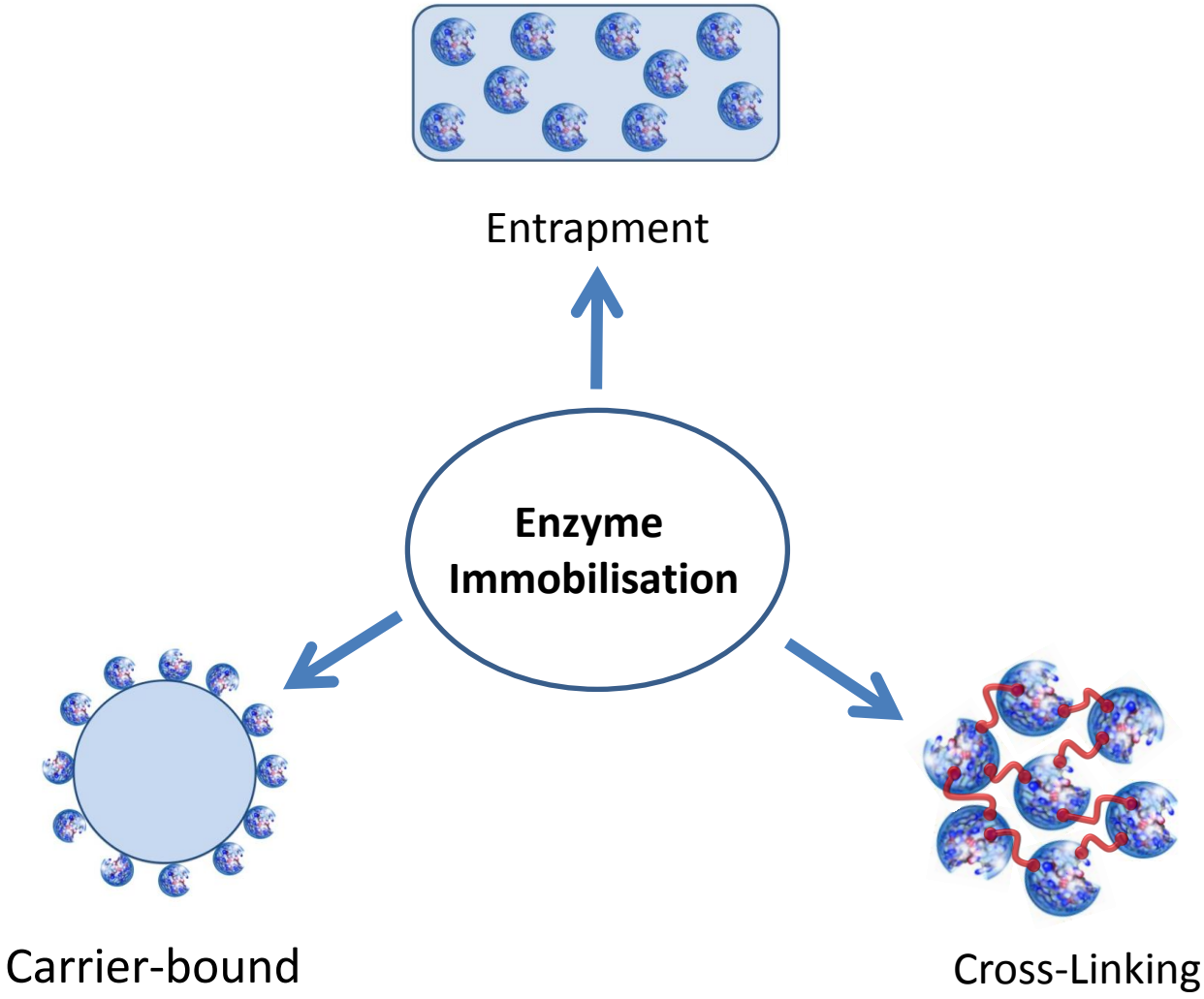
Electronic Supplementary Material (ESI) for Chemical Society Reviews

Enzyme Immobilization: Why, What and How

Roger A. Sheldon

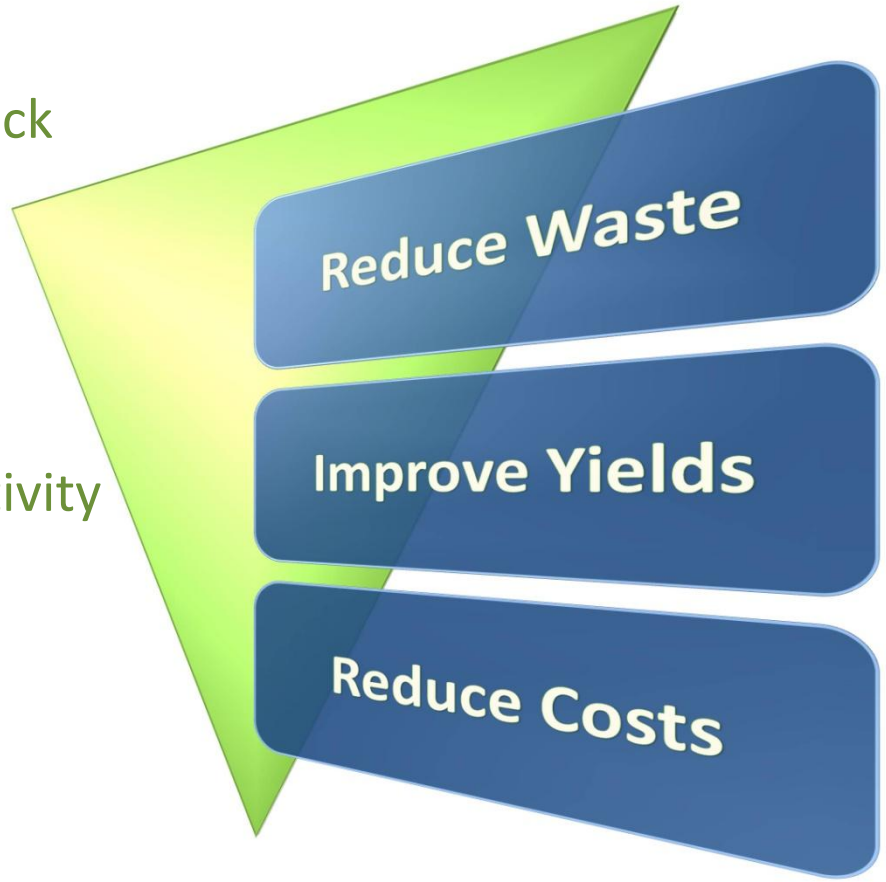


Why use enzymes?

- Renewable, biodegradable feedstock
- Mild conditions (pH, T & P)

- High rates
- Higher quality product
- High chemo, regio & enantioselectivity

- No special equipment needed
- Environmentally & economically attractive (GREEN)



The Challenge

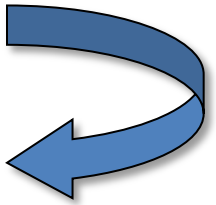
- Disadvantages of enzymes
  - Low operational stability & shelf life
  - Cumbersome recovery & re-use
  - Product contamination
  - Allergic reactions to proteins

- Non viable biocatalytic applications
  - Enzyme costs too high
  - Not practical

The Solution: Immobilization

- Immobilization is Enabling Technology

High waste / low profit

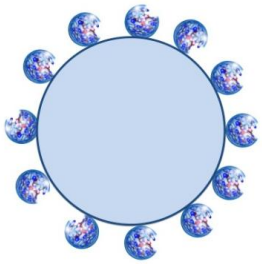


Low waste / high profit

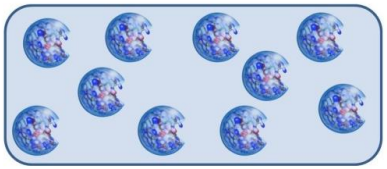
- Advantages
  - stability, stability, stability ...
  - repeated re-use of biocatalyst (batch)
  - easier downstream processing
  - continuous process technology

Types of Immobilization

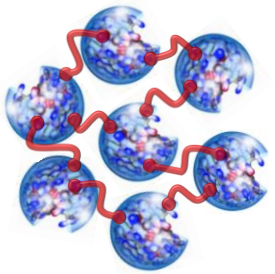
- Binding to a carrier
  - e.g. ion exchange resins



- Entrapment
  - e.g. in silica sol-gel



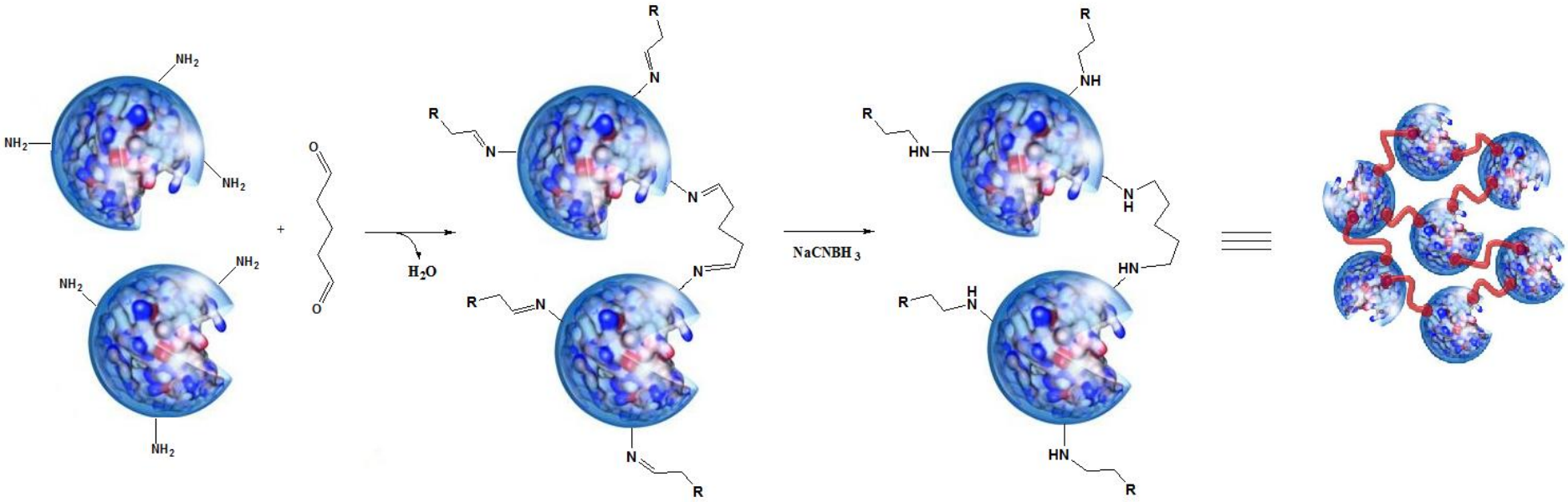
- Cross-Linking
  - e.g. Cross-Linked Enzyme Aggregate (CLEA)



Carrier-bound / entrapped enzymes have inherently low volumetric & catalyst productivities (90->99% non-catalytic mass)

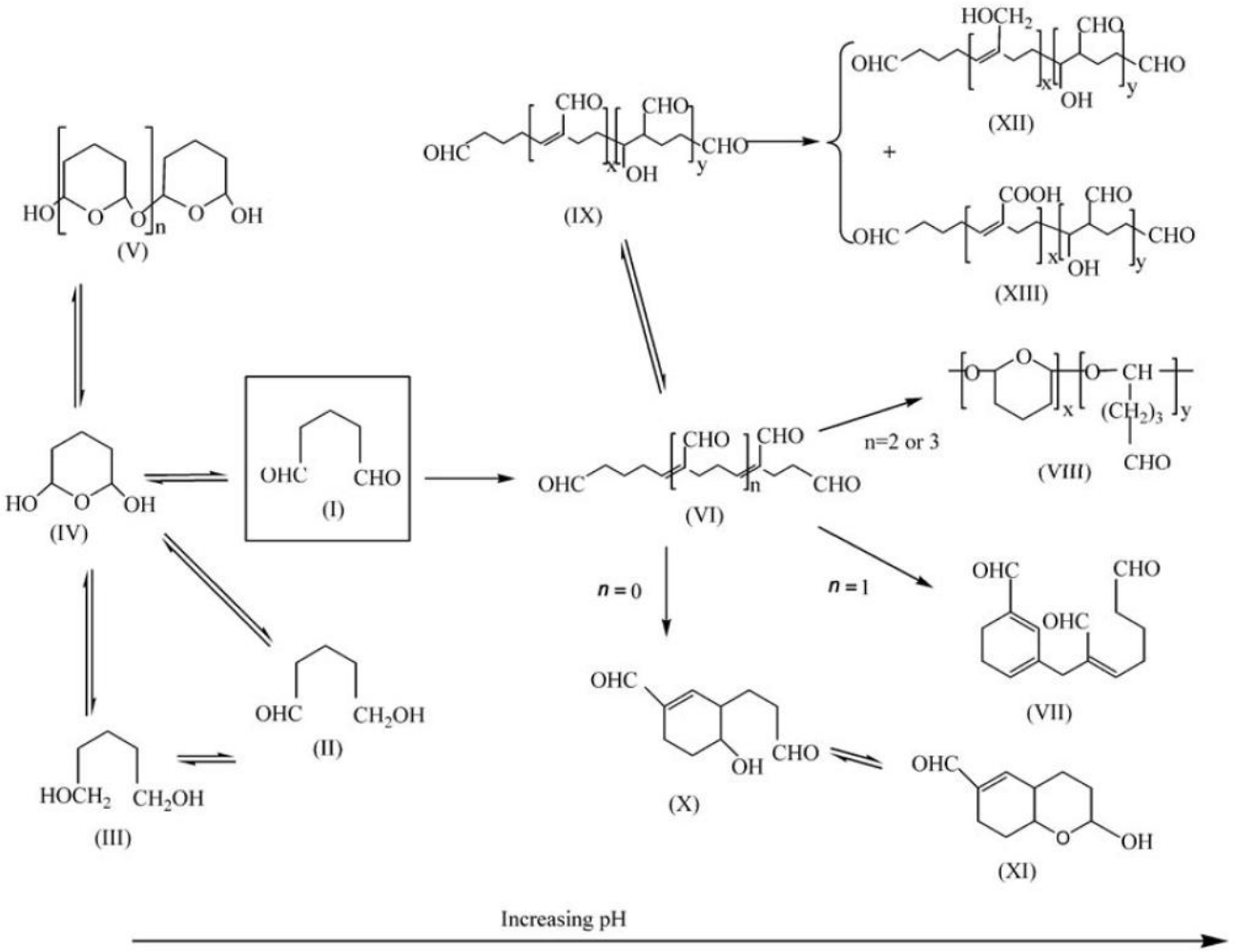
R.A. Sheldon, *Adv. Synth. Catal.*, 349 (**2007**) 1289

Cross-Linking with Glutaraldehyde



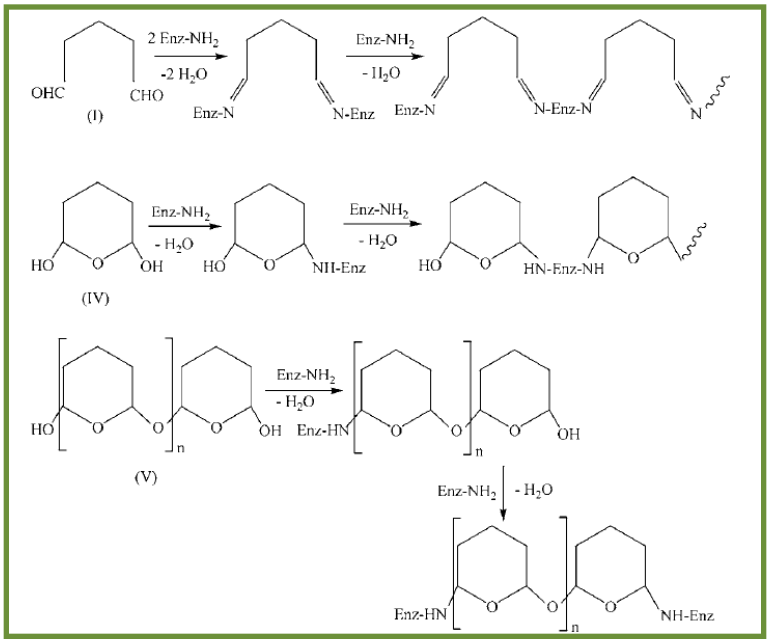
The monomeric structure of glutaraldehyde does not reflect the complexity of glutaraldehyde behavior in solution and its reactivity with proteins!

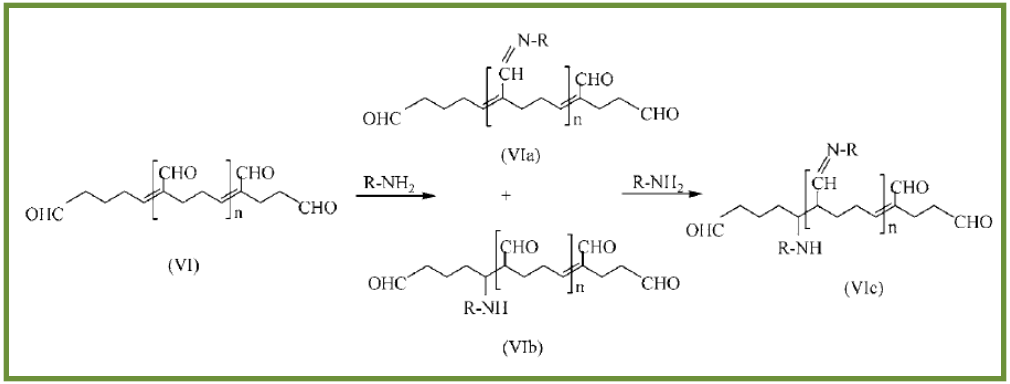
Glutaraldehyde in Practice

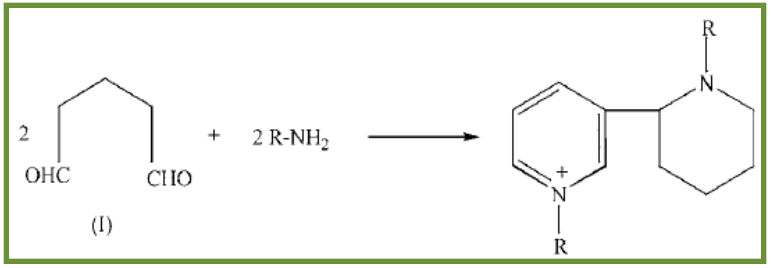


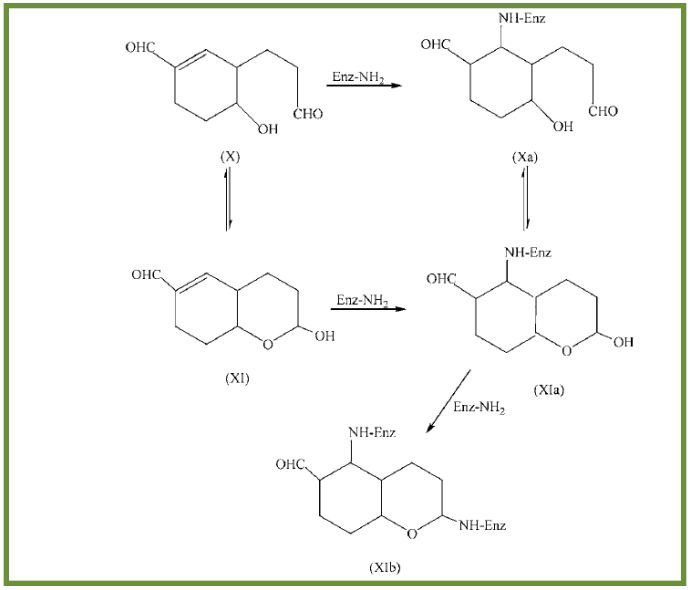
I. Migneault, *BioTechniques*, 37 (**2004**) 790

Glutaraldehyde Reactions with Proteins







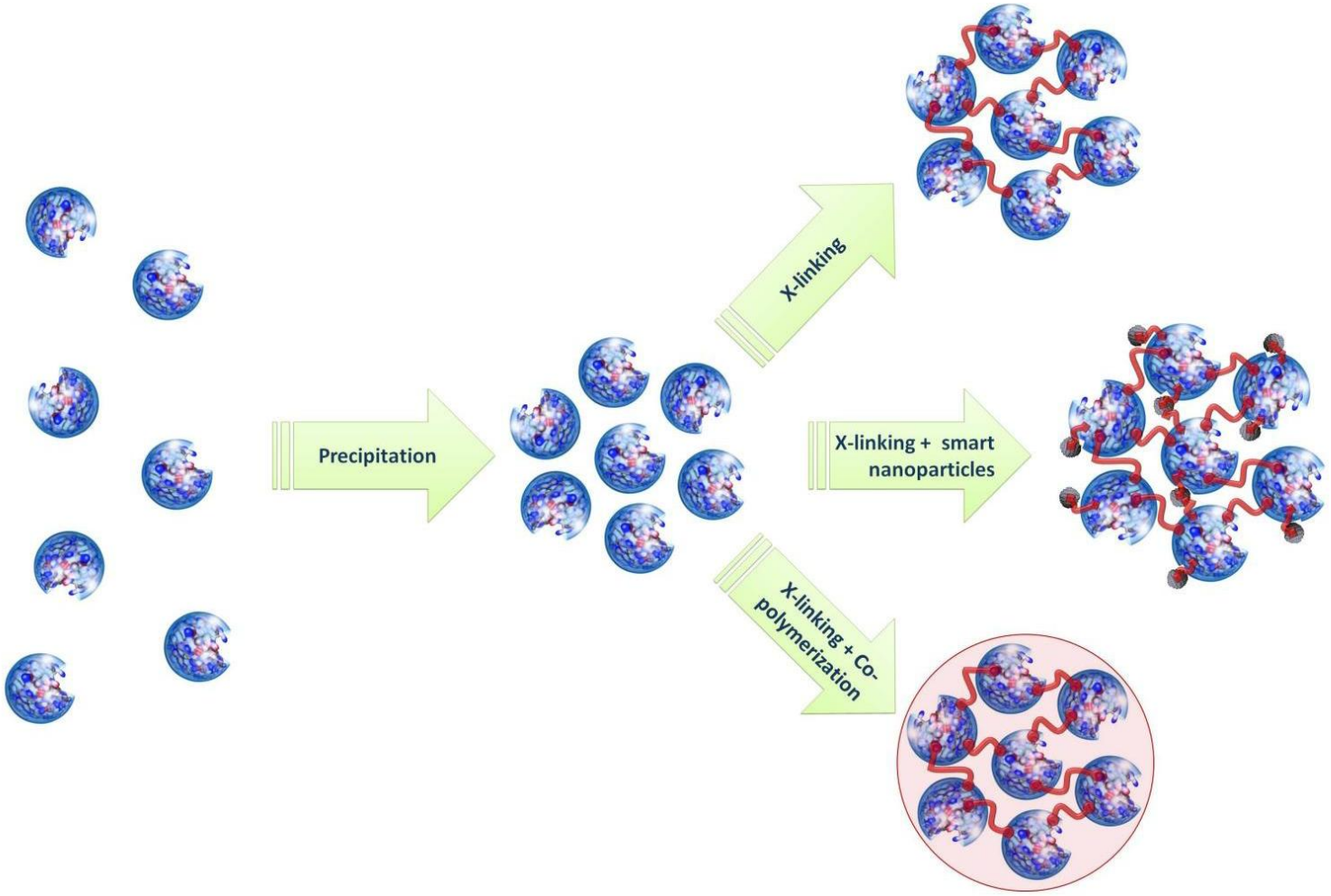


I. Migneault, *BioTechniques*, 37 (**2004**) 790

Cross-Linking with Glutaraldehyde

- Common, inexpensive and effective protein cross-linking agent
- Cross-linking chemistry still not fully understood
- Type of covalent bond formed depends heavily on glutaraldehyde concentration, amine concentration, pH, and temperature
- Reduction of Schiff bases with $NaBH_4$ or $NaCNBH_3$ usually not necessary
- Other aldehyde cross-linkers, such as dextranpolyaldehyde generally do need a reduction step

The CLEA Technology



Basic CLEA Properties

Very high enzyme loading

Particle size typically 5-50 µm

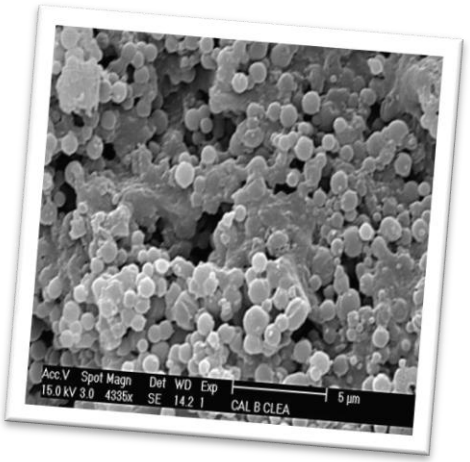
Good filterability and centrifugability

Packed bed possible

Mechanically robust

Excellent operational stability
heat, organic solvents and proteolysis (autolysis)

Tuneable hydrophobicity/hydrophillicity



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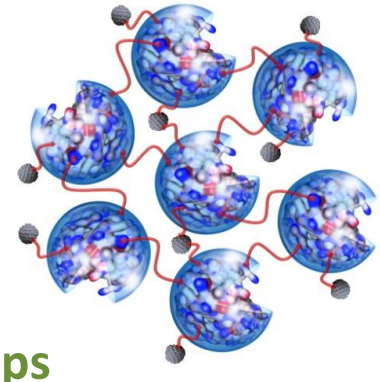
Advantages of CLEAs

1. Improved properties
    - Better storage and operational stability
    - Hypoallergenic
    - No leaching of enzyme in aqueous media
2. Cost-effective
    - No need for pure enzyme (crude cell lysate sufficient)
    - Easy recovery and recycle (easier DSP)
    - High productivities (kg product/kg enzyme)
3. Broad scope & short time to market

Electronic Supplementary Material (ESI) for Chemical Society Reviews

Magnetic CLEAs



- Synthesis of magnetic nanoparticles in silica
- Functionalisation of nanoparticles with aminopropyl groups
- CLEAtion: cross-linking the enzyme and the nanoparticles

Characteristics

- Magnetic decantation
- Magnetic strength can be adjusted
- No change in CLEA activity
- e.g. hydrolases, oxidoreductases, nitrile hydratases

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Additional Properties of mCLEAs

Separation of the enzyme catalyst by magnetic decantation

Magnetic strength of the mCLEA can be adjusted for the particular application

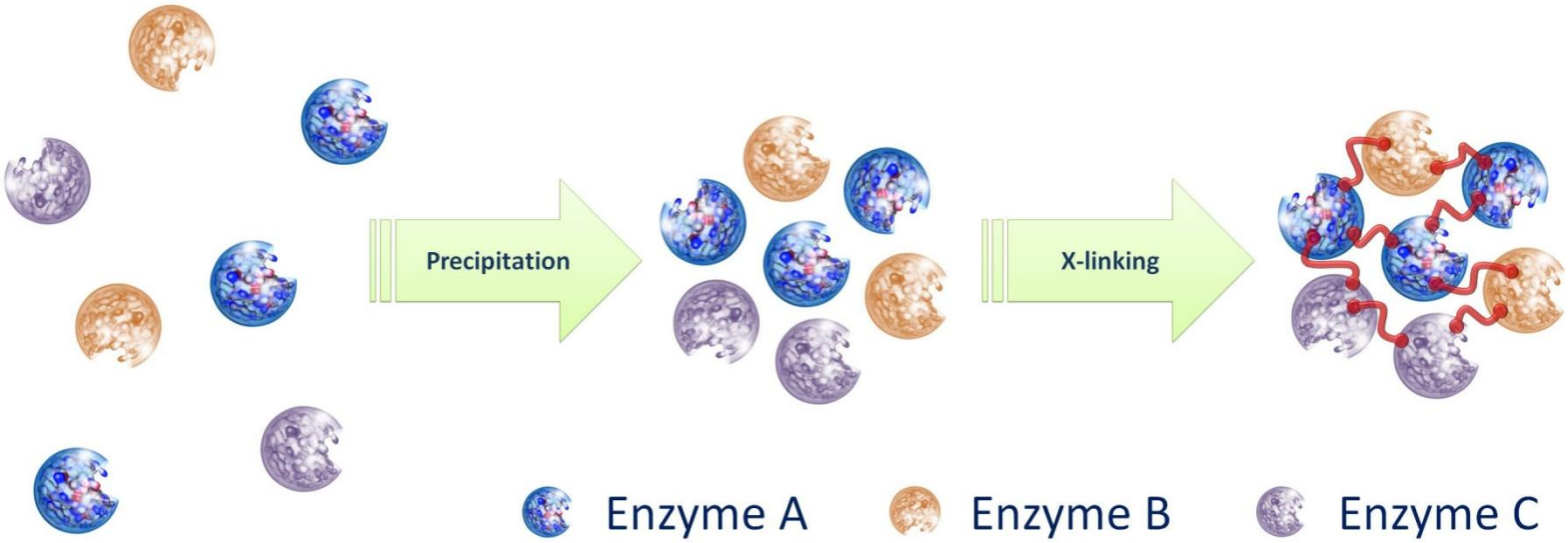
No changes in the structure by the introduction of magnetic particles

No changes in enzyme activity of the immobilised enzyme by the introduction of magnetic particles

mCLEA of any enzyme can be manufactured – currently examples with hydrolases and oxidoreductases

Potential application in the pharmaceutical, food and feed industries, and diagnostics

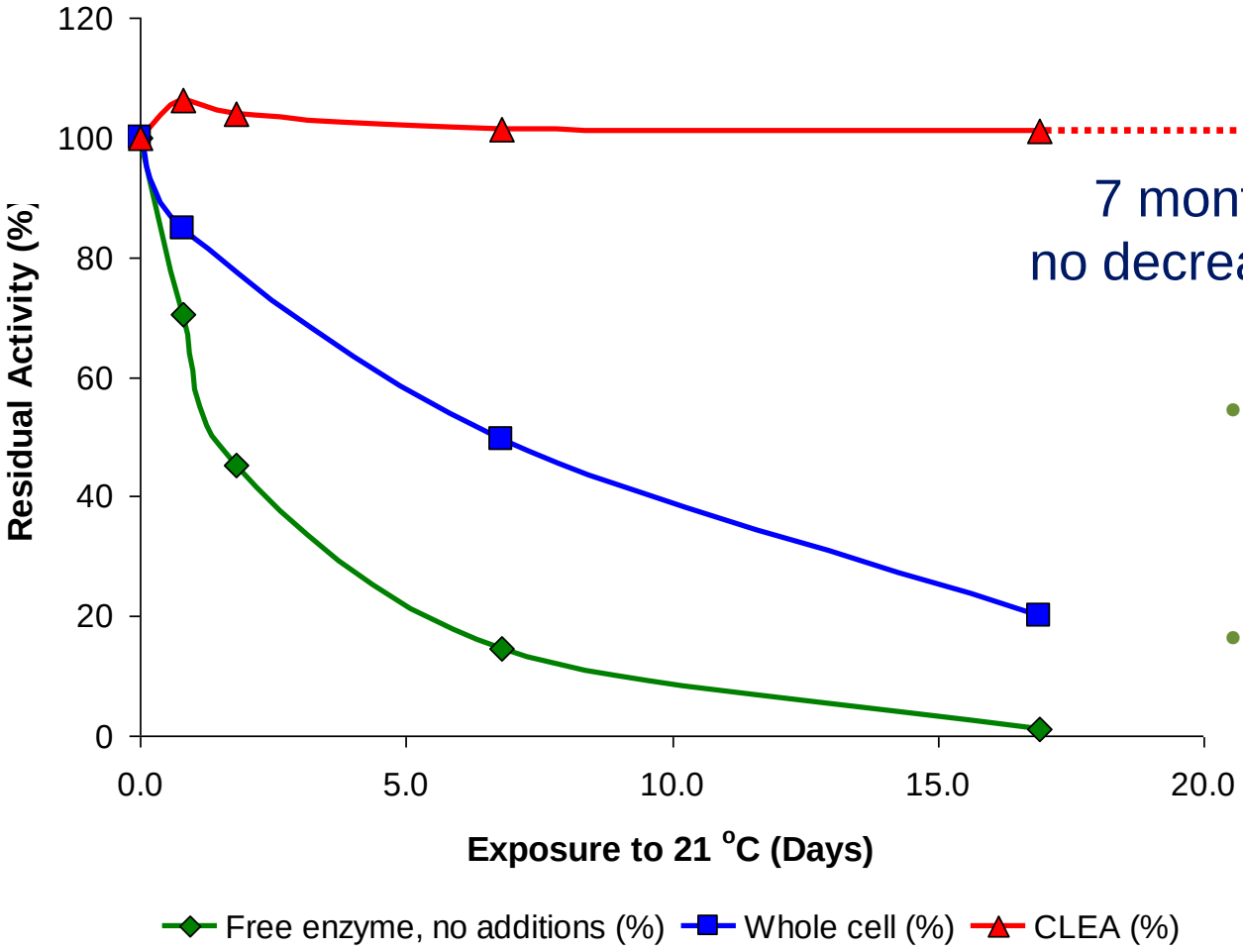
Combi CLEAs



Synthesis of CombiCLEAs

- Two or more enzymes in one CLEA
- Used for cascade reactions

Storage Stability – NHase CLEA



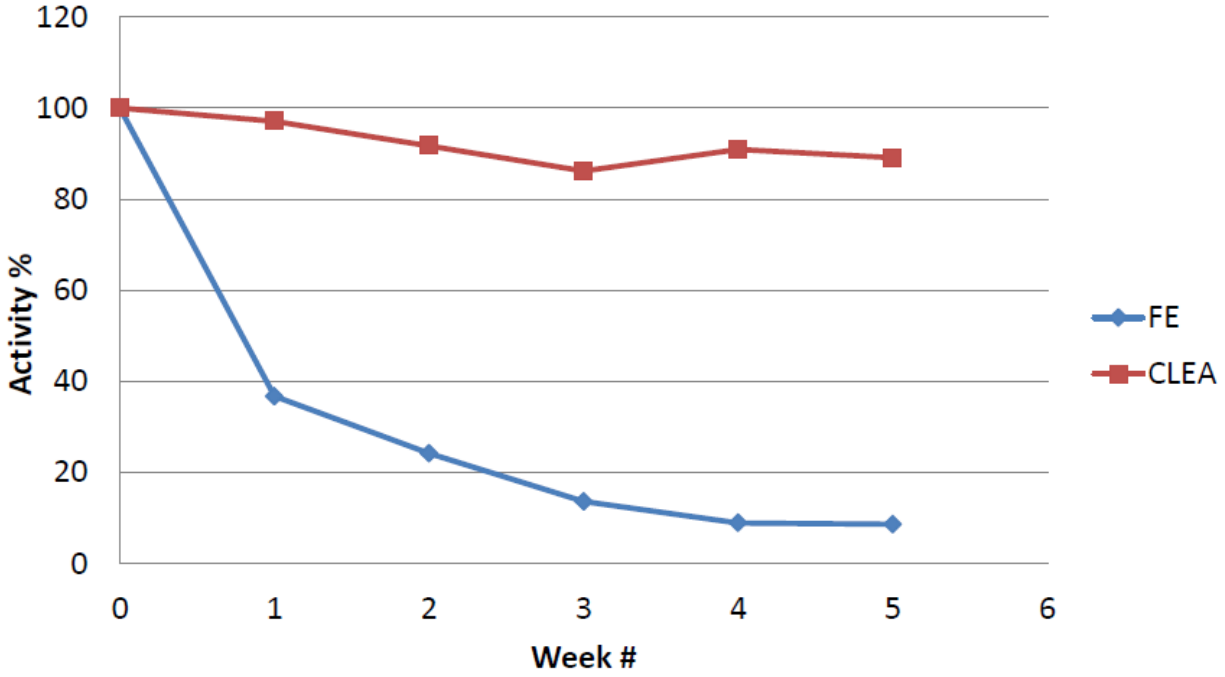
7 months storage
no decrease in activity!

- Storage in 0.01M TRIS buffer pH 8, no additions at room temperature
- Instability of free enzyme due to dissociation of multimeric enzyme

van Pelt, *Green Chem*. 10 (**2008**) 395-400

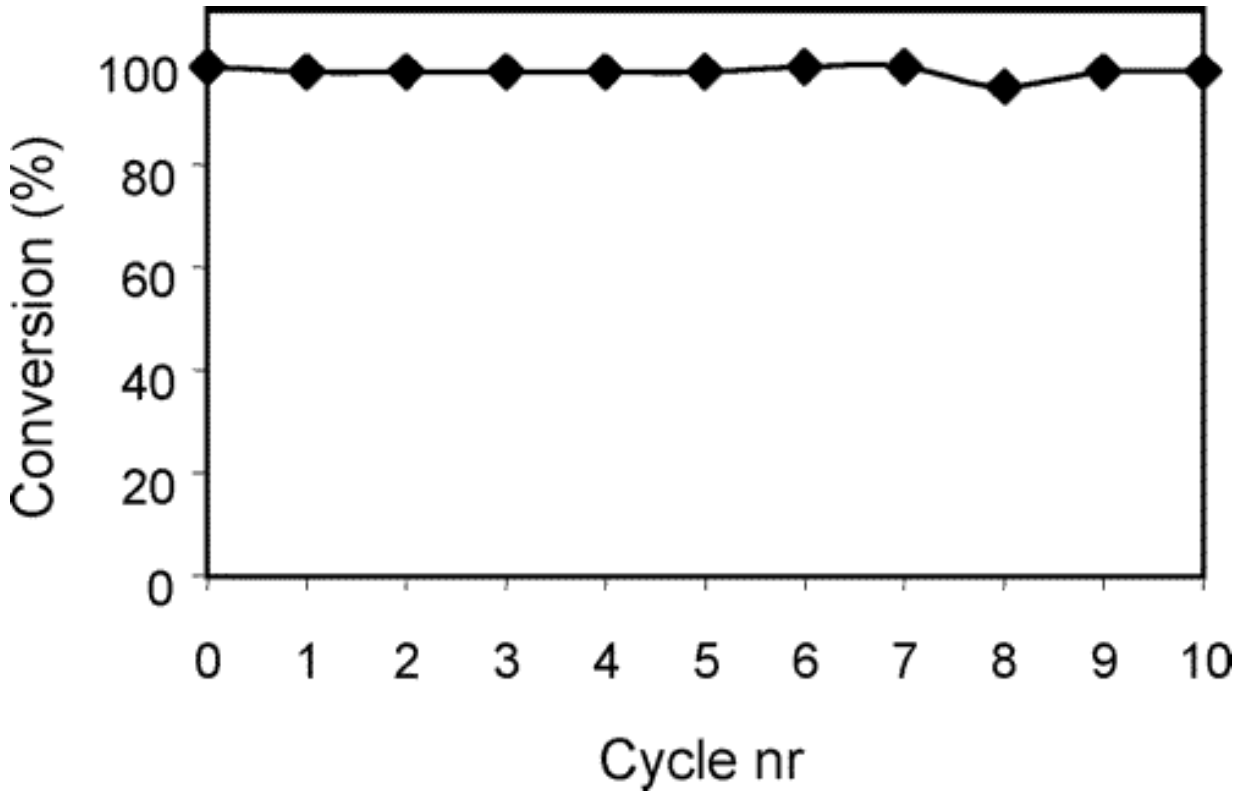
Thermostability – Papain CLEA

- Papain (protease from *C. papaya*) incubated at pH 7 and 50 °C.



Recyclability – PaHNL CLEA

- Effect of recycling on the performance of (R)-oxynitrilase CLEA in the hydrocyanation of *o*-chlorobenzaldehyde



Scope of the CLEA Technology

Hydrolases

- Pen. Acylases (2)
- Lipases (19)
- Esterases (3)
- Proteases (9)
- Nitrilases (5)
- Aminoacylase
- Phytase
- Galactosidase
- Carbonic anhydrase

Oxidoreductases

- KRED
- FDH
- Glucose oxidase
- Galactose oxidase
- Amino acid oxidase
- Laccase (3)
- Catalase
- Chloroperoxidase
- HRP

Lyases

- *R*- & *S*- HNLases (5)
- PDC
- DERA
- Nitrile hydratase (9)

Transferases

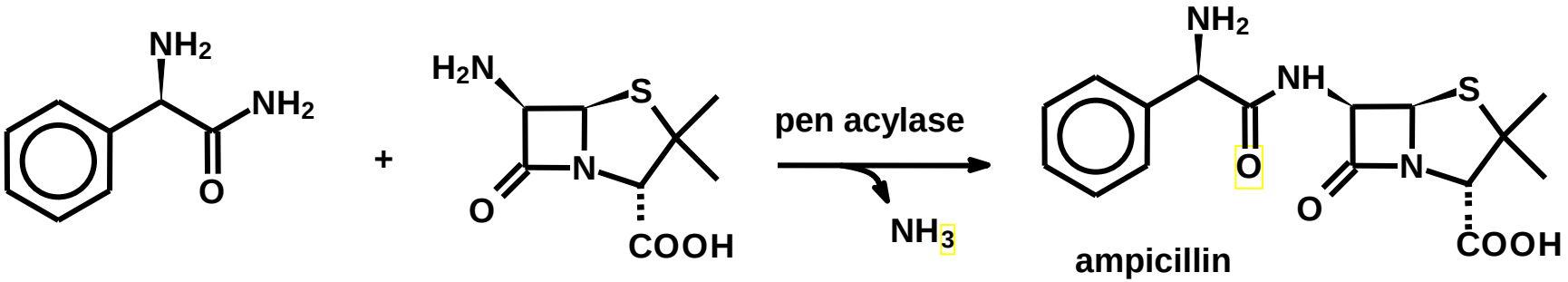
- Transaminases
  - (R) selective (**3**)
  - (S) selective (**5**)

Scope of the Technology

Hydrolases

- **Pen. Acylases** (2)
- **Lipases** (19)
- Esterases (3)
- **Proteases** (9)
- Nitrilases (5)
- Aminoacylase
- Phytase
- Galactosidase
- Carbonic anhydrase

Hydrolases – Pen. Acylase



Biocatalyst	Conv. (%)	S/H ratio	Rel. Productivity
Free enzyme	88	2.0	100
T-CLEA	85	1.58	151
PGA-450	86	1.56	0.8

Conclusion – High productivity and S/H

L.Cao, L.M.van Langen, F. van Rantwijk, and R.A.Sheldon, *J. Mol. Catal. B:Enzym*. 11 (**2001)** 665

Hydrolases – Protease

- Alcalase CLEA : *B. licheniformis* protease
- Savinase CLEA: *B. clausii* protease
- Esperase CLEA: *B. lentus* protease
- BS CLEA : *B. subtilis* protease
- Papain CLEA : *C. papaya* protease
- Protease CLEA Discovery Platform

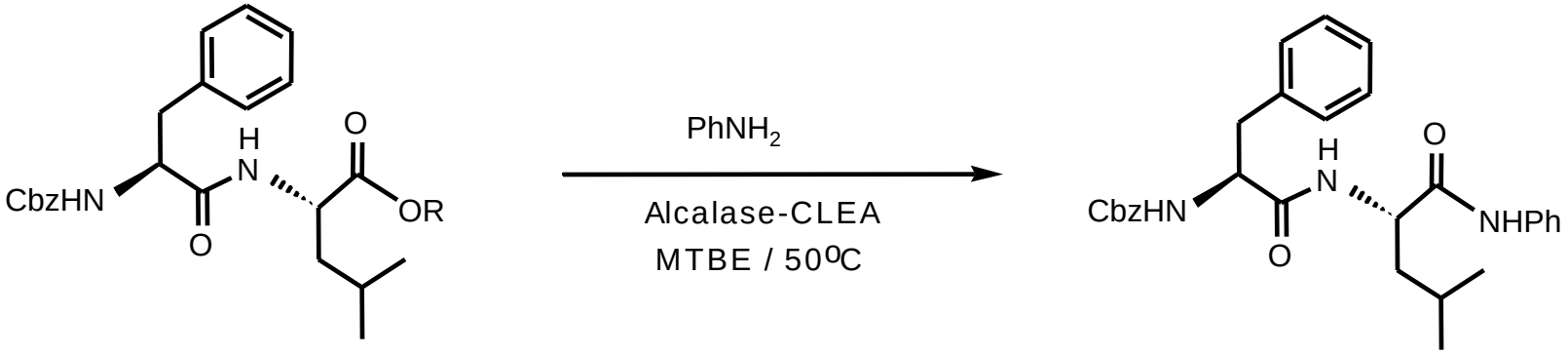
Hydrolases - Proteases

Antifouling Agent in Paint

- To replace toxic organotin compounds (banned in the EU since 2008)
- Cross-linked enzyme aggregates (CLEAs) of proteases were tested in artificial seawater (ASW) both as it is and as a component of the paint.
- It is found that all CLEAs have tolerance to xylene and have great stability in dried paint.
- The maximum increase in relative activity was found for CLEA *B.licheniformis*.
- CLEA *B.licheniformis* has shown 900% activation during storage in ASW.
- In the paint, non-modified subtilisin lost more that 90% of activity in 28 days.

Hydrolases – Proteases

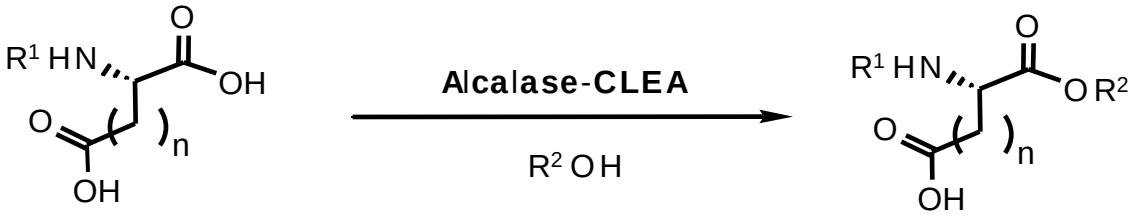
Amidation in Organic Media



R	Yield (%)
CH_3	93
$PhCH_2$	94
H	93

Nuijens, Cusan, Kruijtzer, Rijkers, Liskamp, Quaedflieg, *J. Org. Chem.* 74 (**2009)** 5145

Regioselective Esterifications



n = 1 or 2

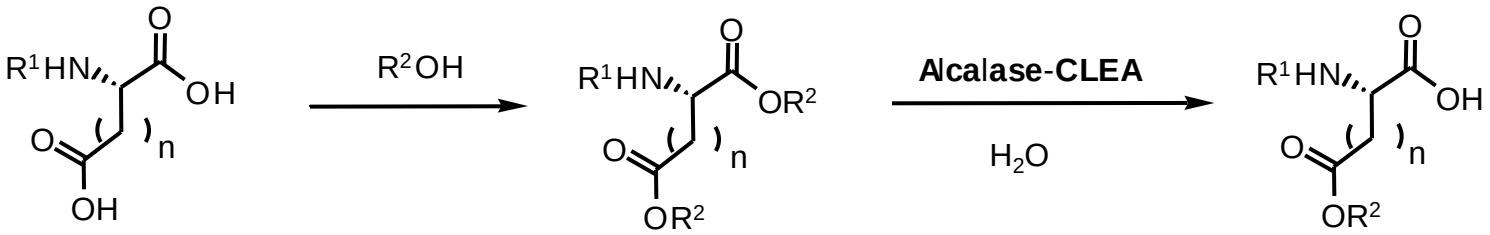
R^1 = Cbz, Boc,Fmoc

R^2 = allyl, $Me_3SiCH_2CH_2$-

92-98% Yield

84-89% Isolated yield

Nuijens, Cusan, Kruijtzer, Rijkers, Liskamp, Quaedflieg, *Synthesis* (**2009)** 809



n = 1 or 2

R^1 = CBz, BOC, FMOC

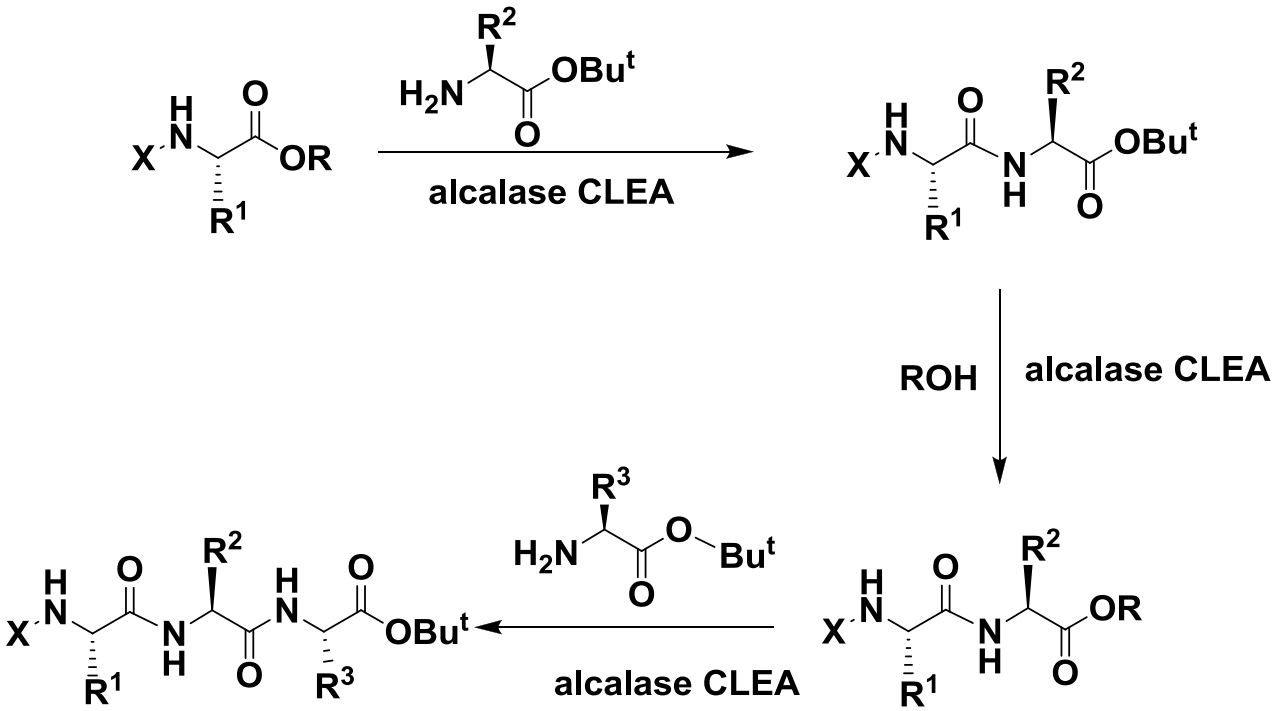
R^2 = allyl or $(CH_3)_3SiCH_2CH_2$-

95-98% Yield

77-87% Isolated Yield

Nuijens, Kruijtzer, Cusan, Rijkers, Liskamp, Quaedflieg, *Tetrahedron Lett*. 50 (**2009)** 2719

Peptide Synthesis

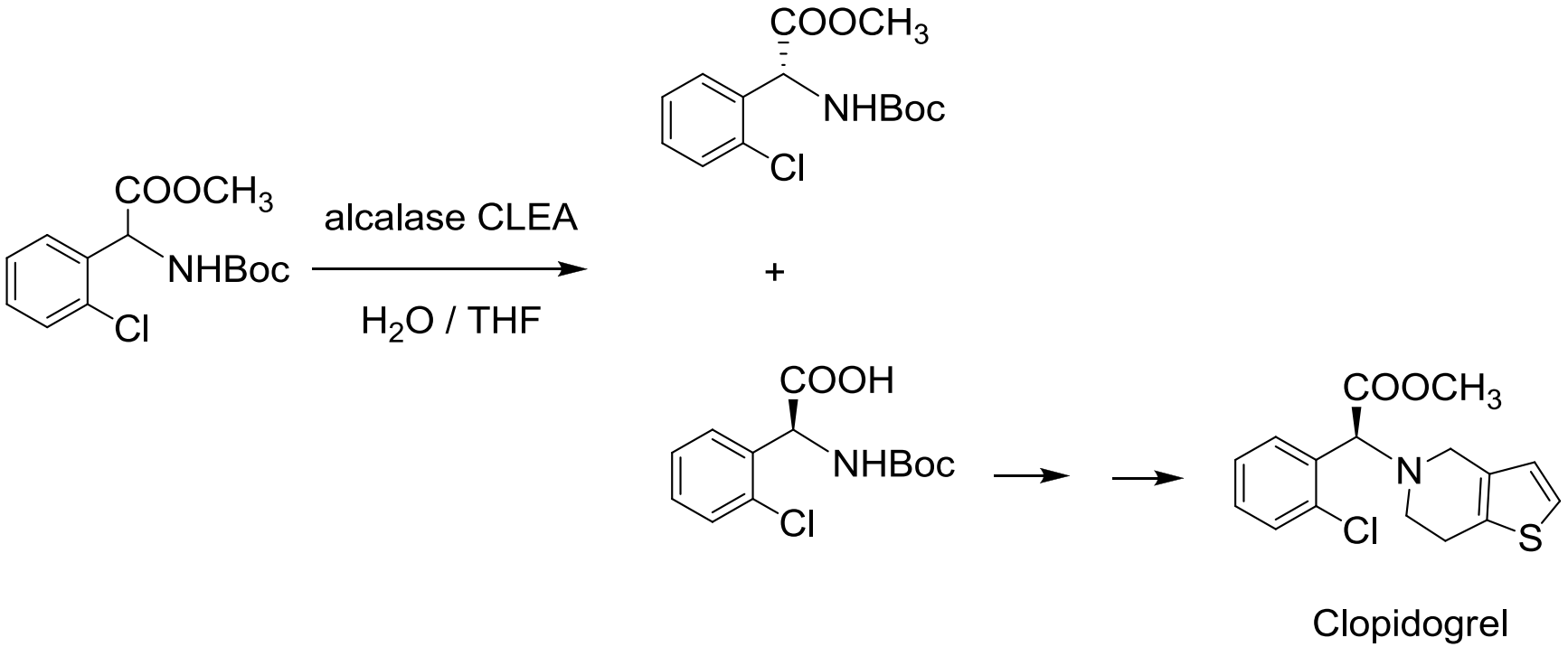


X = N-protecting group

R^1, R^2, R^3 = amino acid side-chains

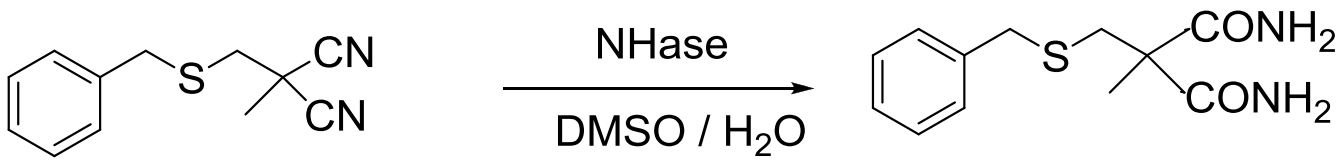
Nuijens et al, *Advan. Synth. Catal*. 352 (**2010)** 2399 – 2404

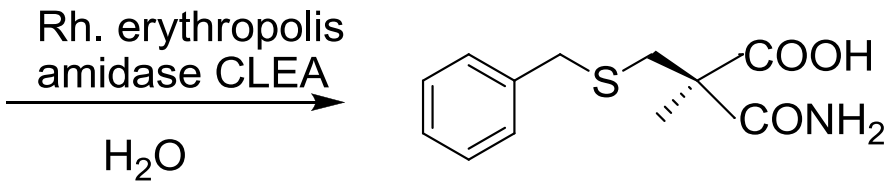
Resolution of Amino Ester with Alcalase-CLEA



Ferraboschi, P., De Miere, F., Galimberti, F. *Tetrahedron Asymm*. (**2010)** 21, 2136.

Rhodococcus erythropolis amidase CLEA: Enantioselective Hydrolysis (Astra Zeneca)



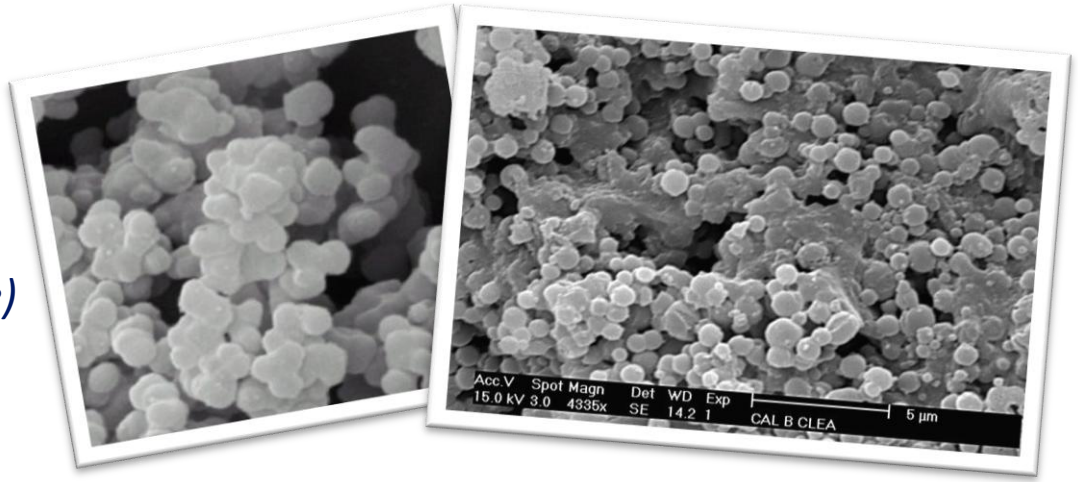


85% yield
93% ee

A. Wells, presented at the SCI Meeting on *Biocatalysis & Biotransformations*, London, October 14, 2010

Hydrolases - Lipases

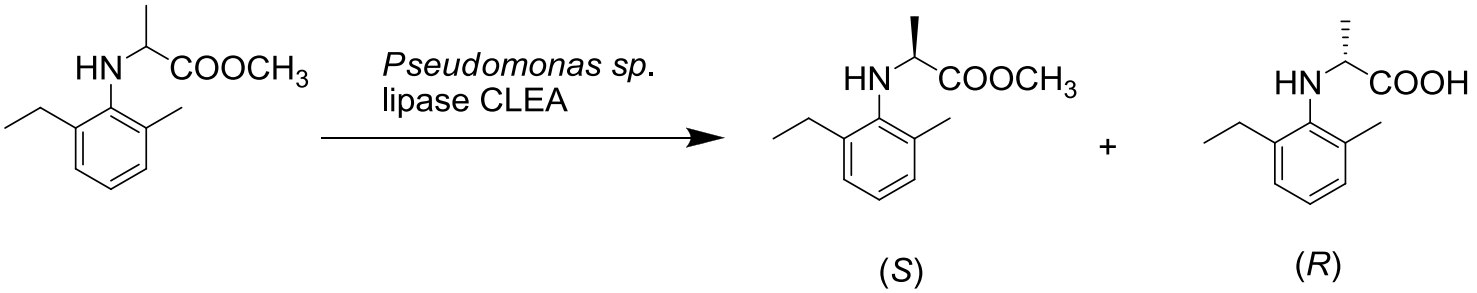
- *Candida antarctica lipase B (CaLB)*
- *Candida antarctica lipase A (CaLA)*
- *Thermomyces lanuginosus (Lipolase)*
- *Rhizomucor miehei*
- *Candida rugosa*
- *Alcaligenes sp.*
- *Pseudomonas stutzeri*



Candida antarctica Lipase B CLEA
The only commercially available immobilized form of CaL B completely stable to leaching in water

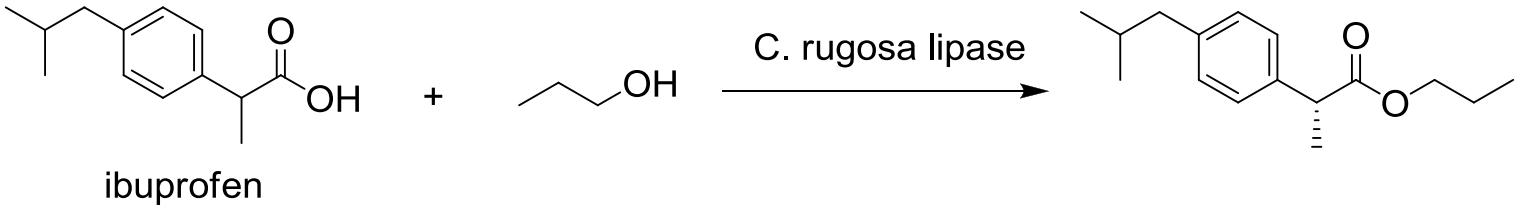
Lipase CLEA discovery platform

Hydrolases - Lipases



E value = > 100

Zhao, L. et al. *J. Mol. Catal. B: Enzymatic* 54 **(2008)** 7

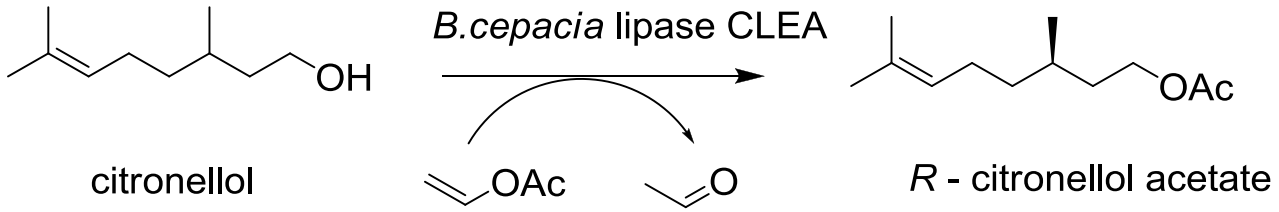


ibuprofen

Free enzyme E = 13
CLEA E = 23

Yu, H. W. et al. *J. Mol. Catal. B: Enzymatic*

Hydrolases - Lipases

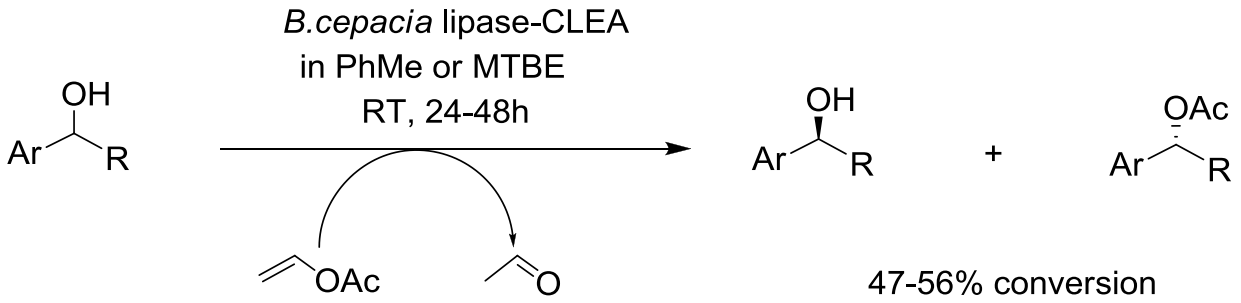


citronellol

R - citronellol acetate

Free Enzyme	E = 19
CLEA	E = 74

Majumder, A. B., Mondal, K., Singh, T. P., Gupta, M. N. *Biocat. Biotrans* 26 (**2008**) 235



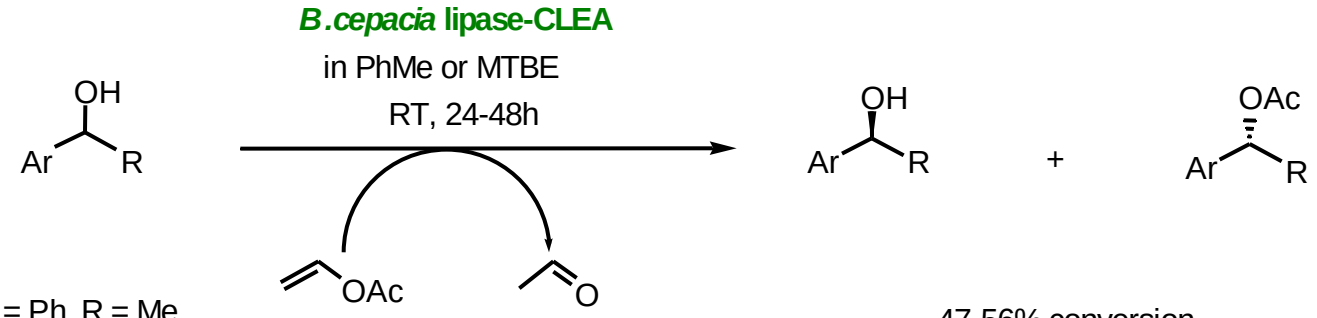
47-56% conversion

E = 35 - >200

Ar = Ph, 2-furyl,
R = Me, $CH_2NHCOPr$

P. Hara, U. Hanefeld, L. T. Kanerva, *J. Mol. Catal. B: Enzymatic* 50 (**2008**) 80

Hydrolases - Lipases

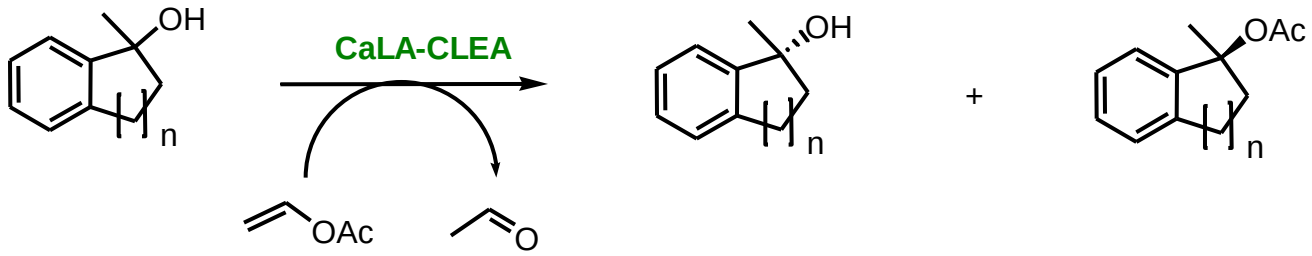


1 Ar = Ph, R = Me
2 Ar = 2-furyl, R = Me
3 Ar = Ph, R= $CH_2NHCO^{P}r$

47-56% conversion

E = 35 - >200

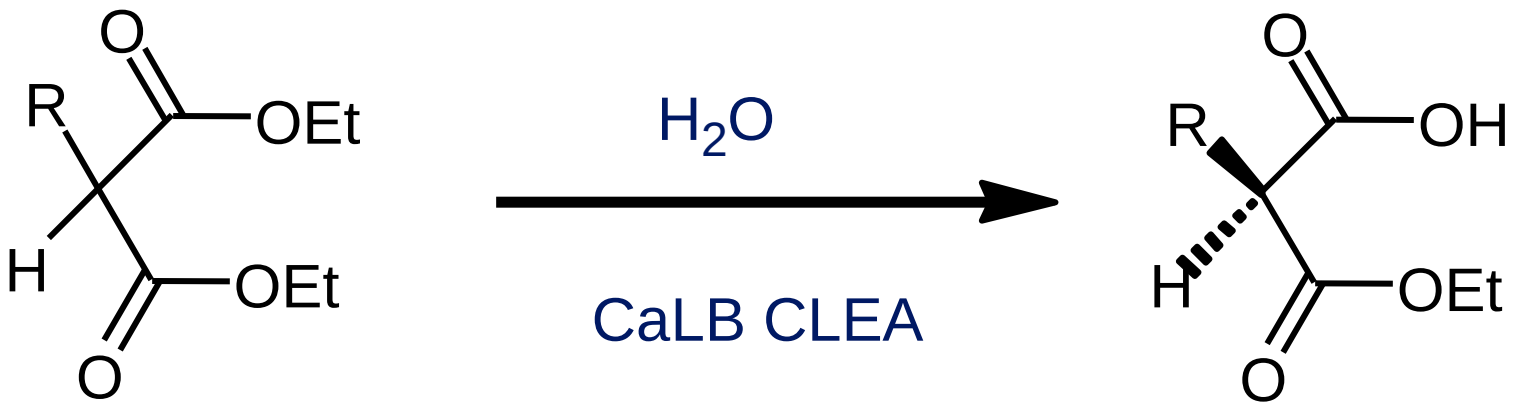
P. Hara, U. Hanefeld, L. T. Kanerva, *J. Mol. Catal. B: Enzymatic* 50 (**2008**) 80



n = 1 or 2

D. Özdemirhan, S. Sezer, Y. Sönmez, *Tetrahedron Asymm*. 19 (**2008)** 2717

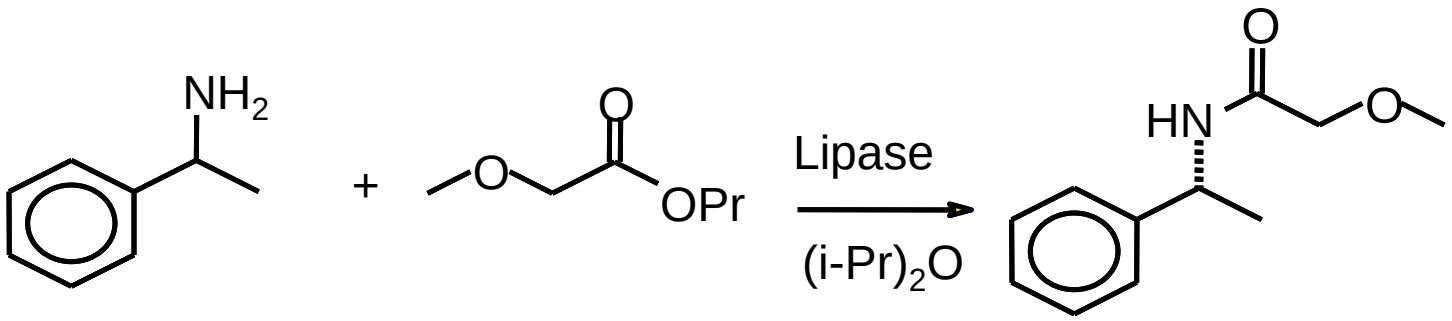
Hydrolases - Lipases



CaL B CLEA in a Fixed Bed Reactor
100% activity after >300 h on stream

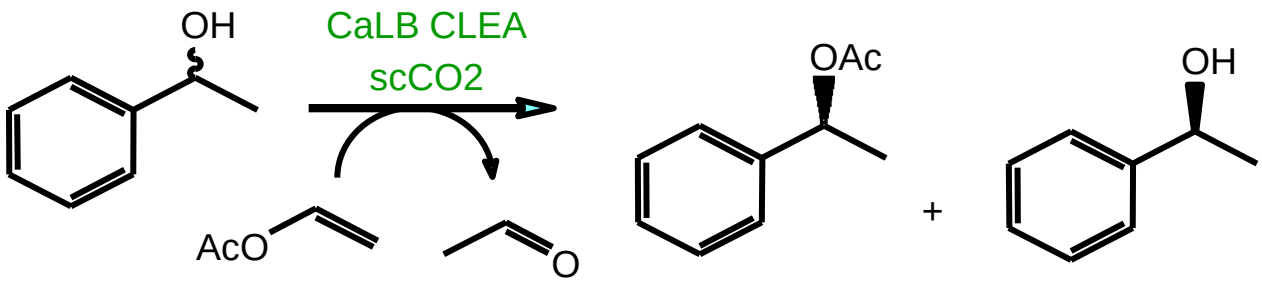
K. Robins, Lonza

CalB CLEA in Organic Media



	Activity in H_2O (U/g)	Activity in $(i\text{-}Pr)_2O$ (U/g)	Ratio
CaL B CLEA–ST	38000	50	21
CaL B CLEA-OM	31000	1500	760
Novozym 435	7300	250	29

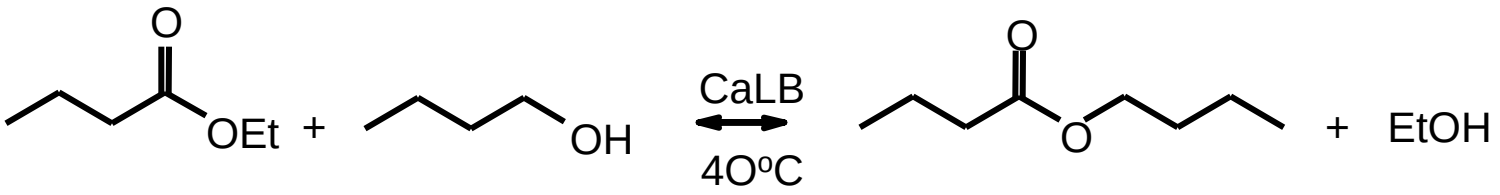
CalB CLEA in $scCO_2$



Catalyst	Conversion (%)	E
Novozym 435	17	280
CaL B CLEA	48	640

H.R. Hobbs, M. Poliakoff, R.A. Sheldon, et al., *Green Chem*. 8 (**2006**) 816

CalB CLEA in Ionic Liquid



Solvent	Lipase	Time (h)	Conv. (%)
t-BuOH	Nov 435	6	83
[bmim][dca]*	Nov 435	24	0
t-BuOH	CaL B CLEA	3	83
[bmim][dca]	CaL B CLEA	6	80

*dca = $(CN)_2N$

A. Ruiz Toral, F. van Rantwijk, R. A. Sheldon et al, *Enz. Microb. Technol*. 40 (**2007**) 1095-1099

Scope of the Technology

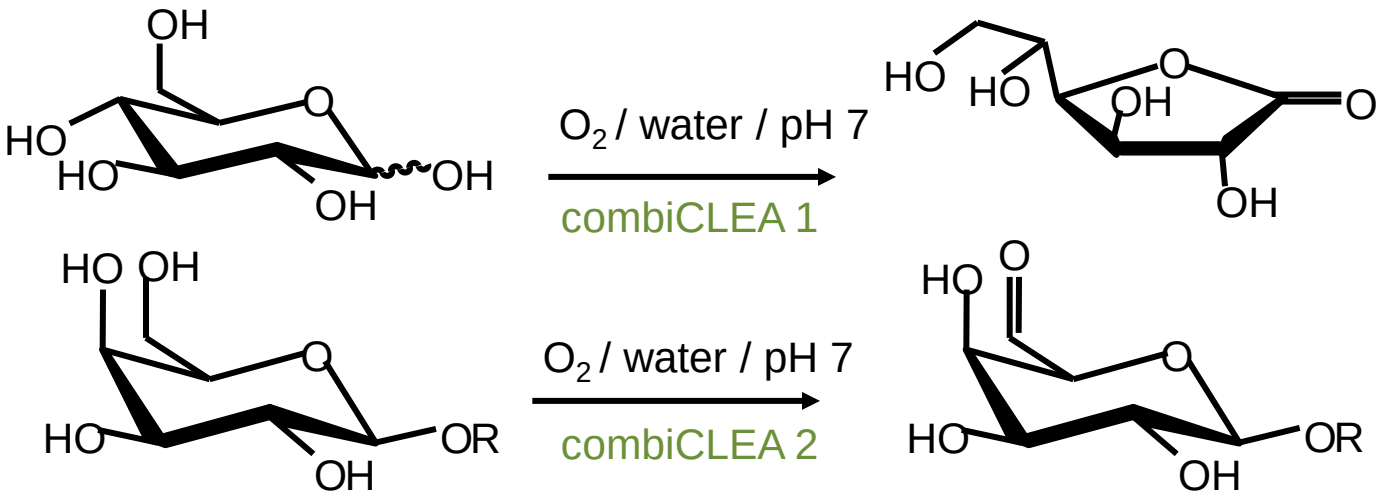
Hydrolases

- Pen. Acylases (2)
- Lipases (19)
- Esterases (3)
- Proteases (9)
- Nitrilases (5)
- Aminoacylase
- Phytase
- Galactosidase
- Carbonic anhydrase

Oxidoreductases

- KRED
- FDH
- **Glucose oxidase**
- Galactose oxidase
- Amino acid oxidase
- **Laccase** (3)
- **Catalase**
- Chloroperoxidase
- HRP

Oxidoreductases – Combi CLEA

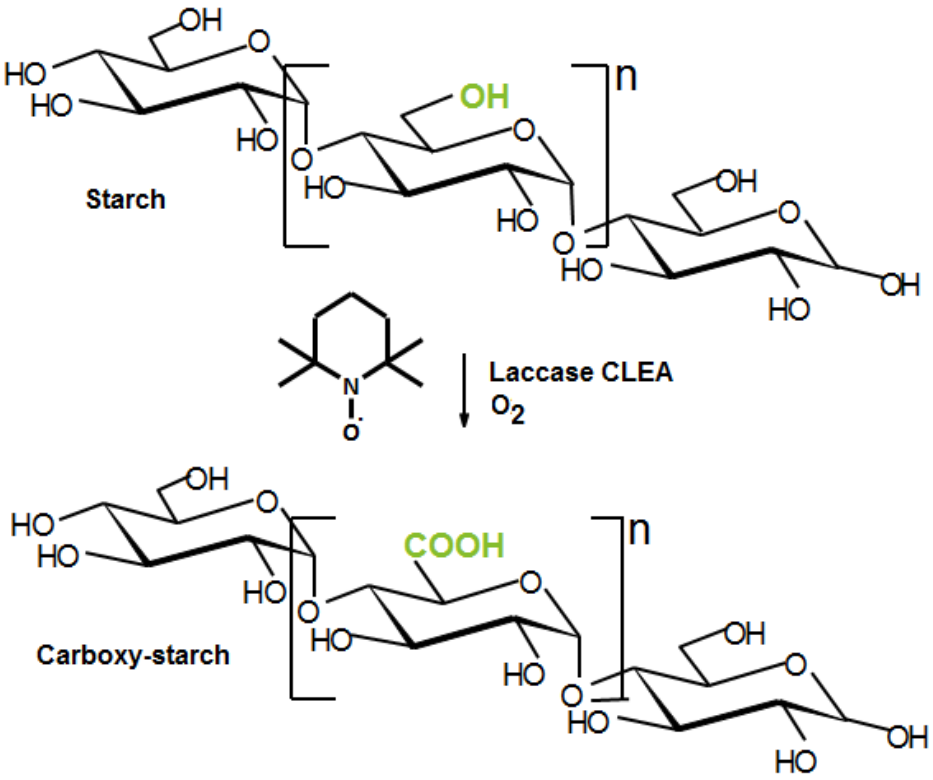


Activity	Free enzyme	CLEA
1st use	100%	100%
2nd use	-	100%

combiCLEA 1 = Glucose oxidase / catalase

combiCLEA 2 = Galactose oxidase / catalase

Oxidoreductases – Laccase



- TEMPO/NaOCl environmentally unfriendly
- Laccase / TEMPO / O_2 :
  - Green Alternative
  - Enzyme costs too high (owing to suicide inactivation)
- Increase operational stability with a laccase CLEA
  - Recycle
- Also with cellulose to carboxycellulose (shampoo)

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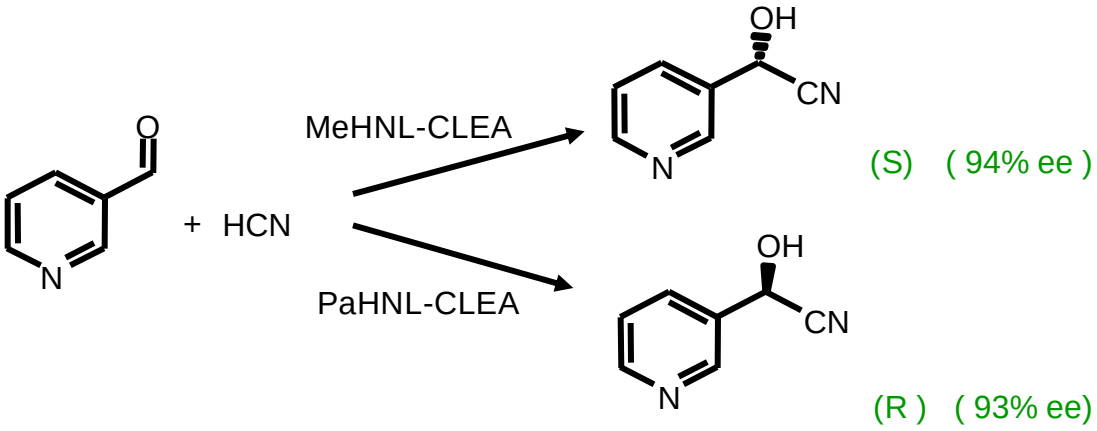
Lyases

- ***R*- & *S*- HNLases** (5)
- PDC
- DERA
- **Nitrile hydratase** (9)

Lyases – Hydroxynitrile Lyases



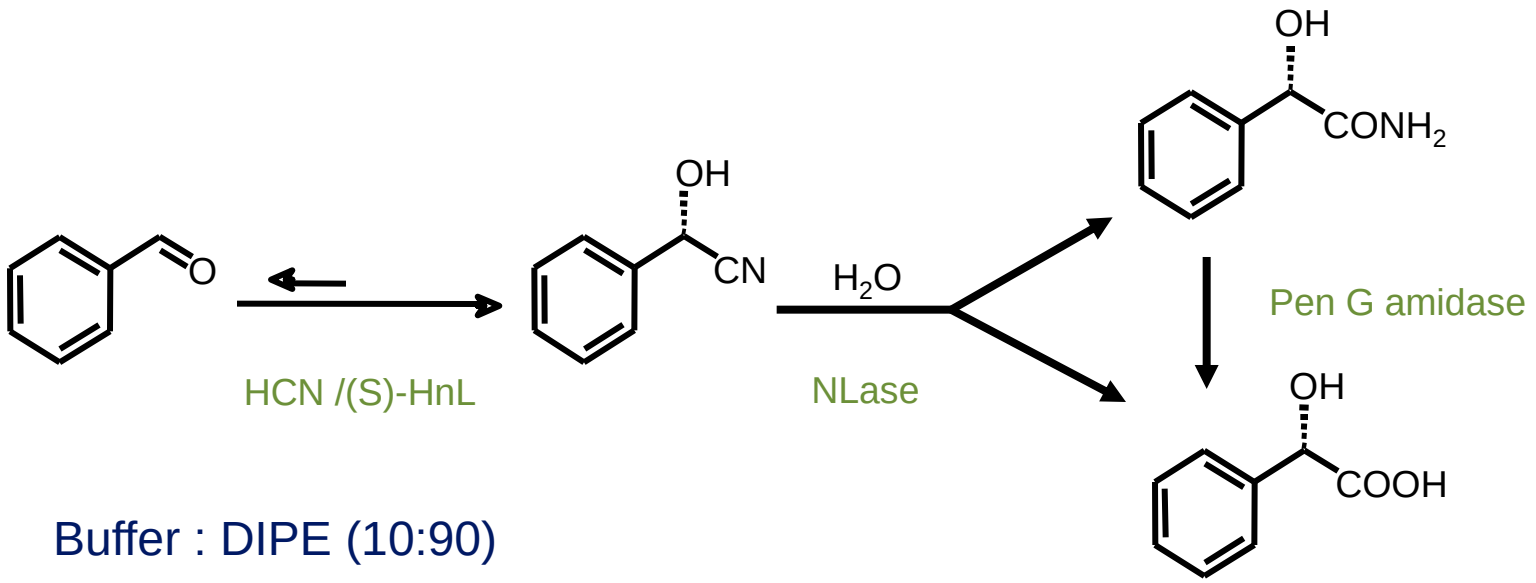
- Low reaction temp. (5°C)
- Microaqueous environment (0.18% H_2O in DCM)
- Immobilization as a CLEA

”The use of a dichloromethane reaction system with enzyme aggregates and free hydrogen cyanide was crucial in improving cyanohydrin stereoselectivity through minimizing background racemic cyanide addition and enzyme-catalyzed racemization of the product.”*

*C. Roberge, F. Fleitz, D. Pollard, P. Devine, *Tetrahedron Letters* 48(8) (**2007**) 1473-1477

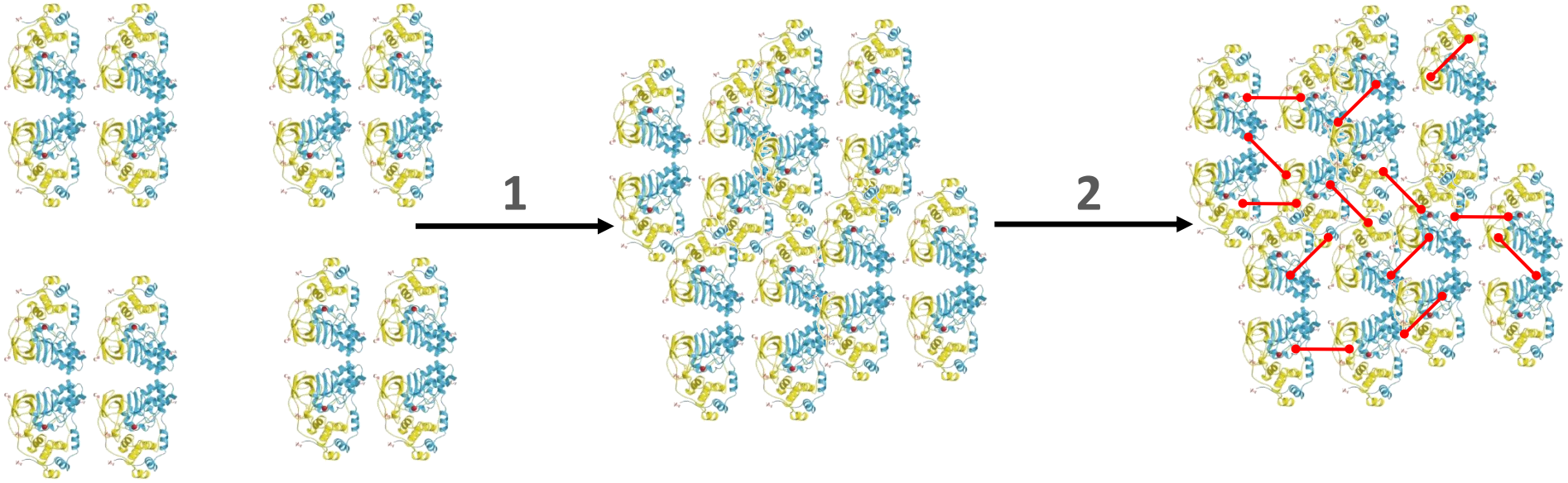
Lyases – Combi CLEA

Step Economy a Tri-enzymatic Cascade with a Triple-Decker Combi CLEA



- Buffer : DIPE (10:90)
- pH 5.5 / RT / < 5h
- HnL/ NLase / Pen.acylase Combi-CLEA
- Conv. 96% / ee >99%

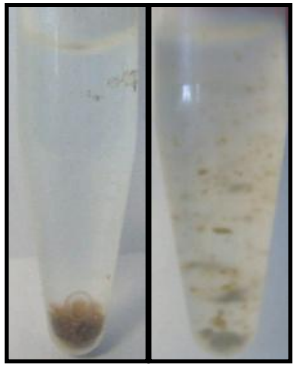
NHase CLEA Formation



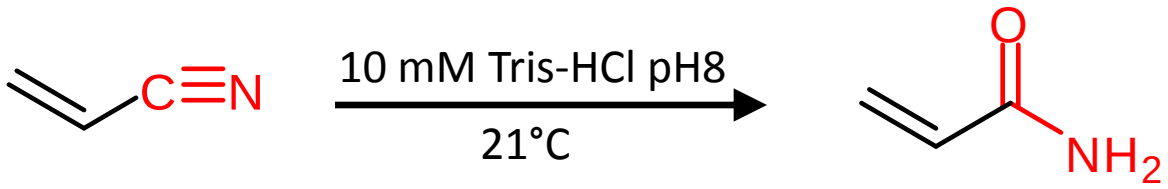
(1) Aggregation/purification using ammonium sulfate

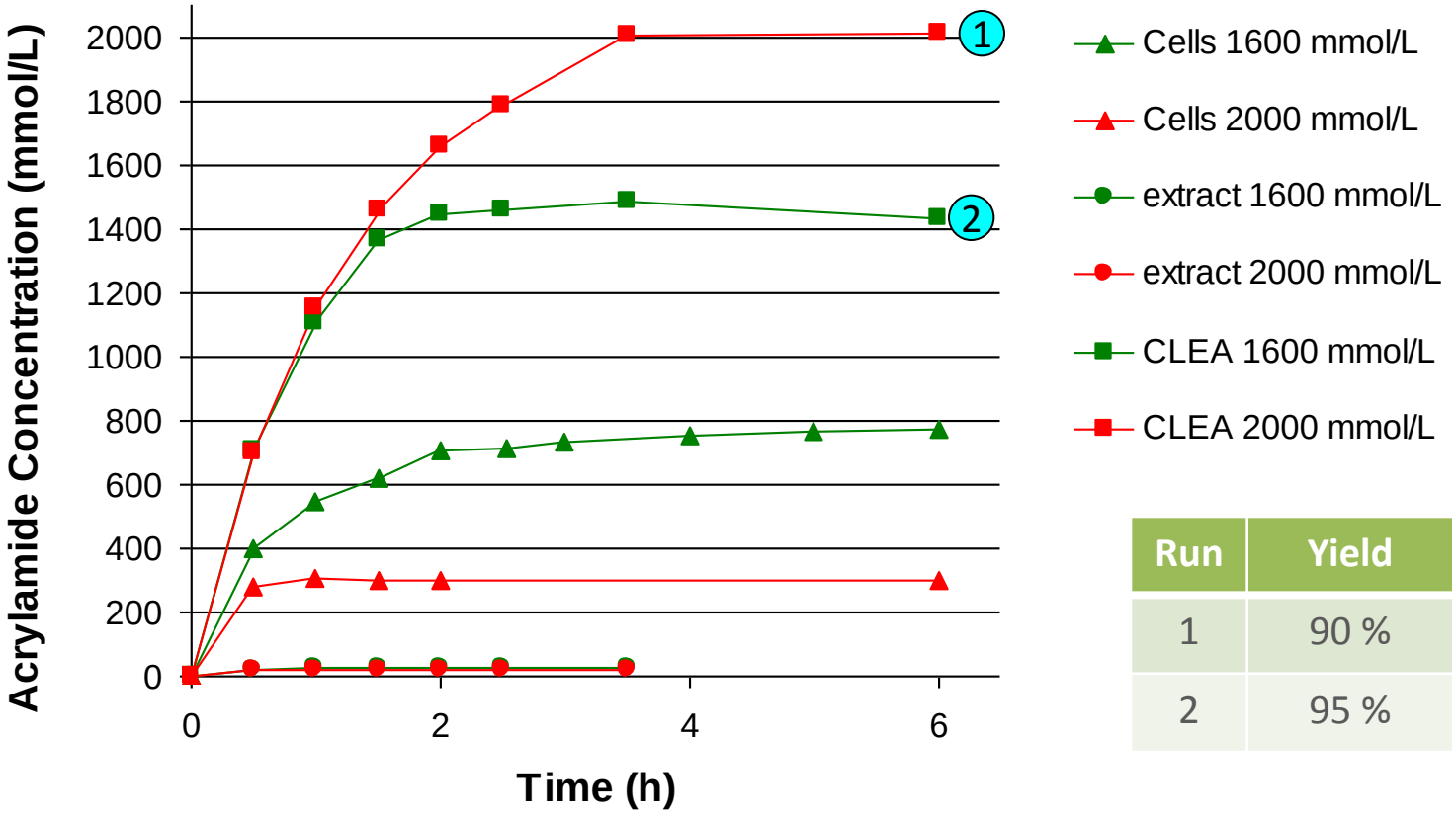
(2) Cross linking using glutaraldehyde

Remaining activity in CLEA: >50%



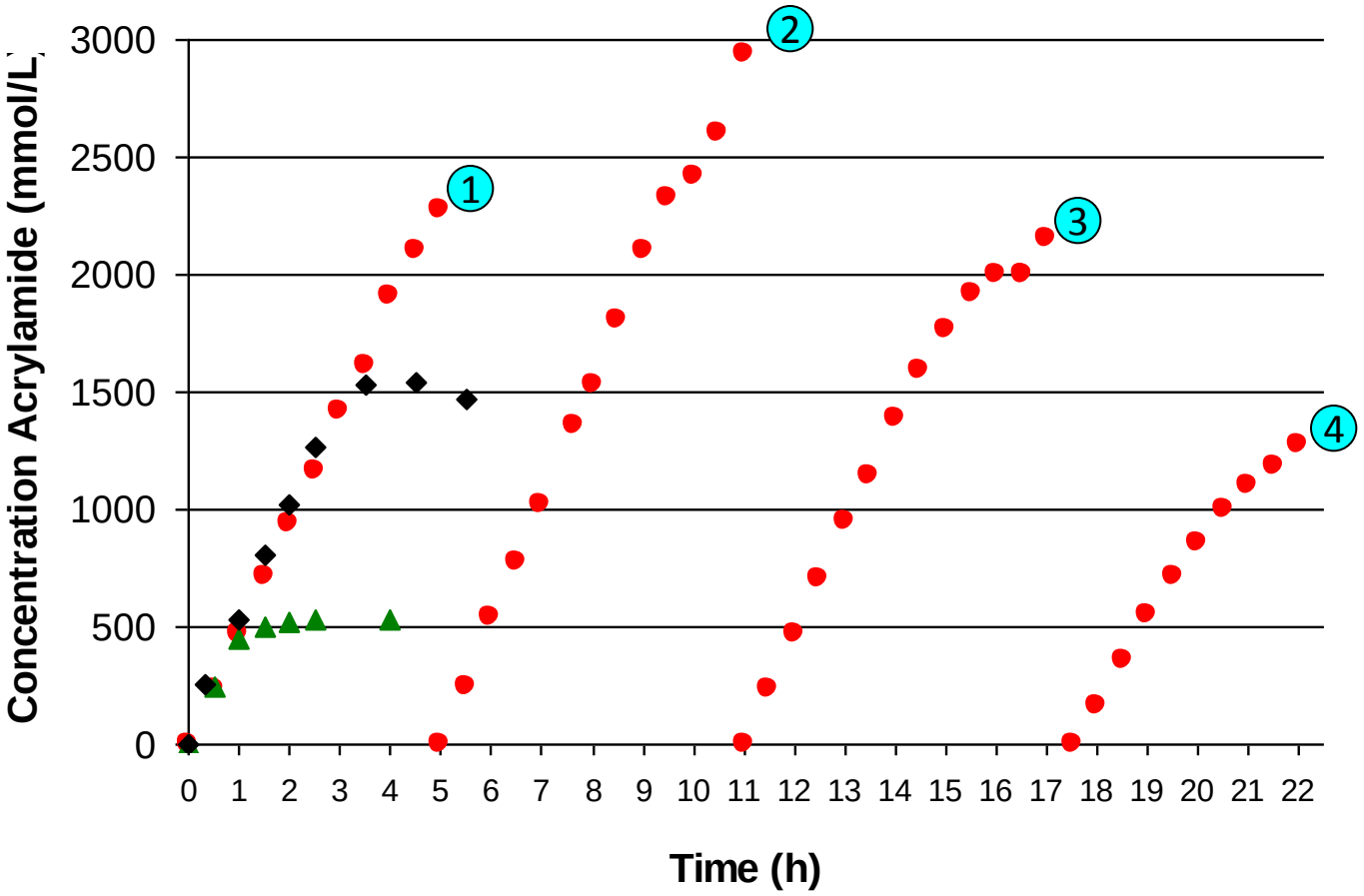
Lyases – Nitrile Hydratase





Run	Yield
1	90 %
2	95 %

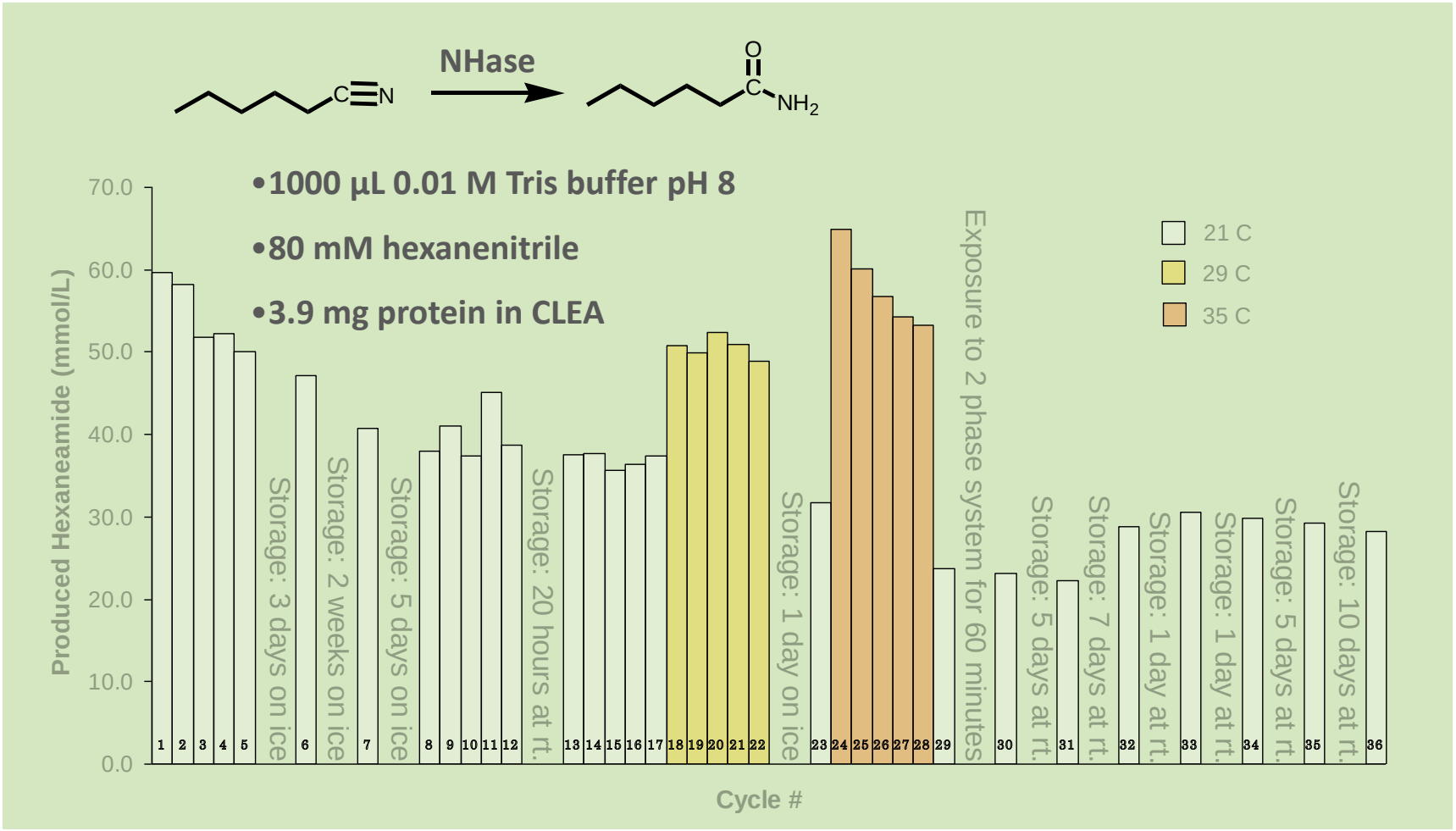
Fed-Batch Production Acrylamide



21 °C

Run	Residual Activity
1	95 %
2	85 %
3	65 %
4	50 %

NHases - Storage and Recycle Stability



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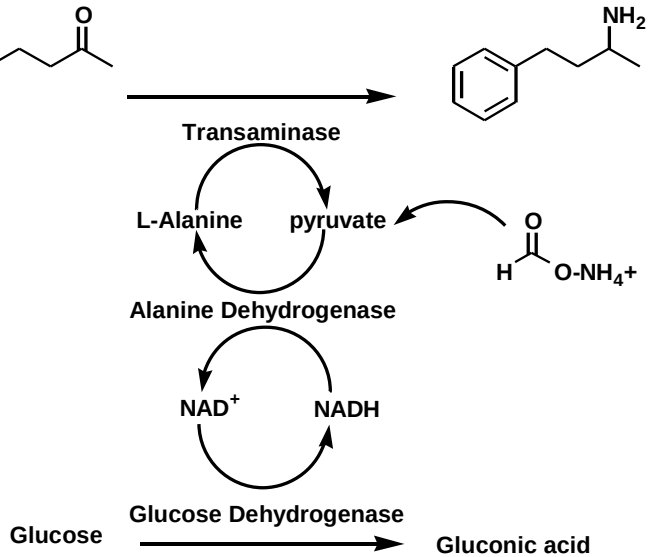
- DERA
- Nitrile hydratase (9)

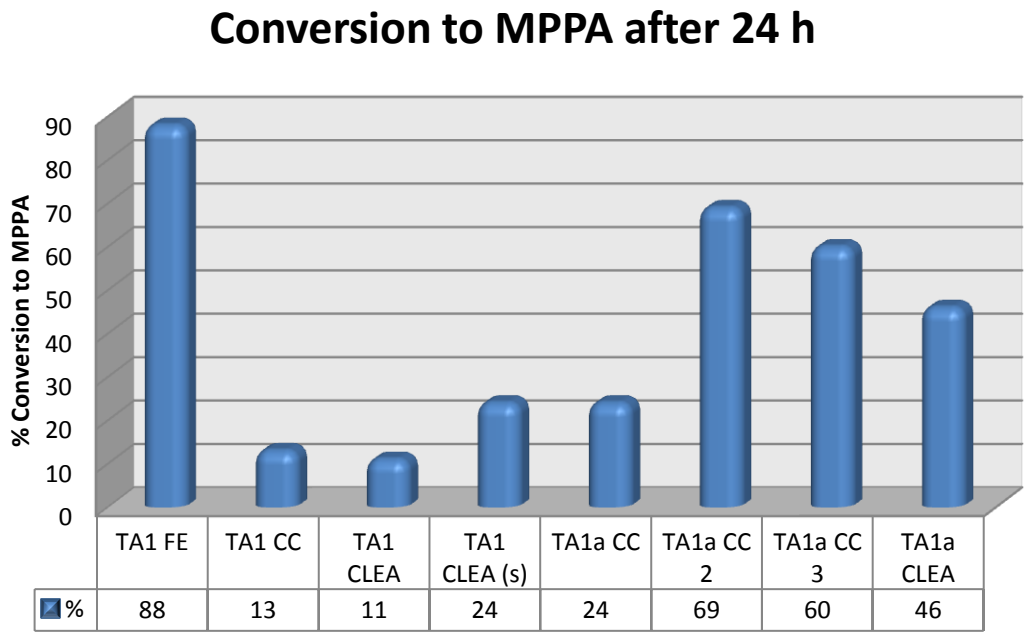
Transferases

- **Transaminases**
  - (R) selective (**3**)
  - (S) selective (**5**)

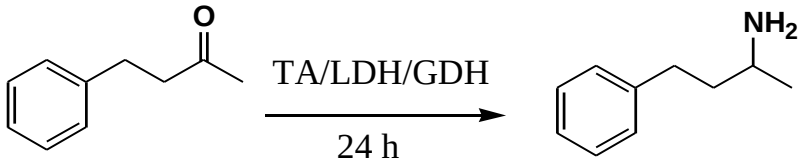
Transferases - Transaminases

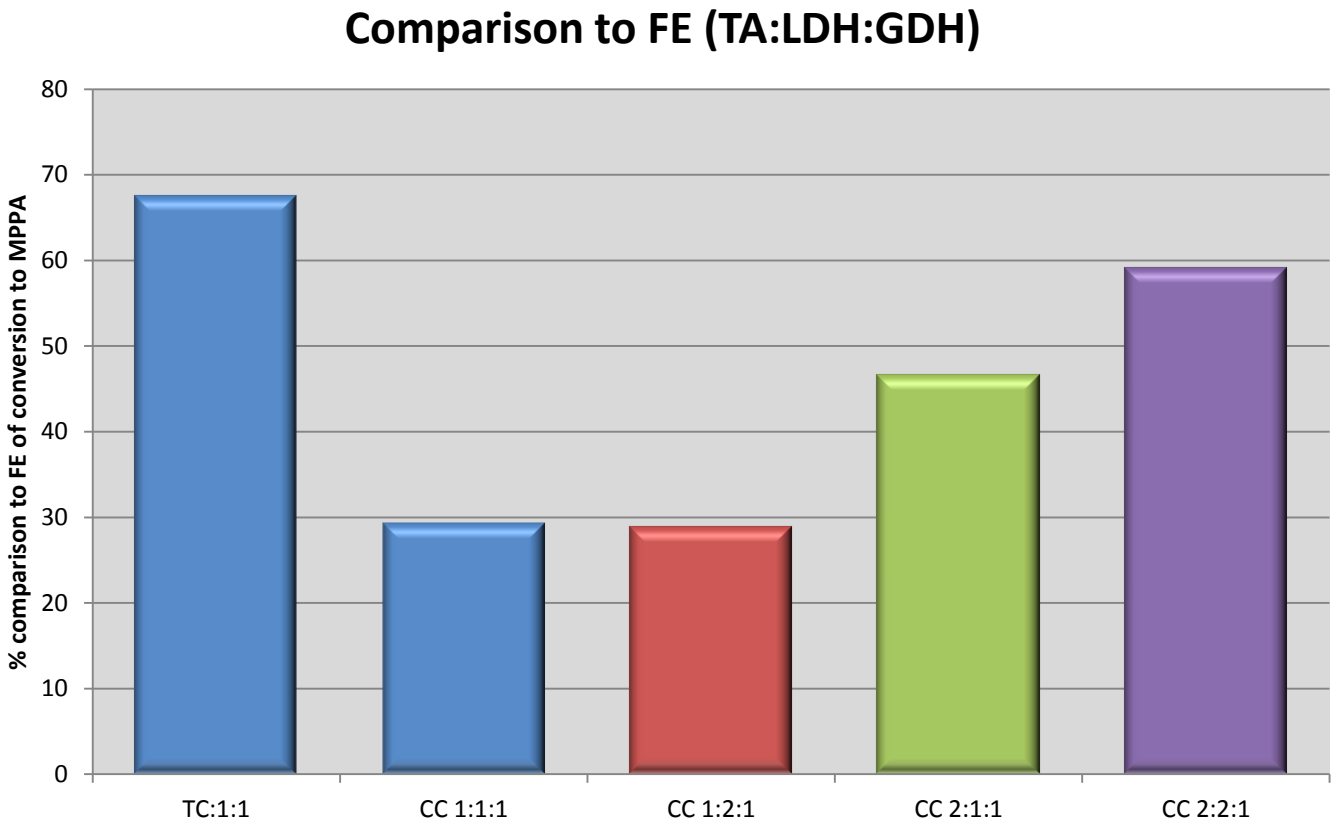


- Combi CLEA – TAm and AlaDH



Triple Combi CLEAs

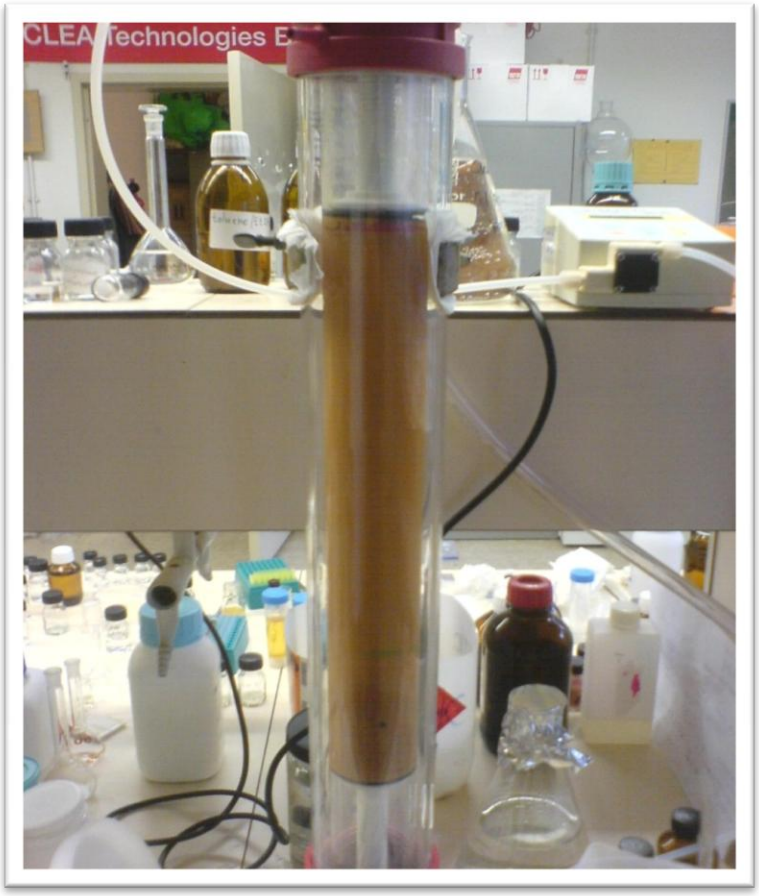




Different TAm : LDH : GDH ratios in the triple combi CLEAs

CLEAs in Reactors

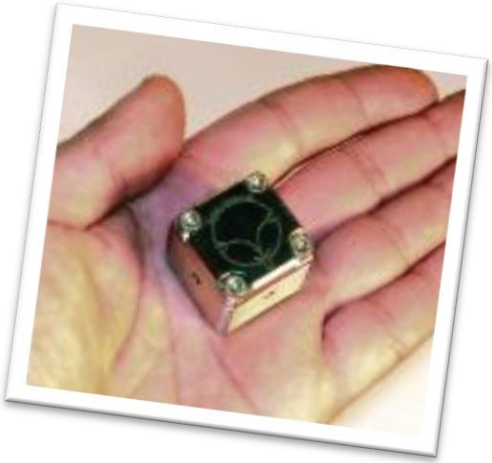
Pen Acylase in Filter Slurry Reactor (FSR)



Alcalase® CLEA in fluidized bed

M. J. Sorgedrager, D. Verdoes, H. van der Meer, R. A. Sheldon, *ChimicaOggi*, 26 (**2008**) 23-25

Microchannel Reactors



- Numbering up vs Scaling up
- 10^2 x larger surface/volume ratio
- Efficient mass & heat transfer
- Rapid screening of enzyme scope

- γ-Lactamase CLEAs in microchannel reactor
  - 100% Activity retention

A. M. Hickey, B. Ngamsom, C. Wiles, G. M. Greenway, P. Watts and J. A. Littlechild, *Biotechnol. J.*, 4(4) (**2009**) 510-516

Conclusions

- Biocatalysis is Green & Sustainable
- Immobilization is a Key Enabling Technlogy
- The **CLEA Technology** has many Benefits
  - Simple, broadly applicable & cost-effective
  - Improved stability & operational performance
  - High productivity & product quality
  - Applicable to crude cell lysates
  - No leaching of the enzyme in aqueous media
  - Combi-CLEAs for biocatalytic cascades
  - Smart CLEAs (e.g. magnetic CLEAs)

Thank you…….

www.cleatechnologies.com