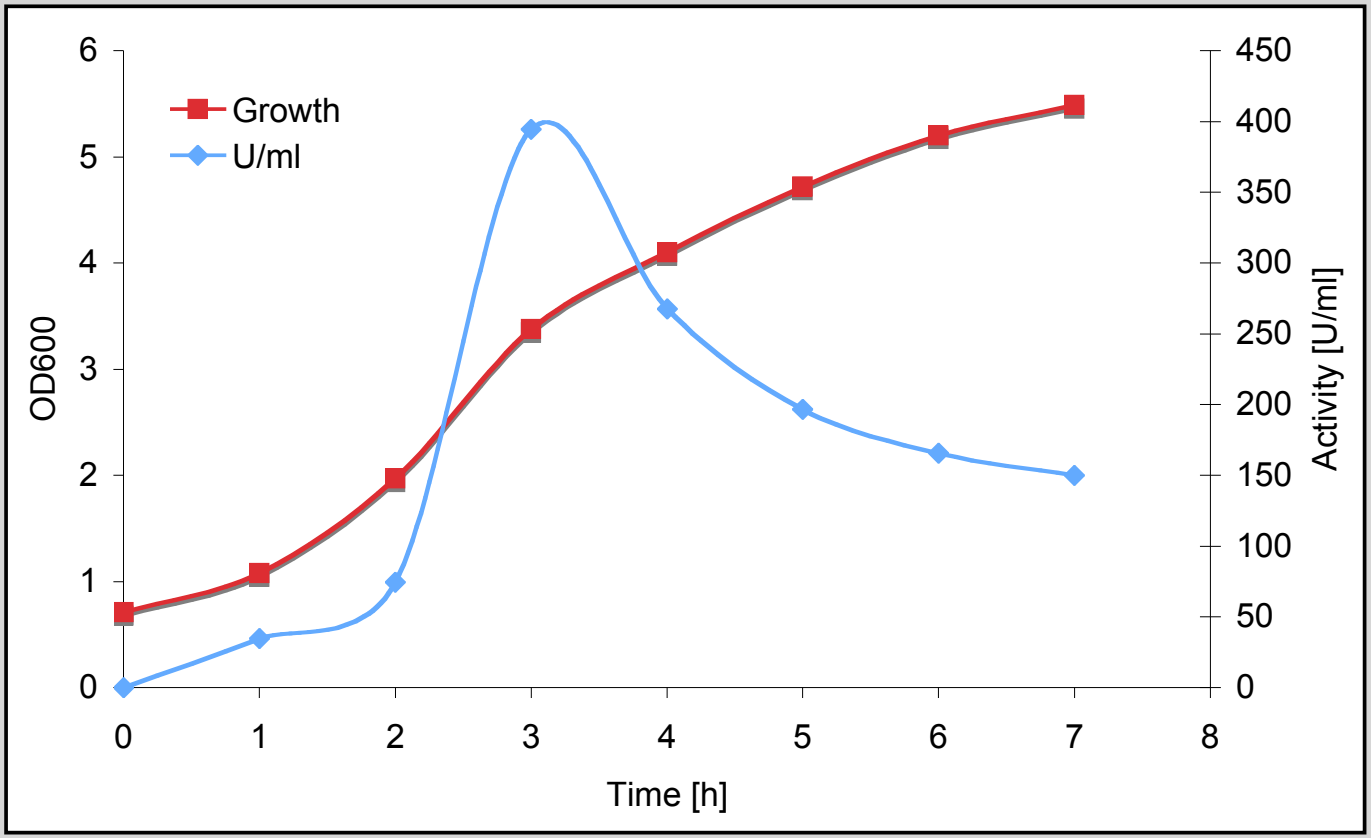
p-Nitrophenylphosphorylcholine

p-Nitrophenolate

Durban, M., Bornscheuer, U.T. (2003) *Eur. J. Lipid Sci. Technol.*, **105**, 633-637
Durban, M., Bornscheuer, U.T. (2007) *Eur. J. Lipid Sci. Technol.*, **109**, 469-473

Screening of Bacillus sp. Wild-Type Strains

Best strain

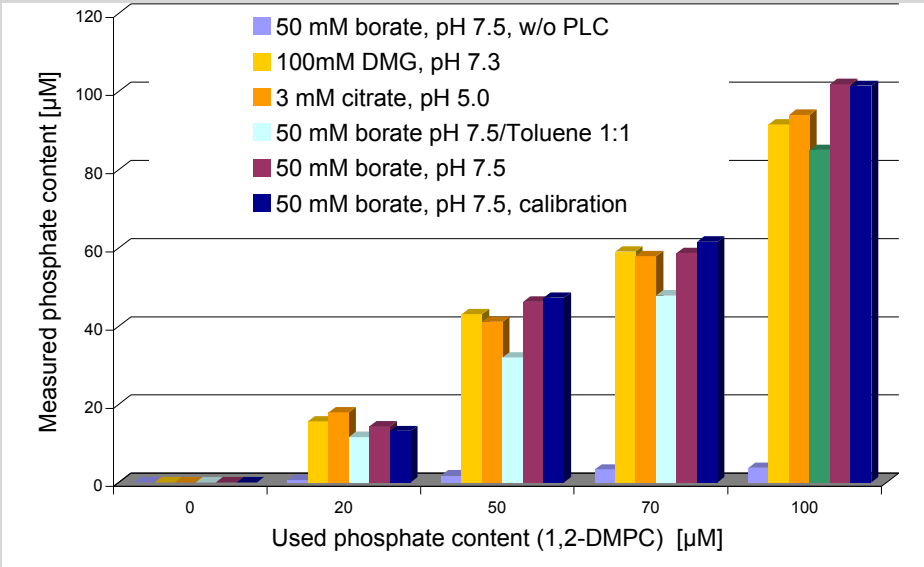


- Up to 8.000 U/mg lyophilized supernatant
- Highly active at acidic pH and up to 70°C
- Efficiently expressed recombinantly in *Bacillus subtilis*

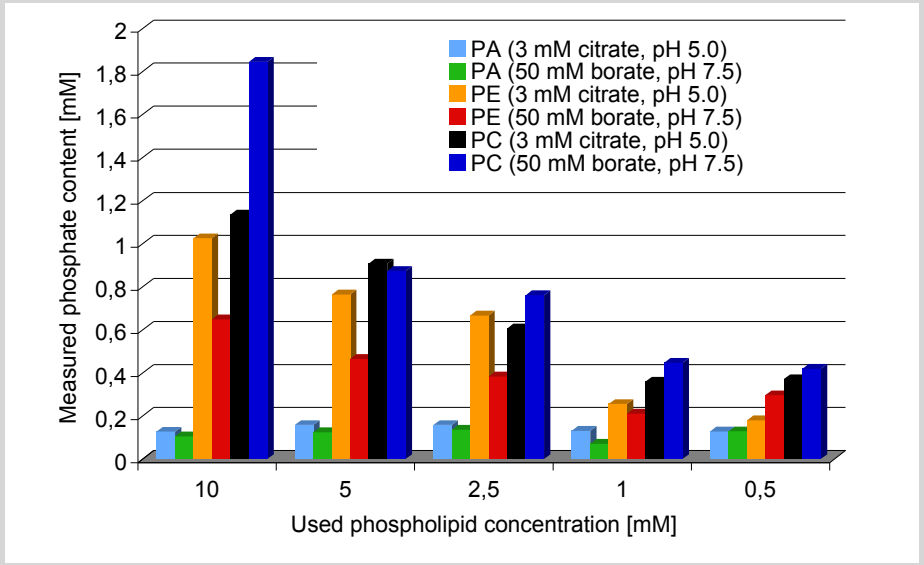
Durban, M., Silbersack, J., Schweder, T., Schauer, F. Bornscheuer, U.T., (2007) *Appl. Microbiol. Biotechnol.*, **74**, 634-639

Application of Novel PLC in Degumming

Model mixture w/ 1,2-DMPC



Rape seed oil spiked w/ phospholipids



- ✓ Complete removal of 1,2-DMPC with PLC up to 0.1 mM
- ✓ Good conversion in rape seed oil spiked with phospholipid mixture
- ✓ PC-PLC also acts on PE and phosphatidic acid (PA)

Durban, M., Bornscheuer, U.T. (2003) *Eur. J. Lipid Sci. Technol.*, **105**, 633-637

Durban, M., Bornscheuer, U.T. (2007) *Eur. J. Lipid Sci. Technol.*, **109**, 469-473

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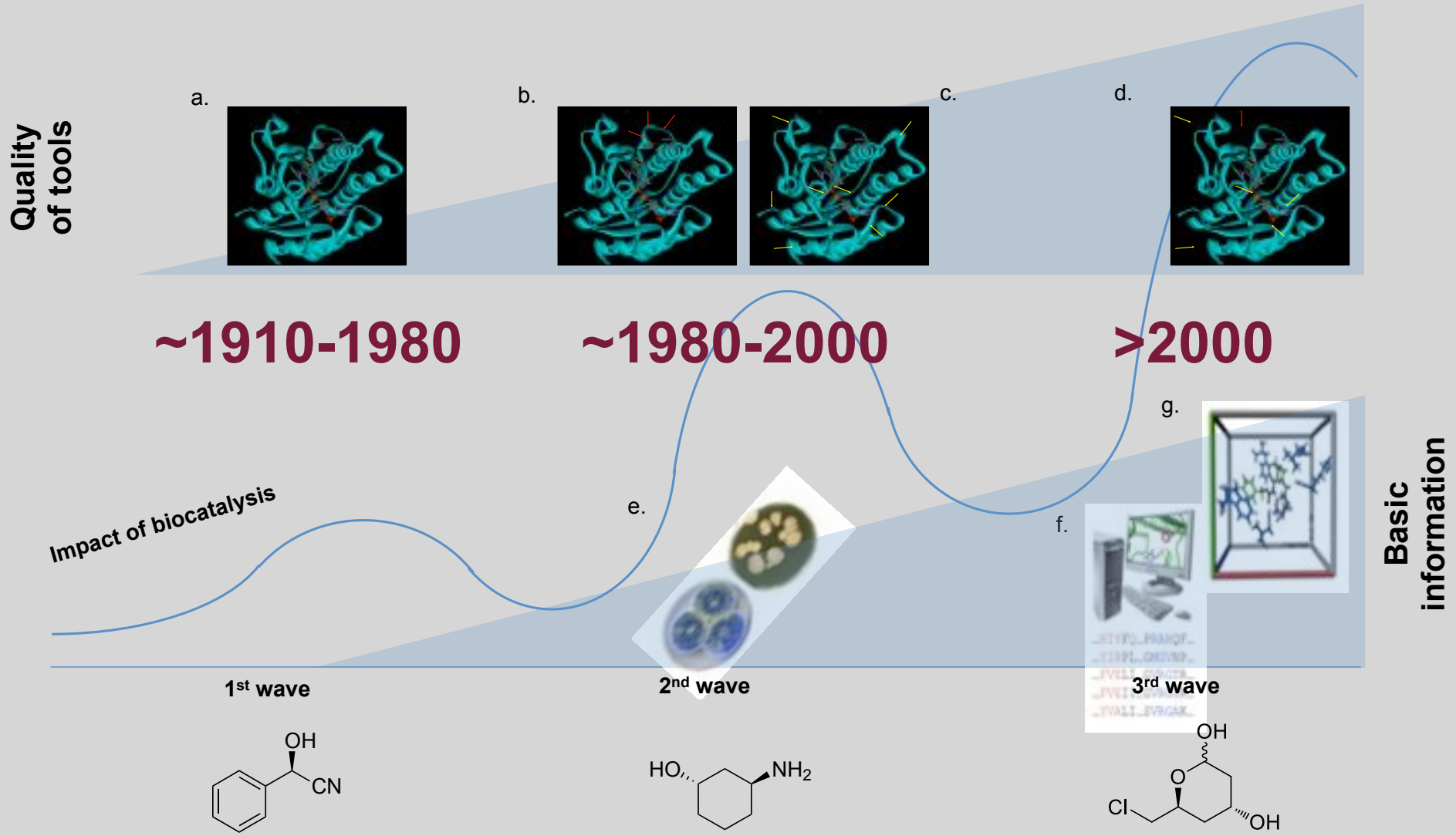
Why Protein Engineering ?

- Understand how proteins function
  - identification of residues involved in catalytic mechanism
  - formation of quaternary structure (i.e., monomer vs. dimer)
- Understand evolutionary relationships between different proteins
  - relationship of proteins in superfamilies
    (i.e. enolase superfamily, α/β-hydrolase fold family)
- Making them better for application
  - enhanced or inverted (chemo-, regio-, stereo-) selectivity
  - broadened or narrowed substrate specificity
  - altered pH- or temperature profile
  - improved stability (temperature, solvent etc.)
  - suppressed substrate or product inhibition
  - enhanced catalytic efficiency

Engineering the Third Wave in Biocatalysis



Source:	Cell extract / microbes	Recombinant enzyme	Recombinant w/ synthetic genes
Issues:	Stability, substrate range etc.	Random mutagenesis / HTS...	Fewer than before
Solution:	Immobilization or no solution	Screen large libraries	Protein/structure database; bioinformatics

Bornscheuer, U.T., Huisman, G. W., Kazlauskas, R.J., Lutz, S., Moore, J.C., Robins, K., *Nature*, **485**, 185-194 (2012)

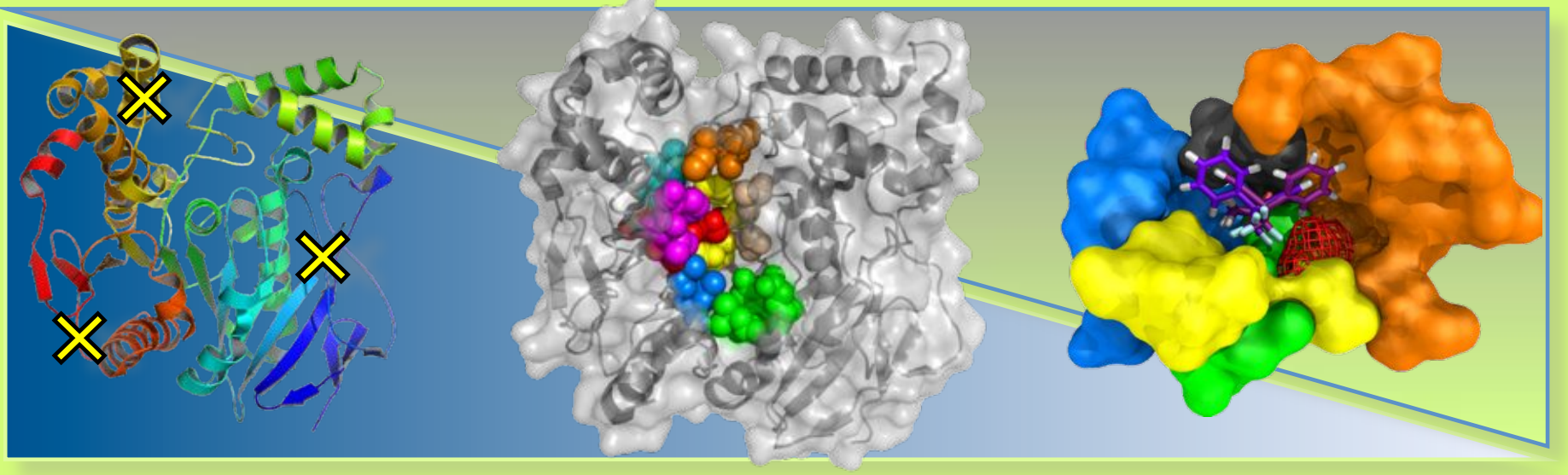
Strategies in Protein Engineering

directed evolution
(random mutagenesis)

combined approaches

rational design
(distinct point mutations)

screening effort



information intensity

epPCR
gene shuffling
insertion/deletion
circular permutation

CASTing/ISM
ProSAR
consensus approach 3DM

homology modeling
+ docked substrates
x-ray structures
+ transition state analogues

Kazlauskas, R.J., Bornscheuer, U.T., *Nature Chem. Biol.*, **5**, 526-529 (2009)
Lutz, S., Bornscheuer, U.T. (Eds.), *Protein Engineering Handbook*, Wiley-VCH, Weinheim (2009)
Kourist, R., Brundiek, H., Bornscheuer, U.T., *Eur. J. Lipid Sci. Technol.*, **112**, 64-74 (2010)
Behrens, G.A., Hummel, A., Padhi, S.K., Schätzle, S., Bornscheuer, U.T., *Adv. Synth. Catal.*, **353**, 2191-2215 (2011)

How to Find the Needle in the Haystack?



Number of possible variants of a proteins by introducing

M substitutions in **N** amino acids = $19^{M}[N!/(N-M)!M!]$

Substitutions (M)	Length of Protein Sequence (N)	
	10	200
1	190	3 800
2	16 245	7 183 900
3	823 080	9 008 610 600
4	27 367 410	8 429 807 368 950

How to Find the Needle in the Haystack?

A rapid and reliable assay system is the key to successful directed evolution experiments

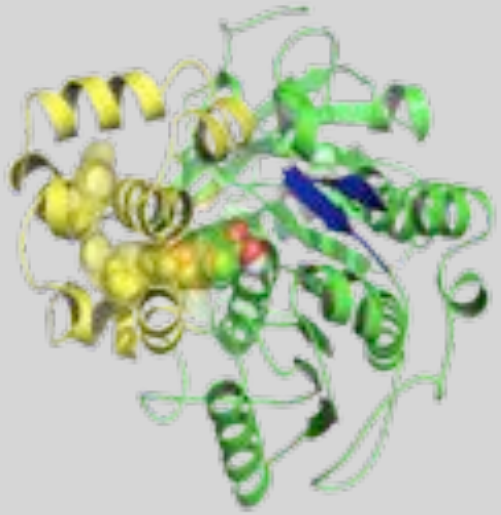
Substitutions (M)	Length of Protein Sequence (N)	
	10	200
3	823 080	9 008 610 600
4	27 367 410	8 429 807 368 950

But the throughput can vary considerably depending on the specific problem / assays at hand

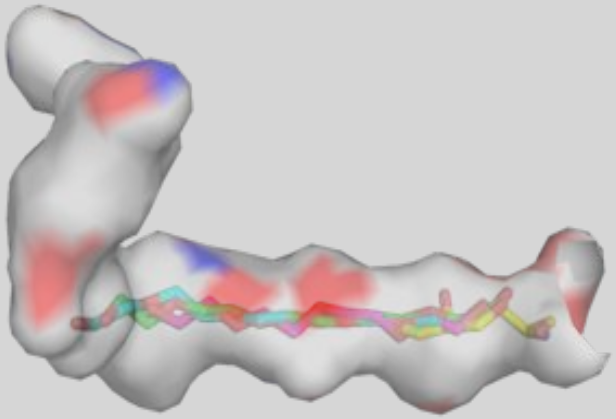
Protein Engineering of CAL-A

Strategy

- Identification of key residues in protein structure for mutagenesis
- Functional expression of gene encoding *C. antarctica* lipase A in *E.coli*
- Development of suitable high-throughput screening method
- Identification, verification and characterization of desired mutants



CAL-A structure
pdb-code: 2veo



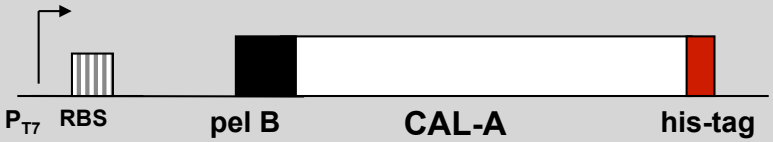
Substrate
binding-tunnel

D. J. Ericsson, A. Kasrayan, P. Johansson, T. Bergfors, A. G. Sandström, J.-E. Bäckvall, S. L. Mowbray, *J. Mol. Biol.* **2008**, *376*, 109-119.

Recombinant Expression of CAL-A in E.coli

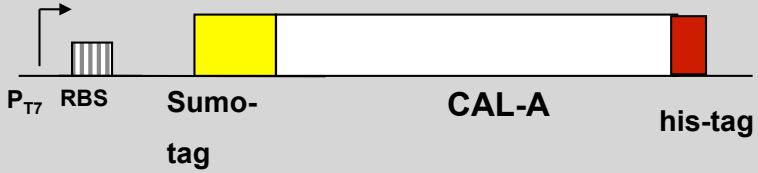
Constructs used (all codon-optimized, synthetic genes)

I. pelB-CAL-A



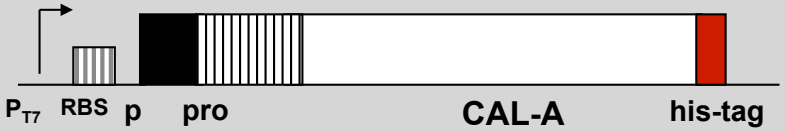
Periplasmic expression

II. Sumo-CAL-A



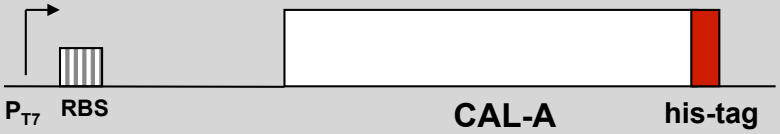
Cytoplasmic expression

III. ppro-CAL-A



Periplasmic expression

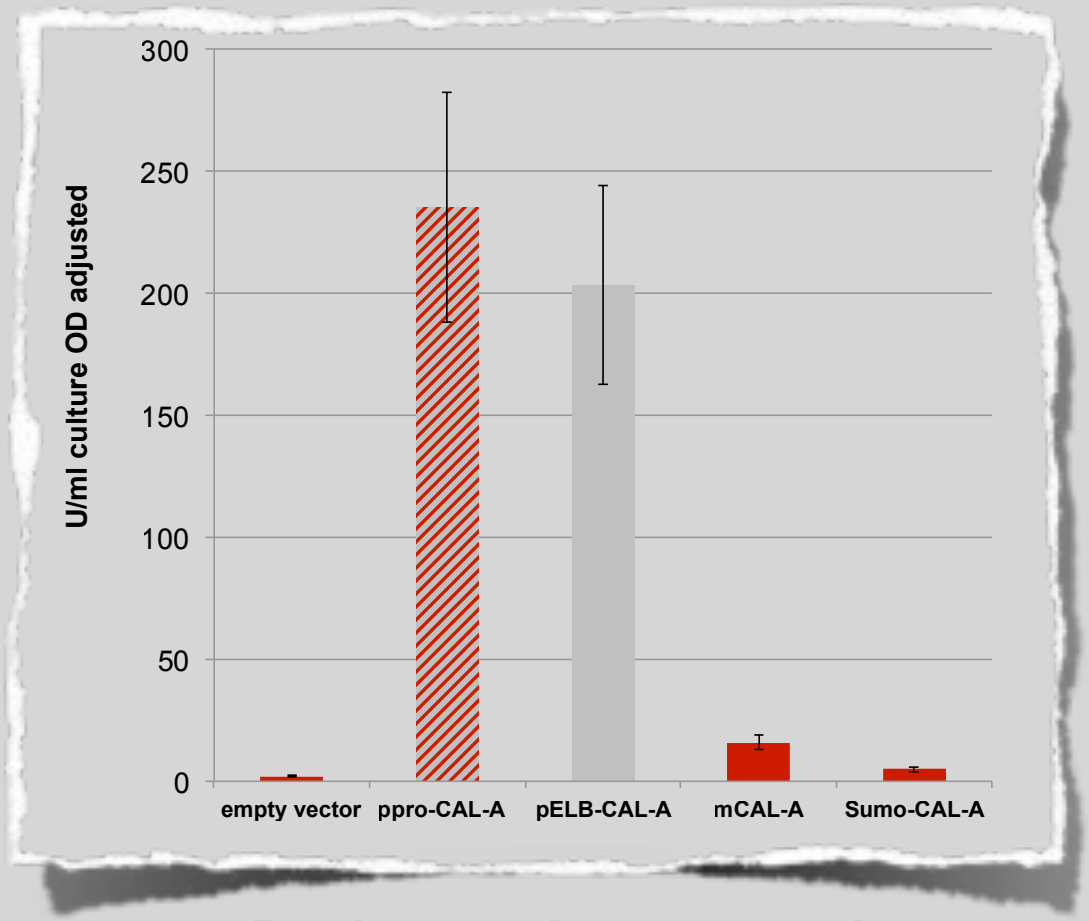
IV. mCAL-A



Cytoplasmic expression

Recombinant Expression of CAL-A in E.coli

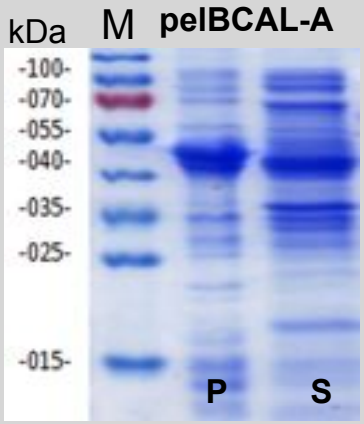
Expression analysis and activity test w/ p-NP-laurate



Periplasmic expression works best !

I. pelB-CAL-A

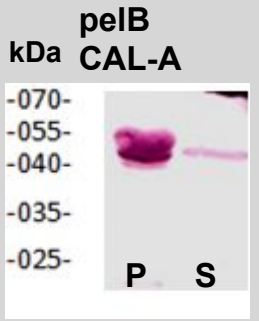
SDS-PAGE:



P = insoluble fraction (pellet)

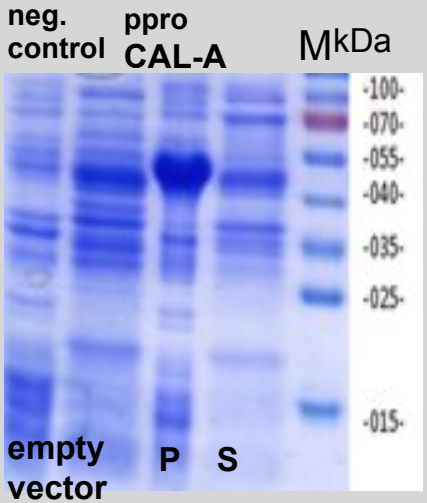
S = soluble fraction (supernatant)

Western-Blot:

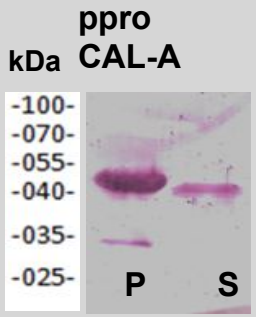


III. ppro-CAL-A

SDS-PAGE:



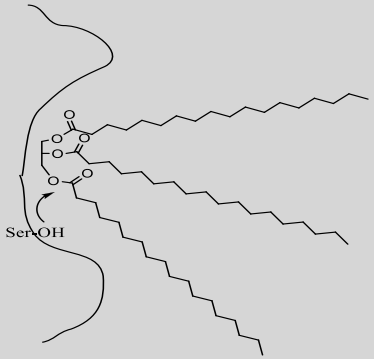
Western-Blot:

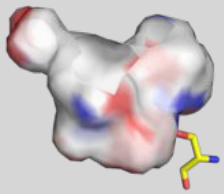


Protein Engineering of CAL-A

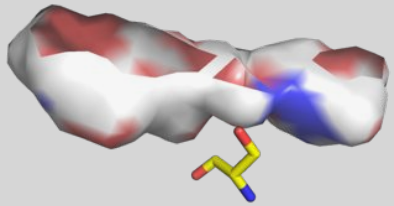
Funnel or cleft structure

"Non-tuning fork binding"

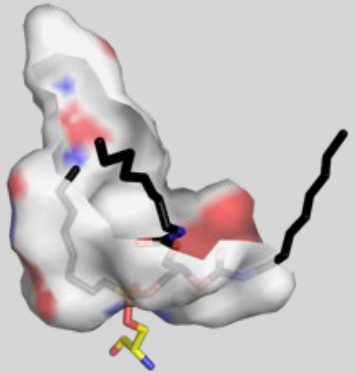




Candida antarctica
Lipase B (CAL-B)



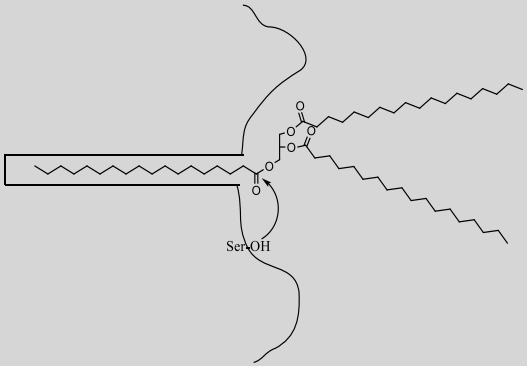
Mucor miehei Lipase

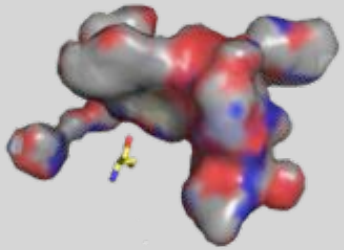


Burkholderia cepacia Lipase

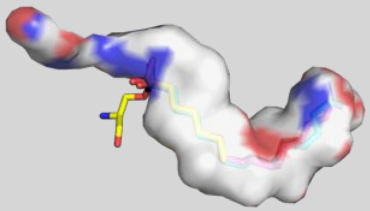
Tunnel structure

"Tuning fork binding"

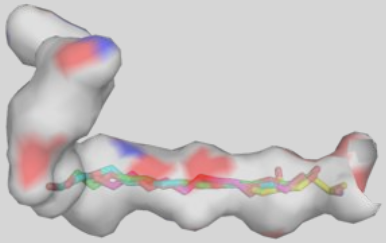




Geotrichum candidum
Lipase (GCL)



Candida rugosa
Lipase (CRL)

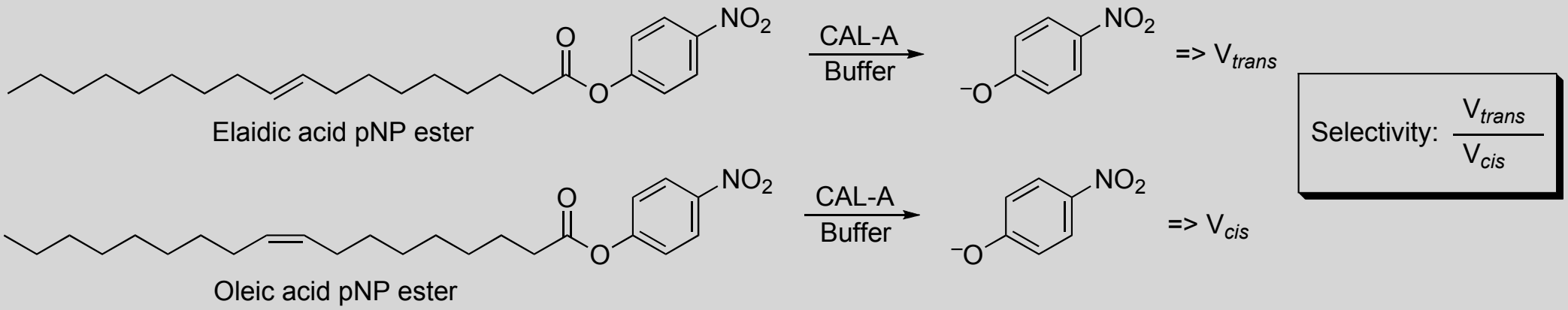


Candida antarctica
Lipase A (CAL-A)

Assays

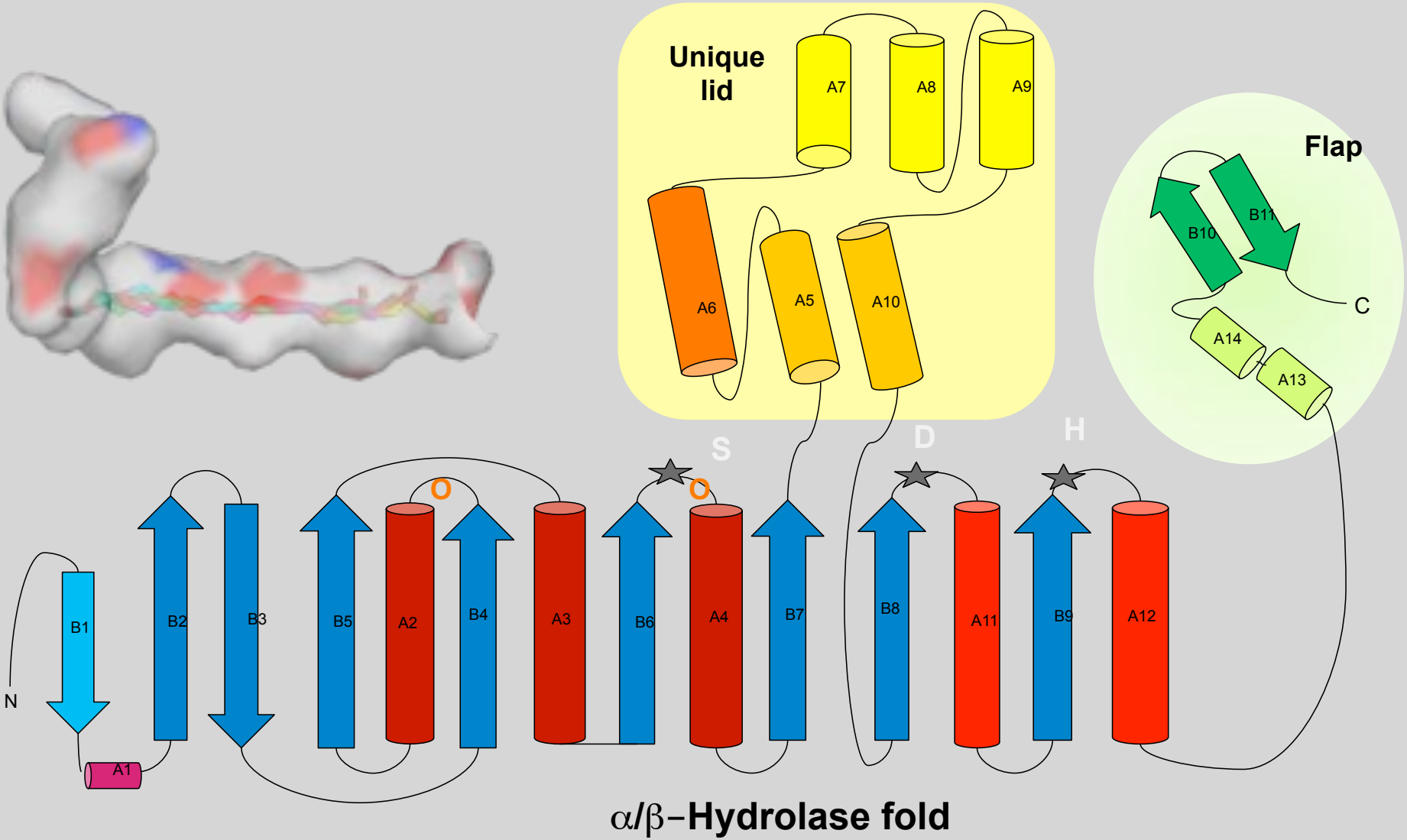
- Selectivity towards medium-chain fatty acids
  - Use of *p*-nitrophenyl esters of varying chain-lengths

- Selectivity towards *trans*-fatty acids
  - Use of pairs of *trans*-/*cis*-*p*-nitrophenyl esters of oleic vs. elaidic acid
  - Verification w/ partially hydrogenated plant oil (hydrolysis, ethanolysis)



Protein Engineering of CAL-A

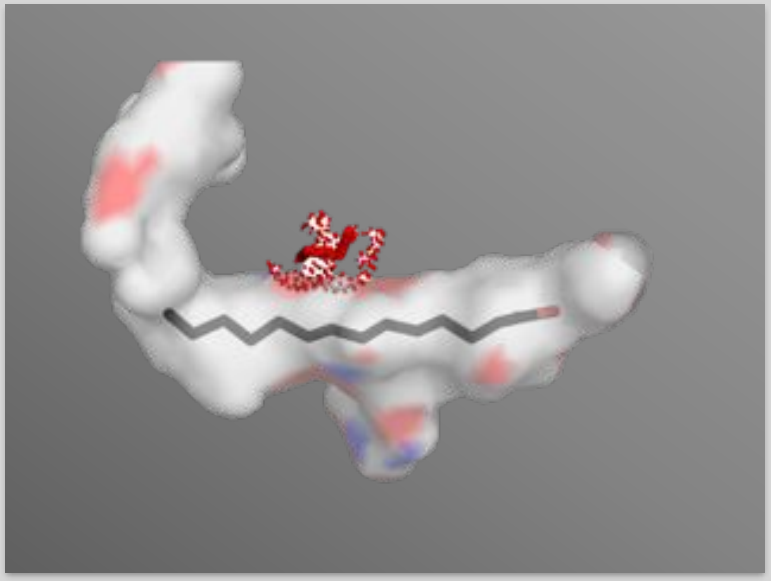
Substrate binding tunnel of CAL-A



α/β−Hydrolase fold

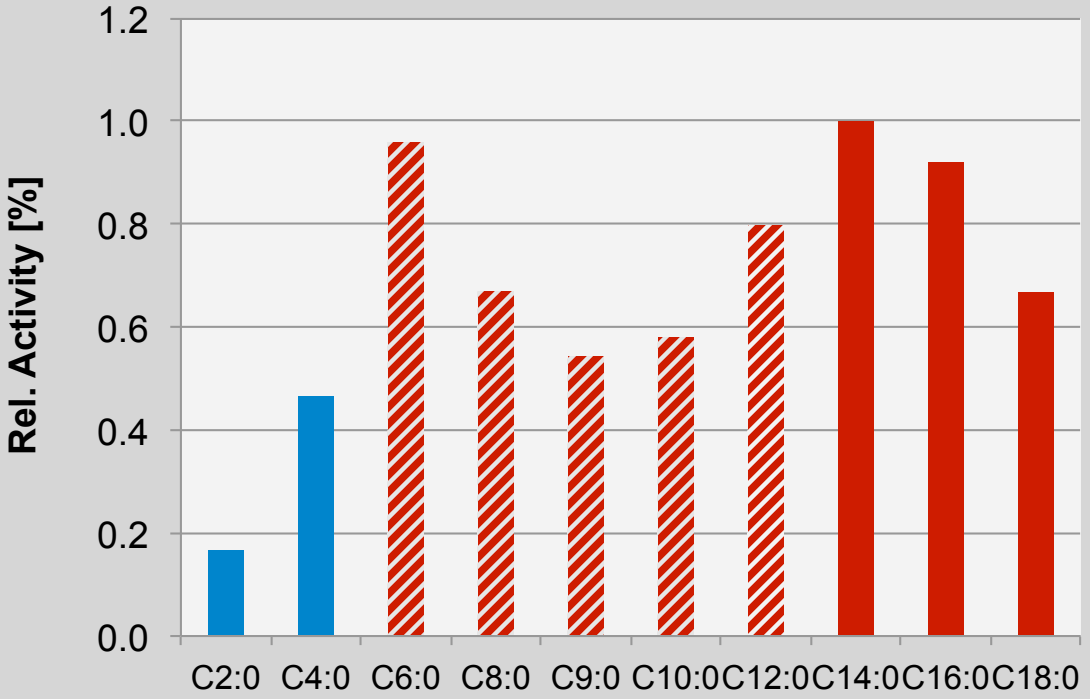
Protein Engineering of CAL-A

CAL-WT



G 237

Chain-length profile CAL-A (WT)

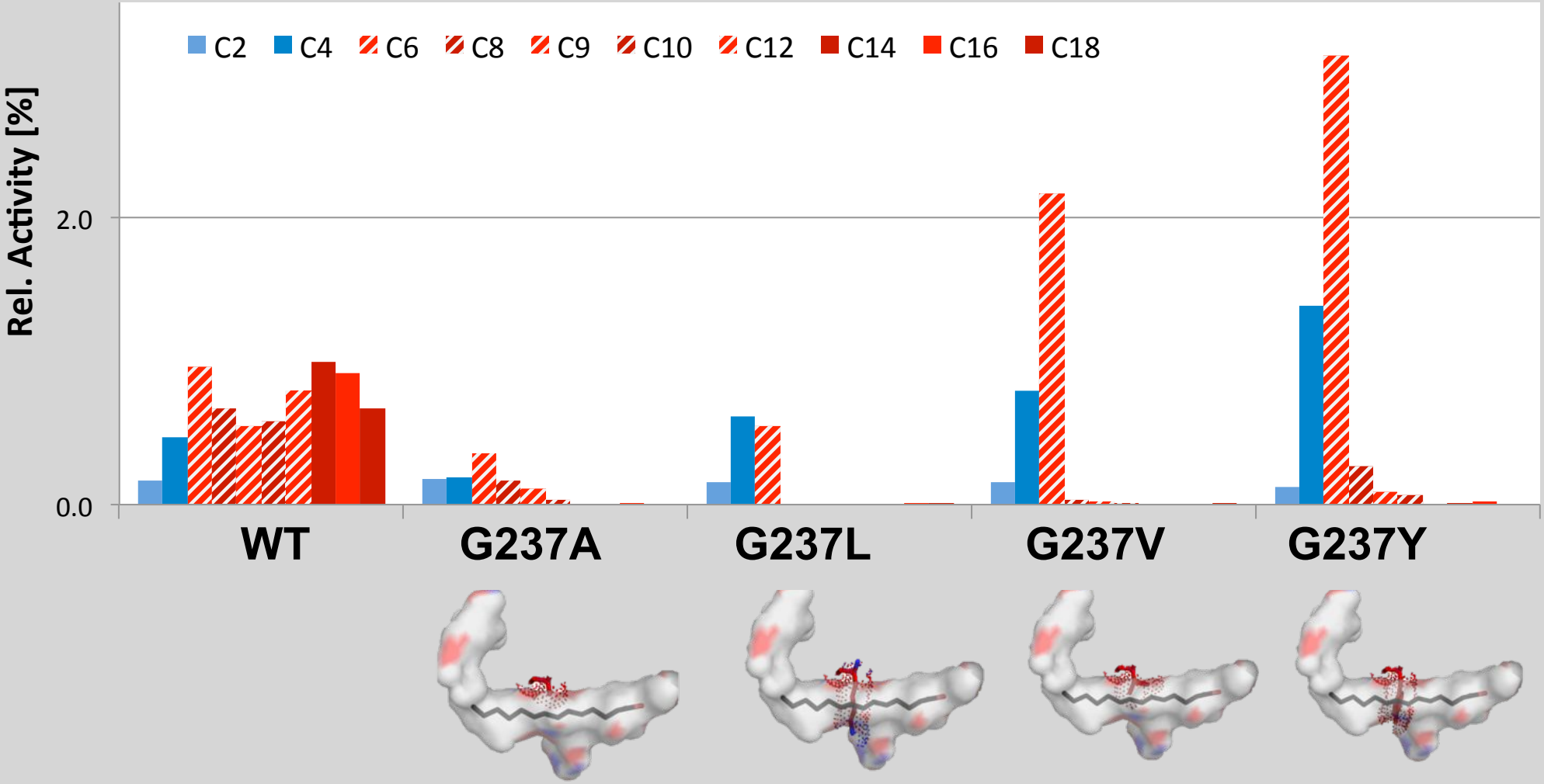


- ✓ Creation of mutants by site-directed mutagenesis
- ✓ Expression in *E. coli*, His-tag purification
- ✓ Activity determination w/ pNP-esters of varying chain-length

Brundiek, H.B., Padhi, S.K., Kourist, R., Evitt, A., Bornscheuer, U.T., *Eur. J. Lipid Sci. Technol*. (2012), online

Protein Engineering of CAL-A

Chain-length profile CAL-A (WT & mutants)



Clear shift to preference for medium chain-length fatty acids

Brundiek, H.B., Padhi, S.K., Kourist, R., Evitt, A., Bornscheuer, U.T., *Eur. J. Lipid Sci. Technol*. (2012), online

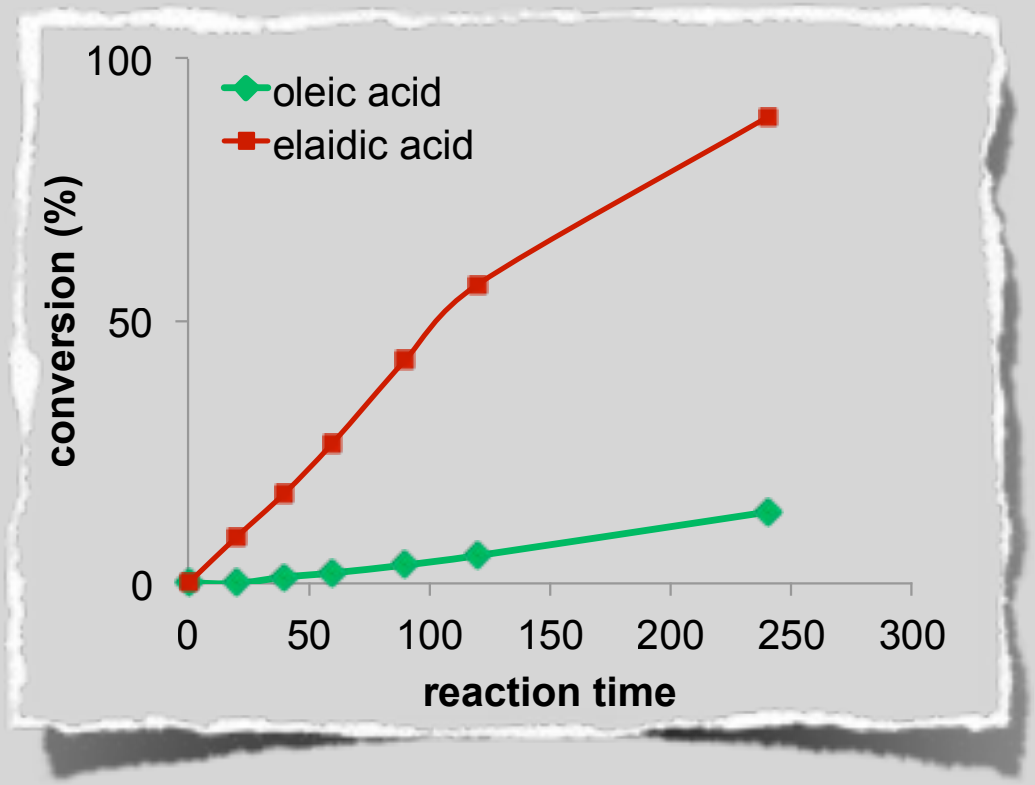
Why Remove Trans-Fatty Acids?

- Certain *trans*-fatty acids bear risks in human nutrition
- They can form during partial hydrogenation of plant oils bearing at least two double bonds
- Several countries have strict regulations or banned *trans*-fatty acids
- Methods for their selective removal are thus important

Protein Engineering of CAL-A

Why CAL-A?

- 3D-structure known
- Wildtype enzyme already shows unique *trans*-selectivity



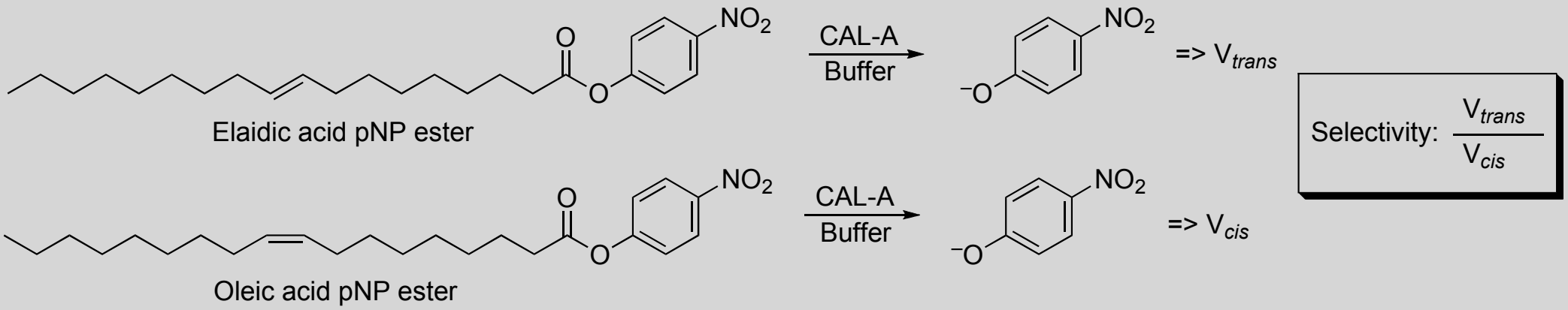
15-fold faster esterification with *n*-butanol for *trans*-fatty acid compared to *cis*-fatty acid

R. Borgdorf, S. Warwel, *Appl. Microbiol. Biotechnol.* **1999**, *51*, 480-485.

Assays

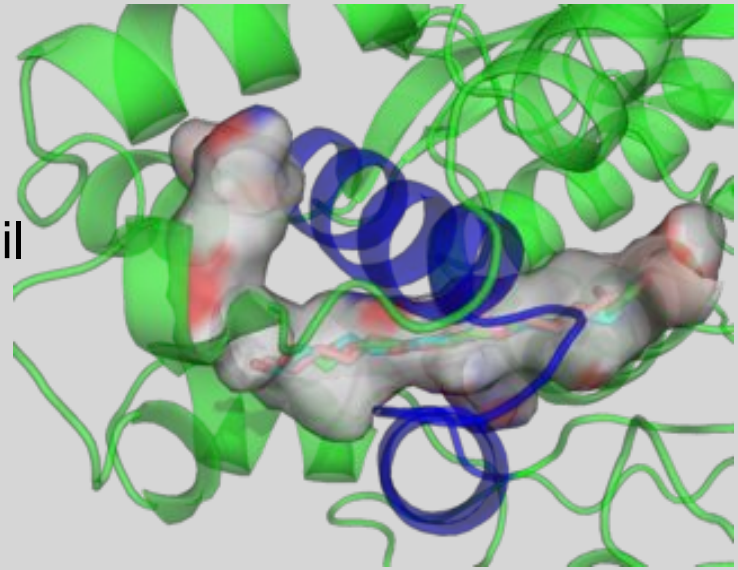
- Selectivity towards medium-chain fatty acids
  - Use of *p*-nitrophenyl esters of varying chain-lengths

- Selectivity towards *trans*-fatty acids
  - Use of pairs of *trans*-/*cis*-*p*-nitrophenyl esters of oleic vs. elaidic acid
  - Verification w/ partially hydrogenated plant oil (hydrolysis, ethanolysis)



Protein Engineering of CAL-A

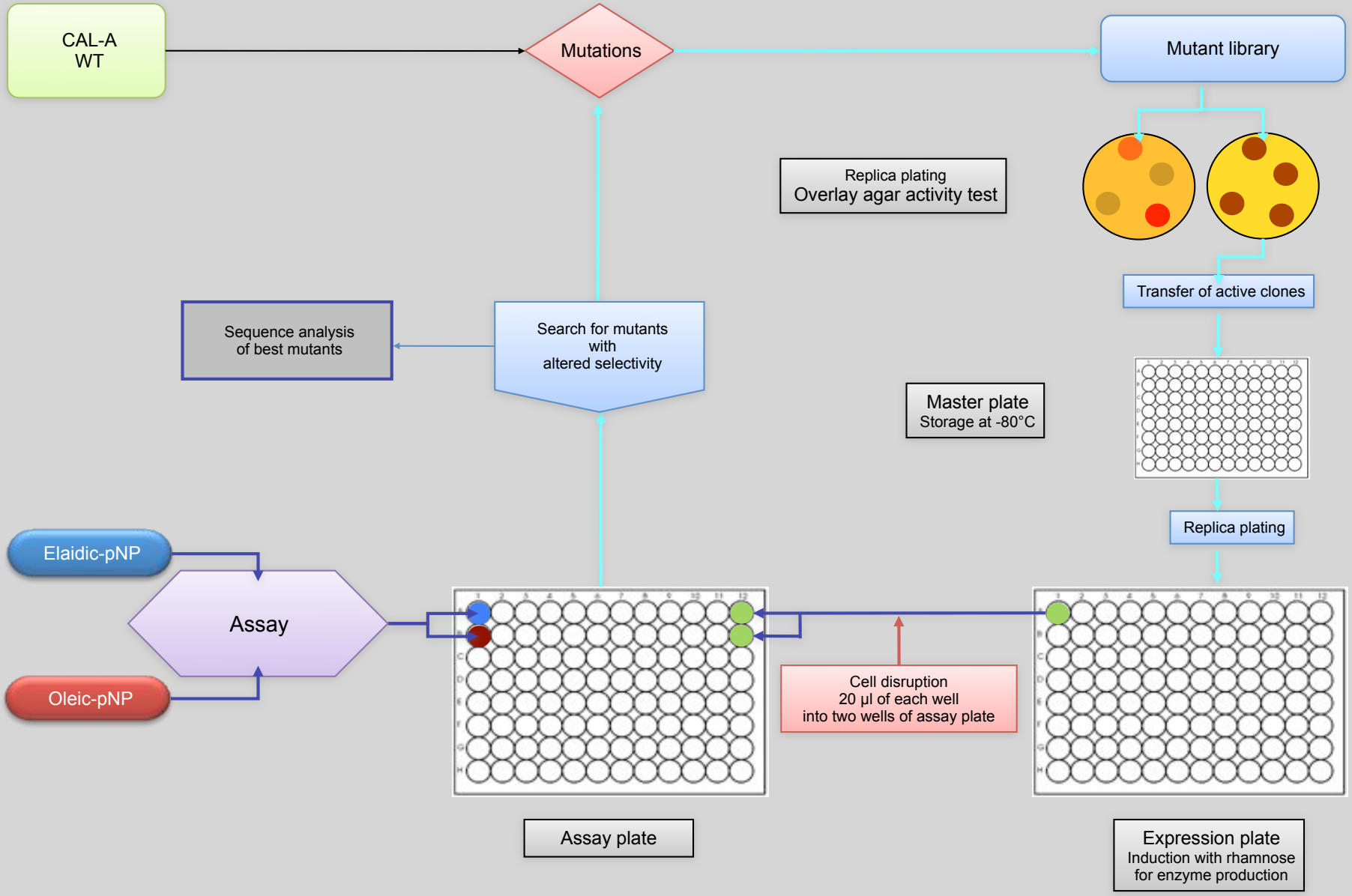
- Selectivity towards *trans*-fatty acids
  - Identification of residues from modeling to possibly deal with selectivity
  - Saturation mutagenesis of residues, expression in *E. coli*, His-tag purification
  - Analysis of *trans*-/*cis*-selectivity w/ pNP-esters
  - Identification of best hits per position
  - Larger scale production of variants
  - Confirmation of selectivity w/ partially hydrogenated oil
  - Combination of best mutants



Residue		F149	I150		A218	T221	F222	L225		F233	G237	L241		M248		I301	L305	

12 sites in CAL-A in 5 Å distance pointing towards fatty acid

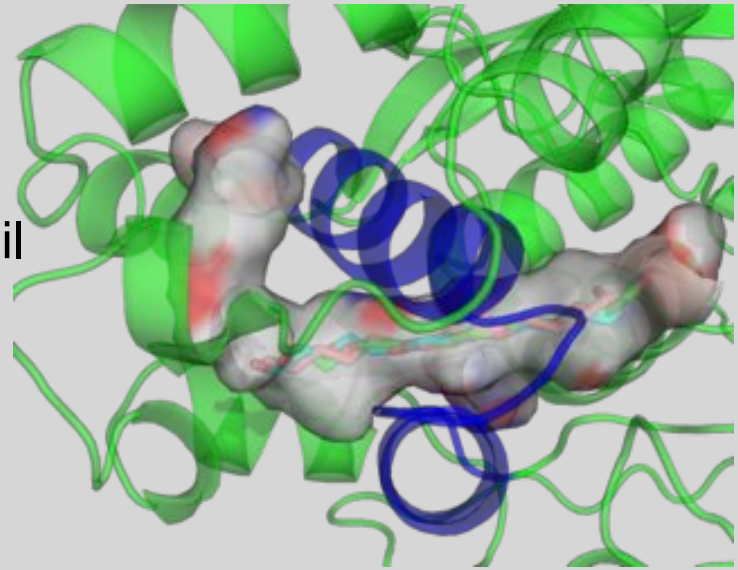
A Typical Screening Procedure



Bartsch, S., Kourist, R., Bornscheuer, U.T., *Angew. Chem. Int. Ed.*, **47**, 1508-1511 (2008)

Protein Engineering of CAL-A

- Selectivity towards *trans*-fatty acids
  - Identification of residues from modeling to deal with selectivity
  - Saturation mutagenesis of residues, expression in *E. coli*, His-tag purification
  - Analysis of *trans*-/*cis*-selectivity w/ pNP-esters
  - Identification of best hits per position
  - Larger scale production of variants
  - Confirmation of selectivity w/ partially hydrogenated oil
  - Combination of best mutants



Residue		F149	I150		A218	T221	F222	L225		F233	G237	L241		M248		I301	L305	

12 sites in CAL-A in 5 Å distance pointing towards fatty acid

Protein Engineering of CAL-A

- Approx. 5,000 variants created by site-directed mutagenesis
- All clones expressed in *E. coli* in deep well plates and subjected to high-throughput screening
- All assayed with *trans*/*cis*-pNP esters to determine selectivity
- Variants with better *trans*-selectivity for pNP esters analyzed in detail with partially hydrogenated soybean oil (PHSO) by GC analysis
- PHSO contains up to 18% *trans*-FA and various individual species

Selectivity	WT	I305N	T221H	I301H	F222C	F149D	F222S
trans/*cis*-pNP	2.5	4	5	6	8	12	15

Brundiek, H.B., Evitt, A., Kourist, R., Bornscheuer, U.T., *Angew. Chem. Int. Ed.*, **51**, 412-414 (2012)

Protein Engineering of CAL-A

- PHSO contains up to 18% *trans*-FA and various individual species
- GC analysis developed to separate & quantify all individual *trans*/*cis*/saturated FA
- Simple data analysis by calculating *trans*/*cis*-rate not possible
- Definition of alpha-selectivity factors required (similar to enantioselectivity)
- Each CAL-A variant had a distinct fatty acid profile
- Two variants found with excellent alpha factor >100:

  no hydrolysis of *cis*-fatty acids observed!

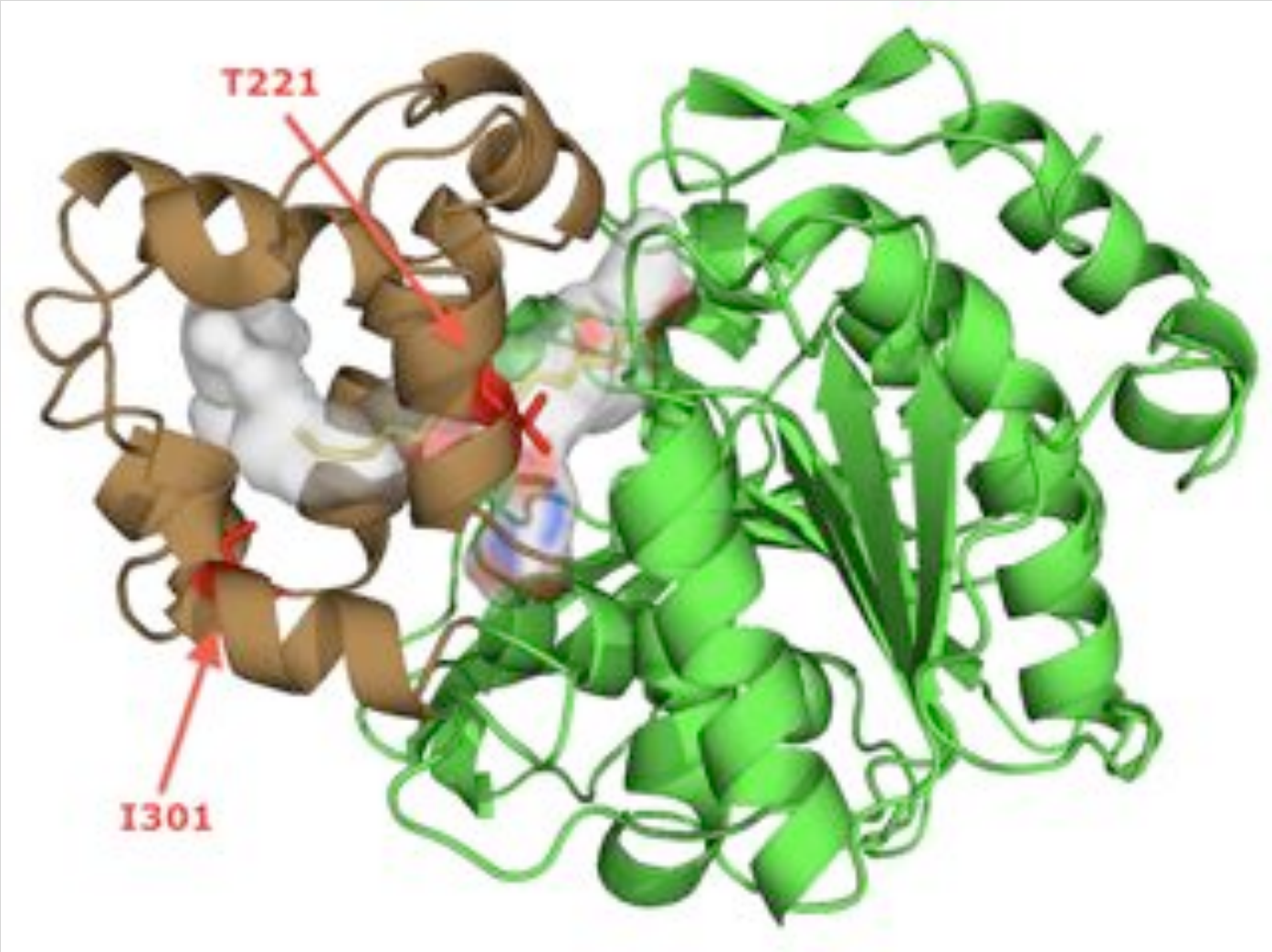
	WT	L305N	F149D	F222S	F222C	T221H	I301H
Conv. [%]	22	14	11	16	20	5	12
alpha[1]	3.5	4.5	4.6	4.5	4.6	9.8	11.2
alpha[2]	6.7	9.6	28.5	32.2	36.6	>100	>100

[1]: Selectivity: *trans*/(*cis*&sat); [2]: Selectivity: (*trans*&sat)/*cis*

Brundiek, H.B., Evitt, A., Kourist, R., Bornscheuer, U.T., *Angew. Chem. Int. Ed.*, **51**, 412-414 (2012)

Protein Engineering of CAL-A

Location of best mutations in 3D-structure



Alternatives to CAL-A ?

CAL-A is very unique, only two sequences w/ >40% homology in databases

Lipase	Amino acids	kDa	Homology [%]
ppro-CAL-A	462	49	100
LipK	458	49	76
LipUM (putative)	582	63	69

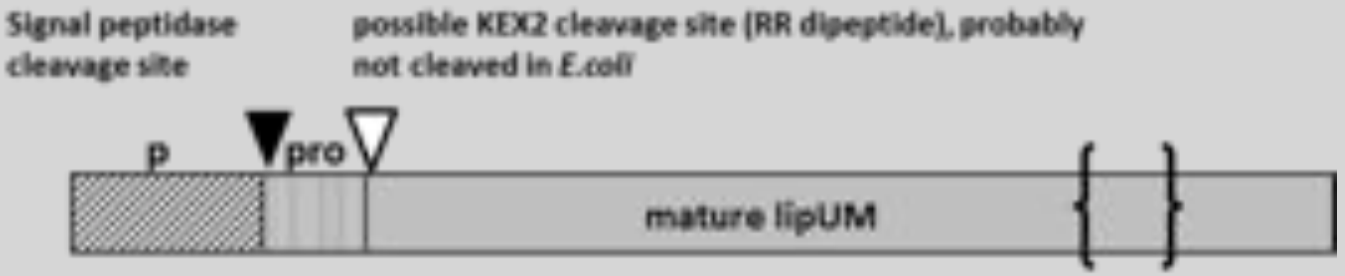
LipK: lipase from *Kurtzmanomyces* sp., expressed and characterized[1]
LipUM: lipase from *Ustilago maydis,* annotated as putative lipase

[1]Kakugawa, K., Shobayashi, M., Suzuki, O., Miyakawa, T. *Biosci. Biotechnol. Biochem.* **66:** 1328-1336 (2002); *ibid.* **66:** 978-985.

Alternatives to CAL-A ?

- LipK shows almost no *trans*-selectivity
- Focus on LipUM, issue: full-length gene has very little lipase activity

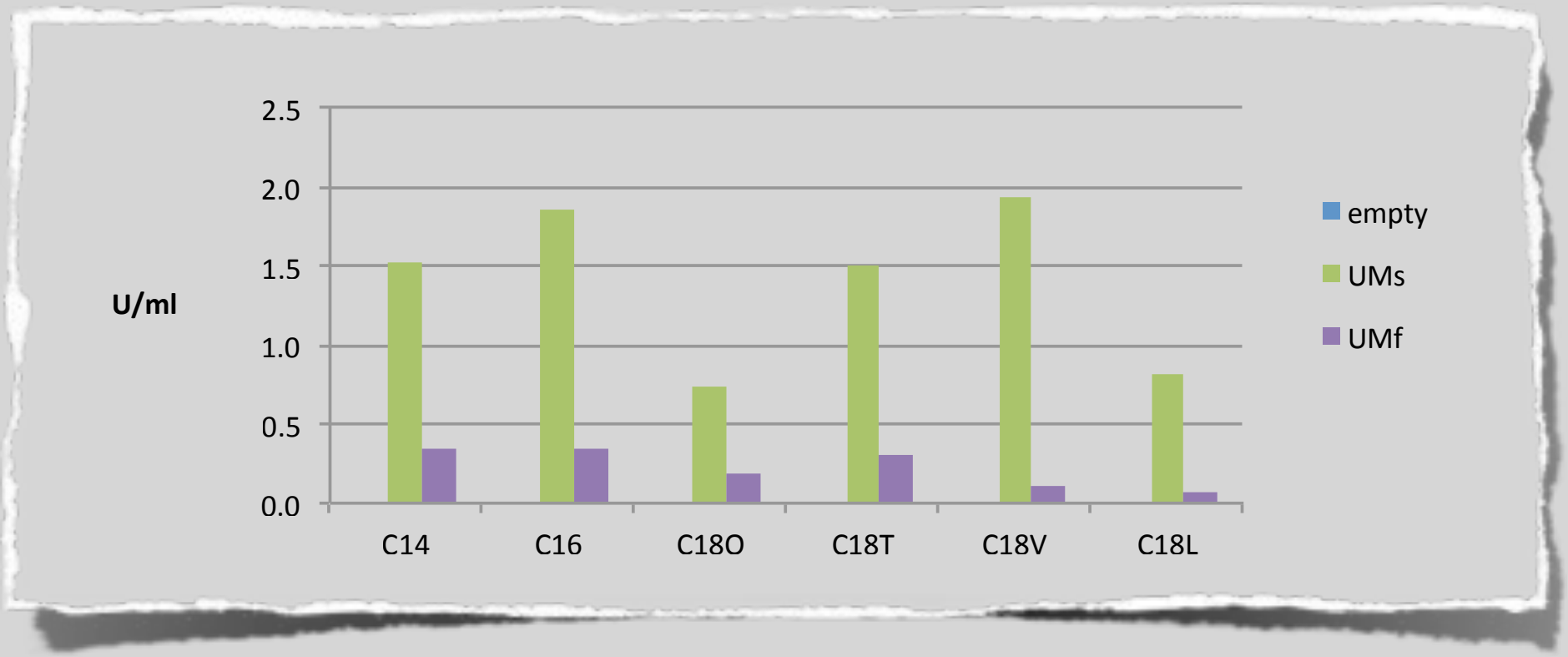
LipUMf (full length lip UM)



LipUMs (short form of lipUM): derived structure

Alternatives to CAL-A ?

- Functional expression of lipUM (short) in *E. coli* and *P. pastoris* achieved
- lipUM also shows *trans*/*cis*-selectivity:
  (1.7-fold for elaidate/oleate-pNP esters; CAL-A: 2.5-fold)
- Protein engineering of lipUM in progress



Brundiek, H.B., Sass, S., Evitt, A., Kourist, R., Bornscheuer, U.T., *Appl. Microb. Biotechnol.*, **94**, 141-150 (2012)

Medium-Chain Fatty Acid Selectivity

- Identification of key residue G237 allowed for blocking the entrance to the fatty acid binding region
- pNP-assay with fatty acids of varying chain length identified mutants with clear shift towards medium chain fatty acids; hydrolysis of long-chain fatty acids suppressed

Trans-Fatty Acid Selectivity

- The mutagenesis strategy identified several mutants with substantially increased *trans*-selectivity (up to alpha>100)
- Results from pNP-assay could be transfered to complex *trans*-fatty acids mixture in partially hydrogenated plant oil
- Combinations of mutations currently under study

Alternatives to CAL-A

- With the lipase from *Ustilago maydis* an interesting alternative to CAL-A could be identified, functionally expressed and characterized

Summary / Future Directions

Summary

- Enzymatic methods are useful for lipid modification
- Lipases are the 'working horses' already applied on industrial scale:
  - in the production of 'healthier' lipids for human nutrition
  - in oleochemistry
- Protein engineering helps to tailor-design biocatalysts
- The metagenome is a useful source of new enzymes

Future Directions

- Focus on oxygenative enzymes (monooxygenases, epoxidases, lipoxygenases etc.)
- Metabolic engineering to use further classes of enzymes in whole-cell lipid modification
- Multi-step biotransformations

Take Home Message

"*In the past, an enzyme-based process was designed around the limitations of the enzyme; today, the enzyme is engineered to fit the process specifications*"

Bornscheuer, U.T., Huisman, G. W., Kazlauskas, R.J., Lutz, S., Moore, J.C., Robins, K., *Nature*, **485**, 185-194 (2012)

Acknowledgements

Thanks For Your Attention !

Announcements

Protein Engineering Conference

Aug. 29./31. (Wed/Fri) 2012

(right before Biocat2012 in Hamburg)

Location: Greifswald, Institute of Biochemistry
Organizers: F. Hollfelder / U.T. Bornscheuer

Topics:

- Computational methods
- Emerging Targets for Protein Engineering
- Metagenome Approach
- Metabolic Engineering / Synthetic Biology
- New Concepts for Protein Discovery/Design

Biocatalysis in Lipid Modification

Sept. 19./21. (Wed/Fri) 2012

(right before EuroFedLipid conf. in Krakow)

Location: Greifswald, Institute of Biochemistry
Organizers: P. Villeneuve / U.T. Bornscheuer

Topics:

- Application of isolated enzymes
- Enzyme discovery and protein engineering
- Whole cell biotransformation
- Understanding lipid biosynthesis in microorganisms
- Metabolic engineering and synthetic biology

European Research Training Network on Directed Evolution of Functional Proteins
ENEF 2008-2012

Euro Fed Lipid