Cross-Linked Enzyme Aggregates (CLEAs) as Industrial Biocatalysts

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Delft University of Technology

CLEA Technologies B.V.

Biocatalysis: Challenges for Pharmaceuticals & Fine Chemicals SCI HQ, London, 14 October 2010

www.cleatechnologies.com

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Why Biocatalysis?

Environmentally Attractive & Cost-Effective:

- **Enzymes are derived from renewable resources and are biodegradable (no heavy metal residues)**
- **Mild conditions: ambient T & P in water (energy efficient)**
- **Largely avoids hazardous solvents & reagents and protection/deprotection steps (less waste)**
- **High rates & highly specific : substrate, chemo-, regio-, and enantiospecific (less waste)**
- **Higher quality product**
- **No special equipment required**

Biocatalysis : why now ?

1. Genome sequencing (> 5000)
   (more enzymes)
2. Directed evolution technologies
   (better enzymes)
3. Recombinant DNA technology
   (better production)
4. Immobilization technologies
   (better formulation)

Disadavantages of Enzymes

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

- **Some applications not viable :**
  - **Enzyme costs too high**
  - **Not practical**

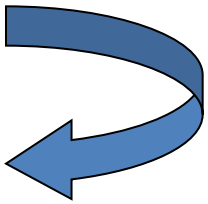
The Solution: Immobilization

Immobilization is Enabling Technology

→ **Reduced 'cost of goods'**

High waste / low profit

Low waste / high profit



Immobilization of Biocatalysts

Advantages

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

Immobilization Methods

1. **Binding to a carrier (support)**

   e.g. on ion-exchange resins
2. **Entrapment**

   e.g. in silica sol-gel or membrane
3. **Cross-linking**

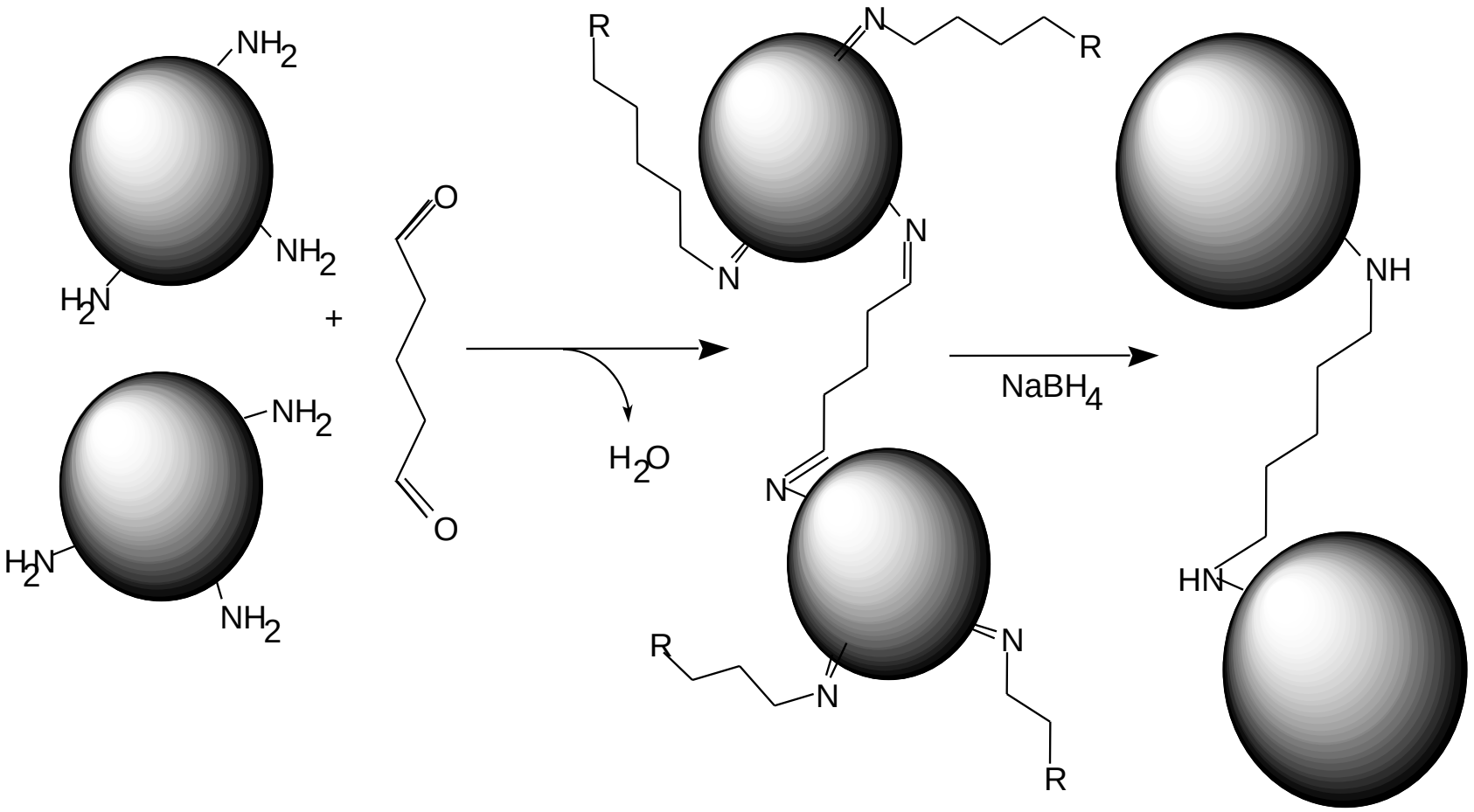
   e.g cross-linked enzyme aggregates (CLEA®)

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

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

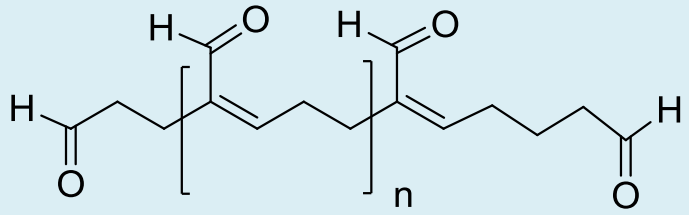
Cross Linking of Enzymes

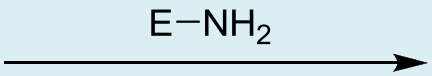
e.g. with glutaraldehyde



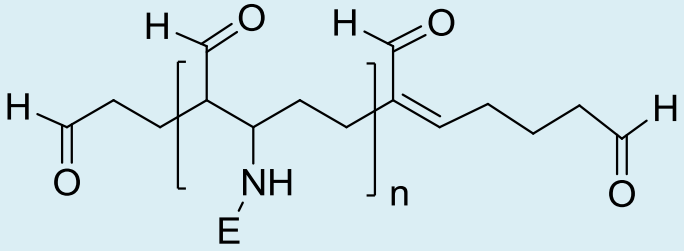
Cross Linking of Enzymes

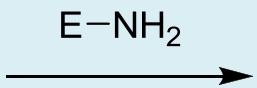
What's in the bottle is not glutaraldehyde

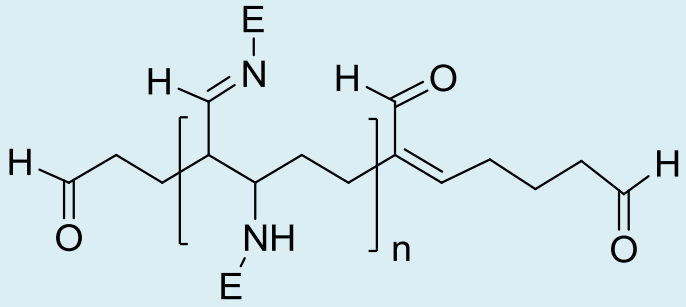




This is what's in the bottle







Classical Cross-linked Enzymes (1960s)

Prepared by mixing an aqueous solution of enzyme with an aqueous solution of x-linker

BUT

- Low activity retention
- Poor reproducibility
- Low mechanical stability
- Difficulties in handling gelatinous material

L.Cao, L.van Langen and R.A.Sheldon,Curr.Opin.Biotechnol., 14, 387-394, 2003

Cross-Linked Enzyme Crystals (CLECs)

- First described in 1964 (Quiocho & Richards)
- Use as biocatalysts pioneered by Altus Biologics (1992)
- CLECs significantly more stable to denaturation by heat, organic solvents and proteolysis.
- Operational stability coupled with ease of recycling and high catalyst and volumetric productivity

But:

- Need to crystallize the enzyme,often a laborious procedure requiring high purity, i.e. very expensive

Cross-Linked Enzyme Aggregates (CLEAs)

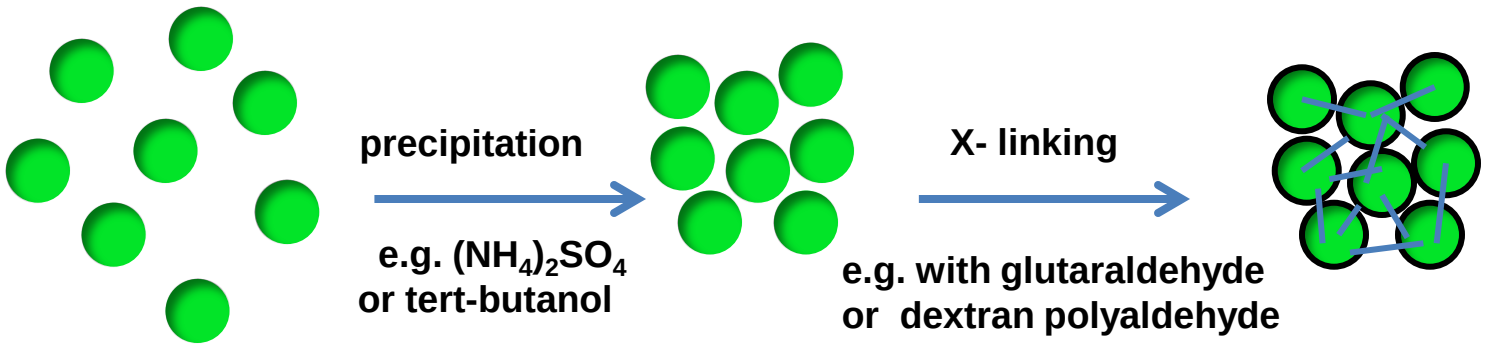
- Can crystallization be replaced by precipitation and cross-linking of the physical aggregates of enzyme molecules?

- Precipitation from aqueous solution by salts , or water-miscible organic solvents or polymers.

- Combines purification and immobilization into one step

L.Cao, F.van Rantwijk and R.A.Sheldon, Org.Lett., 2 , 1831, 2000

Cross-Linked Enzyme Aggregates Part One



- Simple & Broadly Applicable
- Cost-effective (no need for pure enzyme)
- High activity recovery and productivity
- Scalable protocols

L. Cao, F. van Rantwijk and R. A. Sheldon, Org.Lett., 2 , 1831, 2000
EP1807513 (NL 20041027360)

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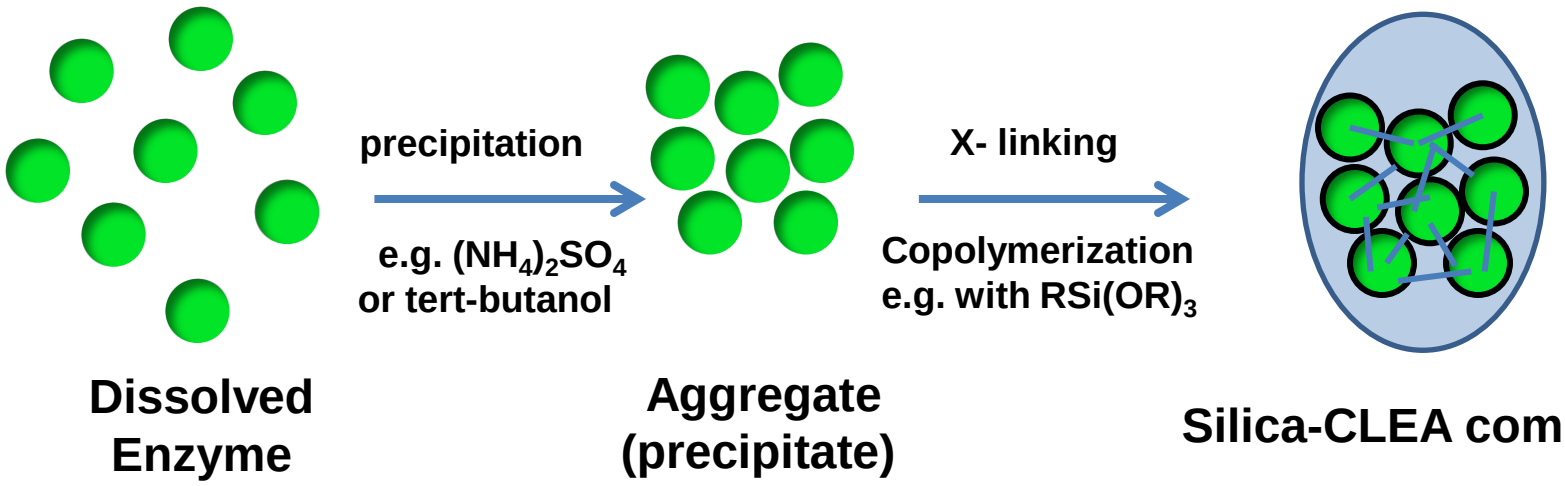
Cross-Linked Enzyme Aggregates Part Two



- Simple & Broadly Applicable
- Cost-effective (no need for pure enzyme)
- Tunable hydrophobicity / hydrophilicity, particle size and mechanical strength

EP1807513 (NL 20041027360) **to CLEA Technologies**

CLEA Technologies

Parameters in CLEA Preparation

- Nature and purity of enzyme
- Nature and amount, of precipitant
- Rate of addition and stirring
- pH and temperature
- Nature of X-linker
- Ratio X-linker / enzyme
- X-linking time (aging effect)
- Washing and drying procedure

Amenable to parallel experimentation: 96 well plate

The Metrics of Immobilization

To measure is to know

1 International Unit (U) = 1µ mole per min.

e.g. 10000 U/g = 10 mmole / min / g biocatalyst

Activity Recovery (AR) = (total enzyme activity out / total activity in) x 100%

e.g. 100 Units in 90 units out = 90% AR

N.B. The mass out could be more or less than the mass in

N.B. Only meaningful comparison if under same conditions

e.g. Tributyrin hydrolysis with free vs immobilized lipase in water

For esterification (non-aqueous medium), e.g. PLU assay
acetone powder vs immobilized enzyme

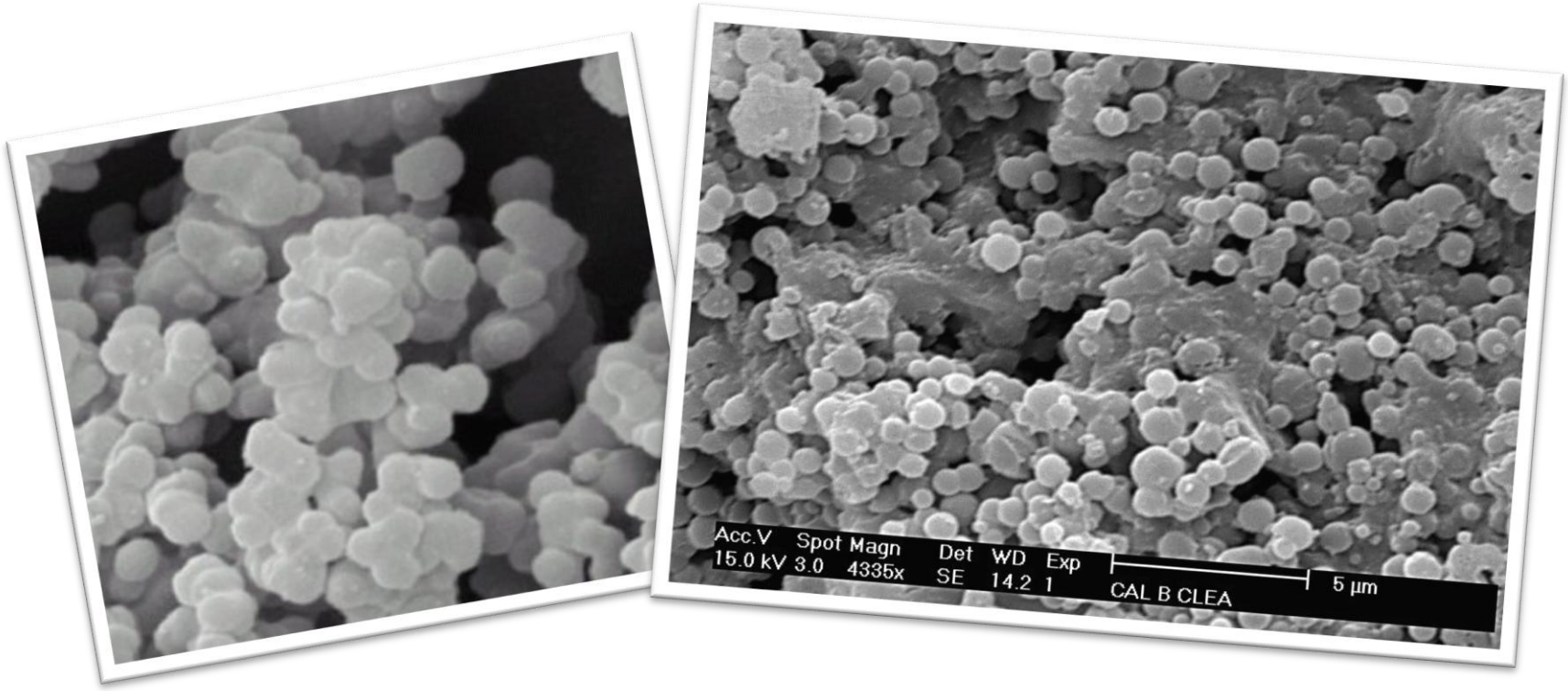
Enzyme Loading = wt enzyme/total wt biocatalyst

Metrics of Immobilization : Price

Factors determining the price of a CLEA:

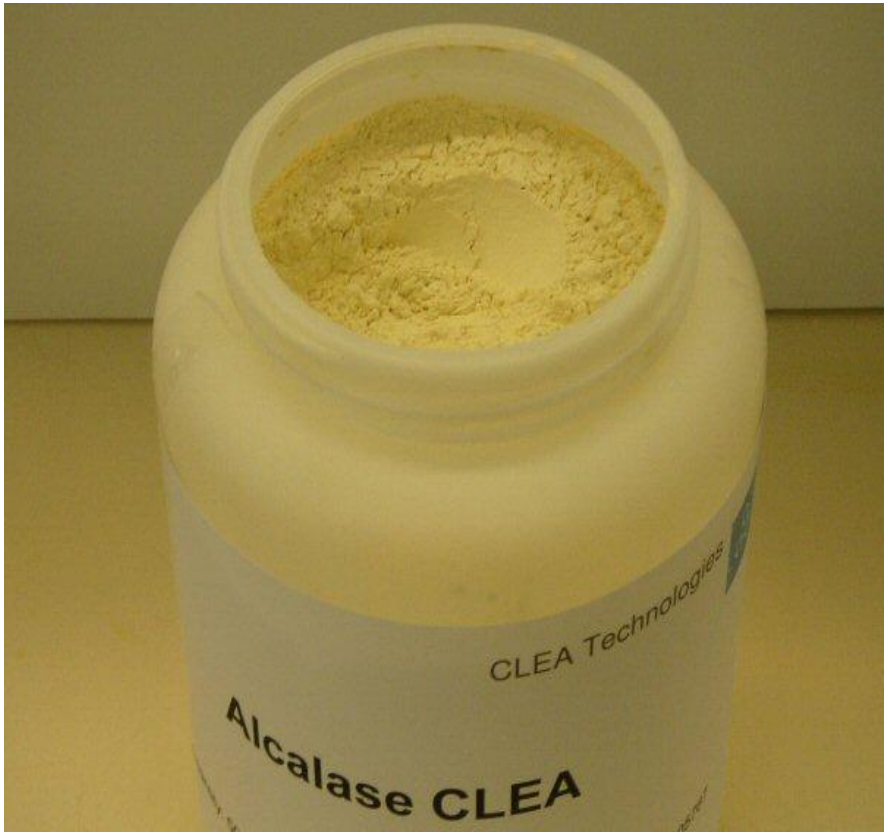
- **Price of free enzyme**
- **Activity recovery (AR)**
- **Amount & price of precipitant & cross-linker**
- **Protein concentration (→ volumetric yield)**
- **Scale of production**
- **Level of equipment sophistication**
- **Number of recycles**

Candida antarctica Lipase B CLEA



The <u>only</u> commercially available immobilized form of CaL B completely stable to leaching in water

AlcalaseCLEA
commercial scale manufacture



- **Free flowing powder**
- **Tunable particle size**
- **Low (no) allergenicity**
- **Excellent thermal stability**

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Advantages of CLEAs

1. **Improved properties**
   - better storage and operational stability
   - (to heat, organic solvents and autolysis)
   - hypoallergenic
   - no leaching of enzyme in aqueous media
2. **Cost-effective**
   - no need for highly pure enzyme (crude enzyme extract sufficient)
   - easy recovery and recycle (no product contamination)
   - high activity recovery and productivity (kg product/kg enzyme)
3. **Broad scope & short time to market**
   - combi CLEAs containing more than one enzyme

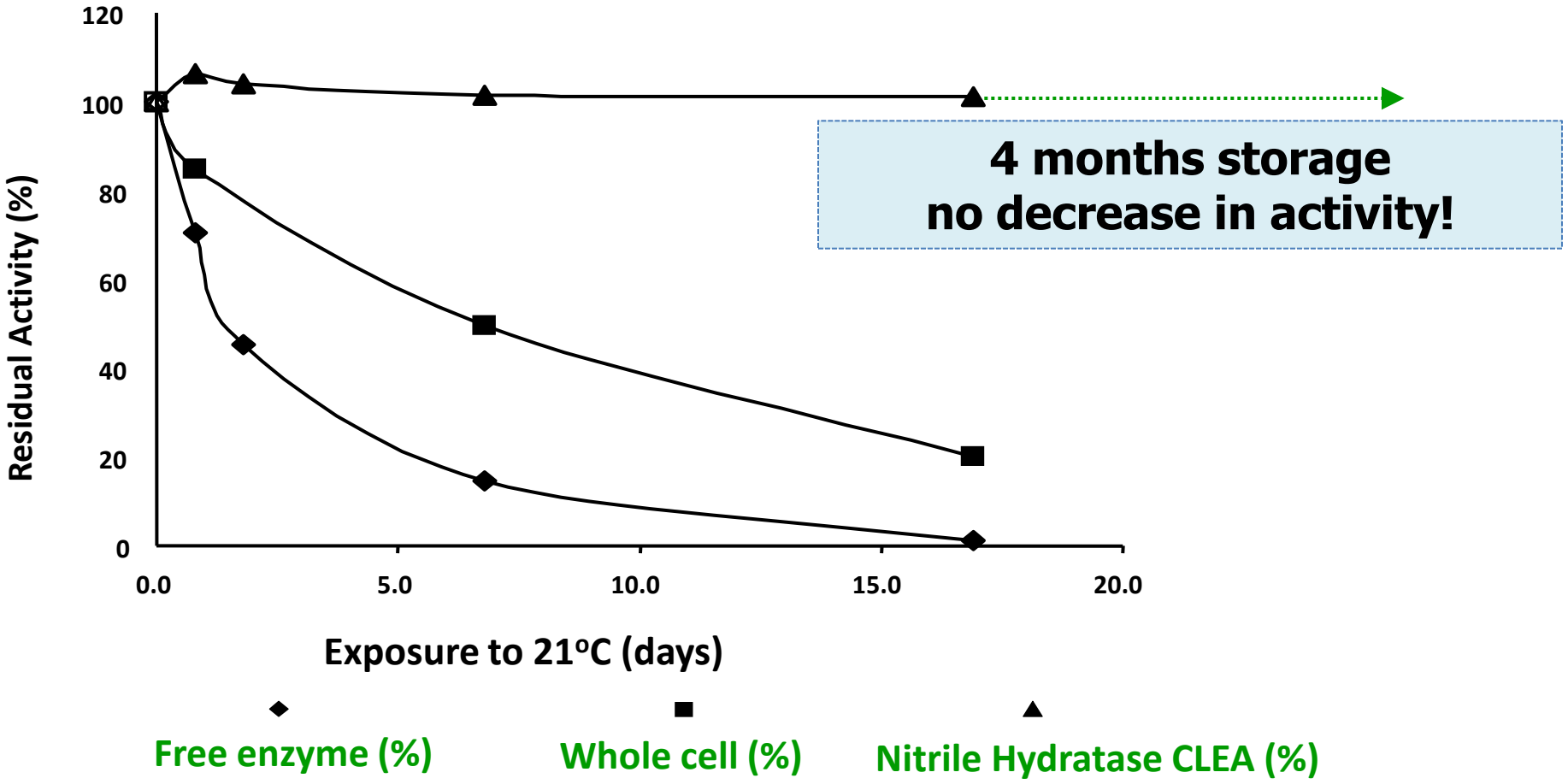
Operational Stability : Peroxidases

Azo Dye Decolorization by a Peroxidase CLEA

CLEA of royal palm (*Roystonia regia*) peroxidase exhibited a 5000 fold increase of stability under operating conditions in treatment of waste water containing azo dyes and could be recycled several times

C. Grateron, O. Barbosa, N. Rueda, C. Ortiz-Lopez, R. Torres, *J. Biotechnol*. **2007**, *131S*, S87.

Dramatic Stability Enhancement of a NHase

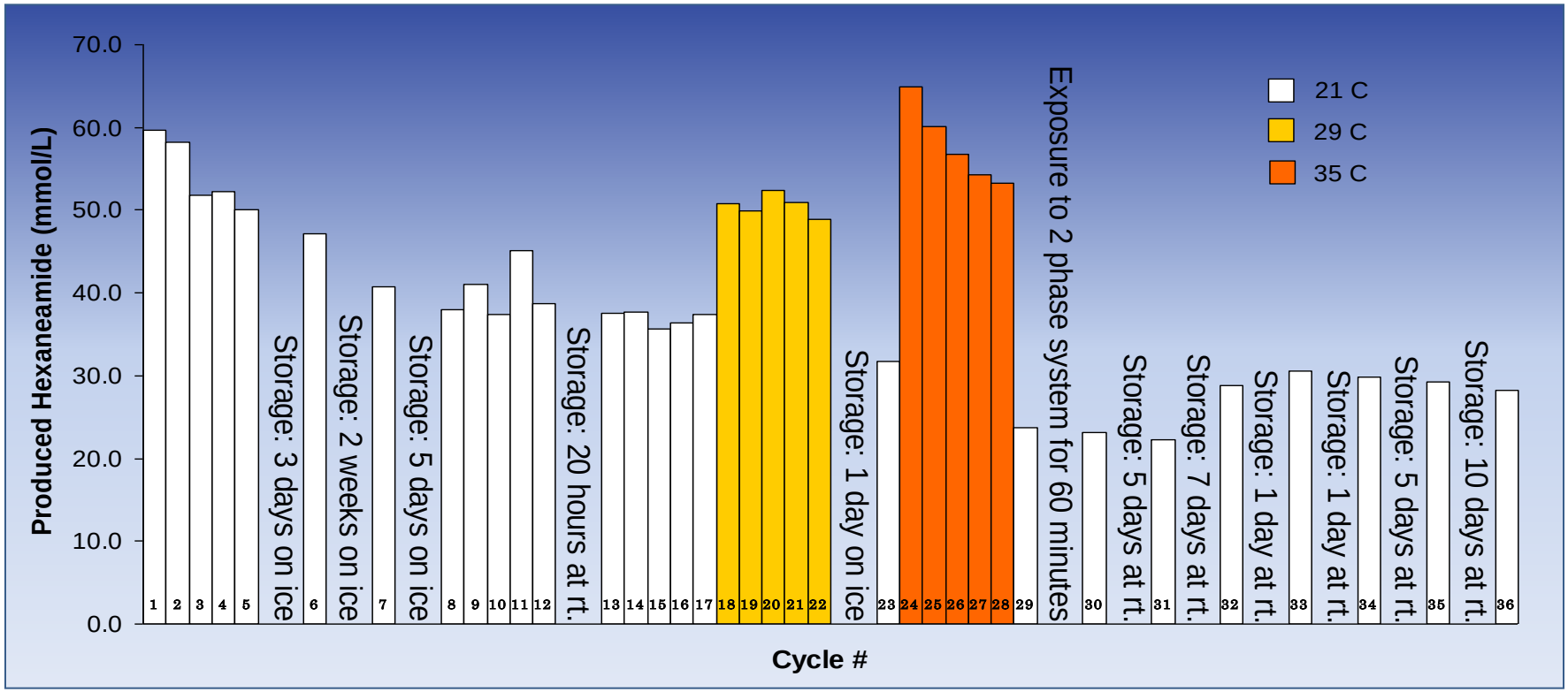


Instability due to dissociation of multimeric enzyme hindered by CLEA formation

van Pelt, Quignard, Kubac, Sorokin, van Rantwijk and Sheldon, Green Chem. 10 (2008) 395-400

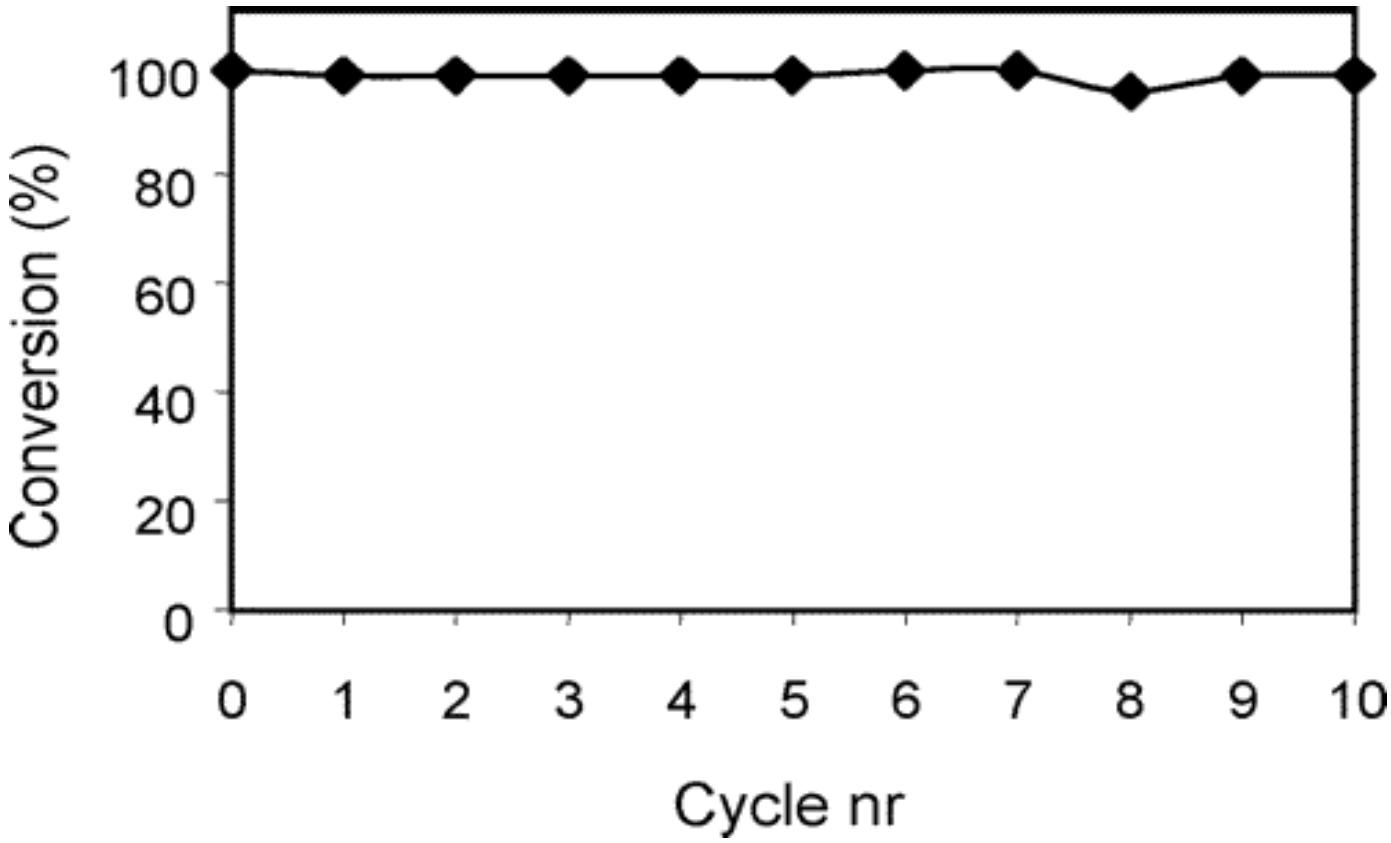
Improvement in Recyclability of a NHase

NHase CLEA in hydrolysis of RCN to $RCONH_2$



Excellent recyclability
- **36 recycles**
- **reduced activity mainly mechanical losses**

Recyclability of PaHNL in Hydrocyanation



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

Scope of the Technology

Examples of Successful Cleation

Hydrolases

- Pen. acylases (2)
- Lipases (15)
- Esterases (3)
- Proteases (7)
- Nitrilases (2)
- Aminoacylase
- Phytase
- Galactosidase
- Carbonic anhydrase

Oxidoreductases

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

Lyases

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

Transferases

- Transaminases

CLEA Technologies: Products & Services

- Off the shelf products
  - **Immobilized Enzymes (CLEAs)**
    - Selection of commercially available Enzymes
  - **Free Enzymes**
    - Guaranteed consistent activity specifications
  - **Discovery Platforms**
    - R&D amounts of a highly relevant selection of biocatalysts in free or immobilized formulation all available on industrial scale
- Custom Immobilization of enzymes on exclusive basis
- Contract research: development of green biocatalytic processes

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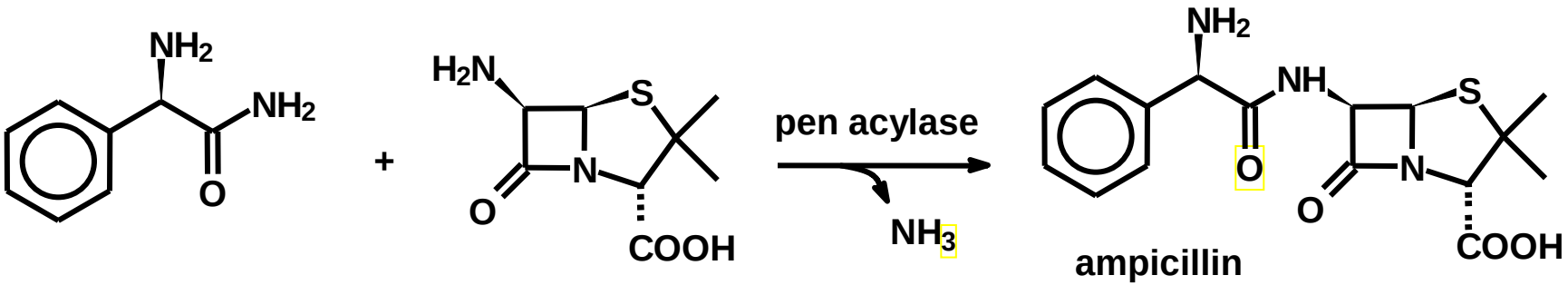
Hydrolase CLEAS

Penicillin acylase CLEA



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, 665, 2000

Protease CLEAs

- Alcalase® CLEA — *B. licheniformis*
- Savinase® CLEA — *B. clausii*
- Esperase® CLEA — *B. lentus*
- BS CLEA — *B. subtilis*
- Papain CLEA — *Carica papaya*

- Protease CLEA Discovery Platform

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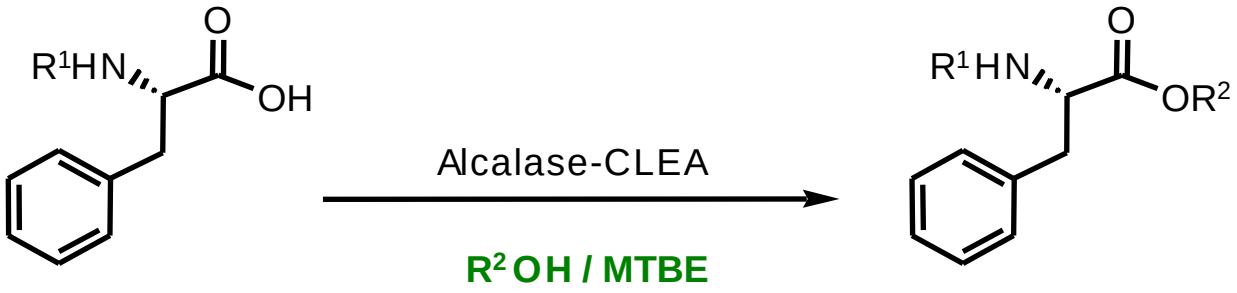
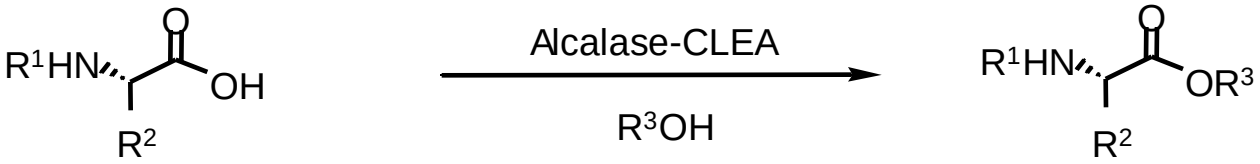
Esterification in Organic Medium



R^1 = Cbz, Boc, Bz, Fmoc, Formyl

R^2 = Me, Et, Bn, t-Bu, allyl

92-99% Yield
83-92% Isolated yield

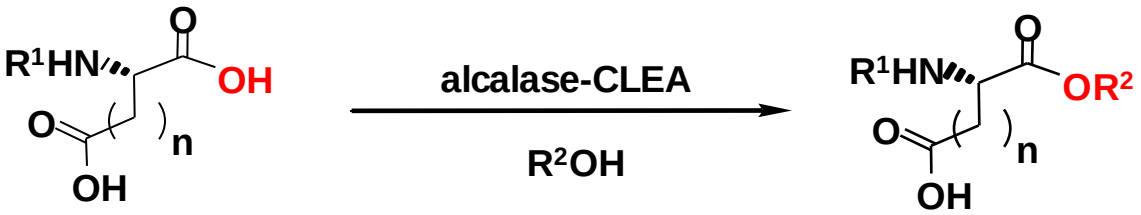


e.g. 1 Cbz-Ala-OBu^t
2 Cbz-Leu-OBu^t
3 Cbz-Ser-OBu^t
4 Cbz-Phe-Leu-OBu^t

83-98% Yield
72-90% Isolated yield

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

Protease CLEAs: Regioselective Esterification

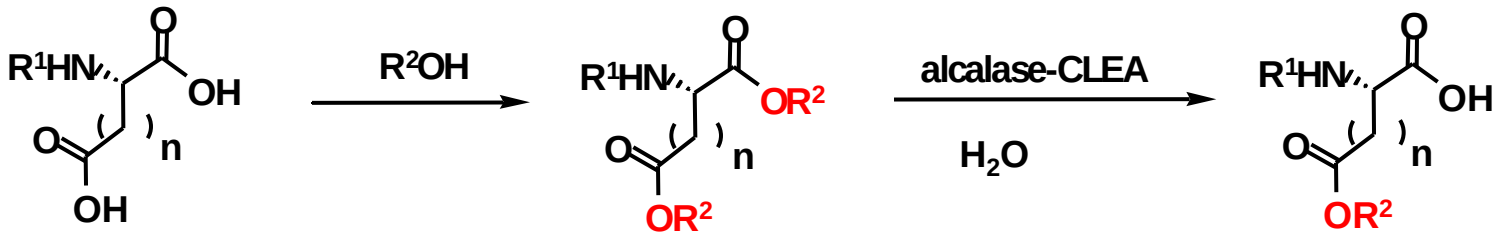


n = 1 or 2

R^1 = Cbz, Boc,Fmoc

R^2 = allyl, $Me_3SiCH_2CH_2$-

92-98% Yield
84-89% Isolated yield



n = 1 or 2

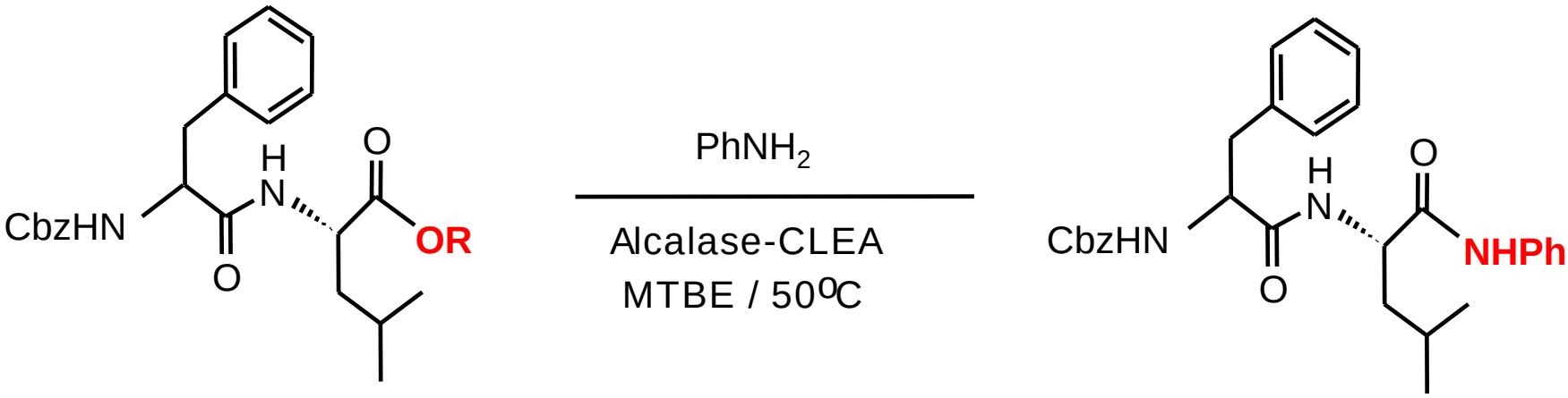
R^1 = CBz, BOC, FMOC

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

95-98% Yield
77-87% Isolated Yield

Nuijens, Cusan, Kruijtzer, Rijkers, Liskamp,Quaedflieg, *Synthesis*, **2009**, 809; *Tetrahedron Lett*. **2009**, *50*, 2719.

Protease CLEAs: Amidation in Organic Medium



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

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

Alcalase CLEA in Peptide Synthesis

BOC-Gly-Phe-Phe-Leu-OBu^t + H_2O

Alcalase-CLEA

NMP /phosphate buffer
pH 8 / 40^{o}C / 17h

BOC-Gly-Phe-Phe-Leu-OH + t-BuOH

> 95% yield

Eggen and Boeriu, WO 2007/082890 (2007) to Organon (Merck)

Lipase CLEAs

Lipase CLEAs

- ***Candida antarctica*** **lipase B (CaLB)**
- ***Candida antarctica*** **lipase A (CaLA)**
- ***Thermomyces lanuginosus*** **(Lipolase)**
- ***Rhizomucor miehei***
- ***Candida rugosa***
- ***Aspergillus niger***
- ***Pseudomonas alcaligenes***

and many more (just ask)

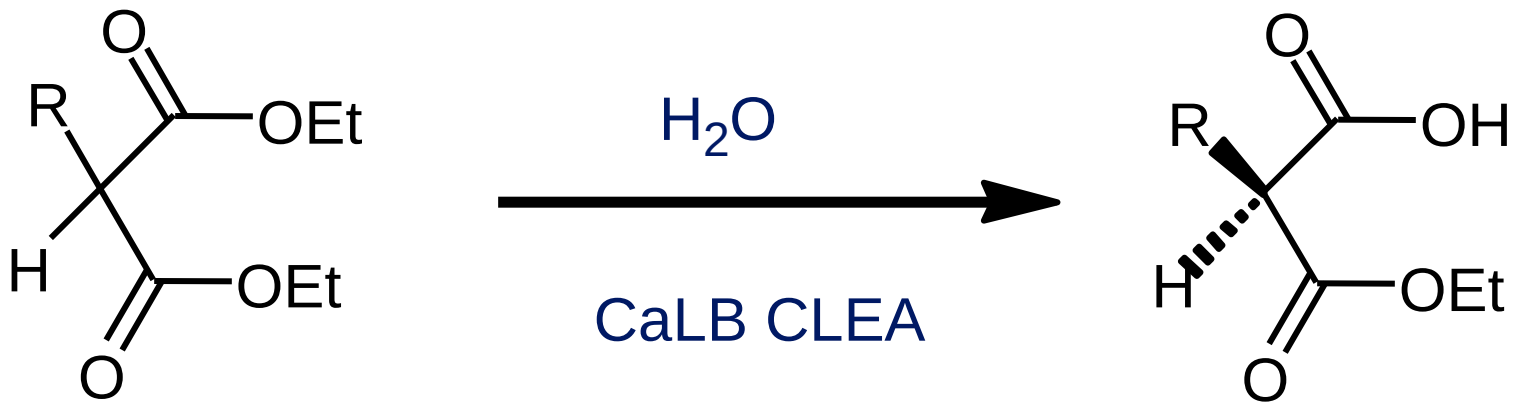
Enzymatic Production: Advantages

- **Process Simplification**
- **Product Quality**
- **Reduced Cost of goods**
- **Sustainability**

Lopez-Serrano,Cao,van Rantwijk and.Sheldon, Biotechnol.Lett.,24, 1379, 2002; NL 1017258 (2002)

CLEA Technologies

Enantioselective Hydrolysis

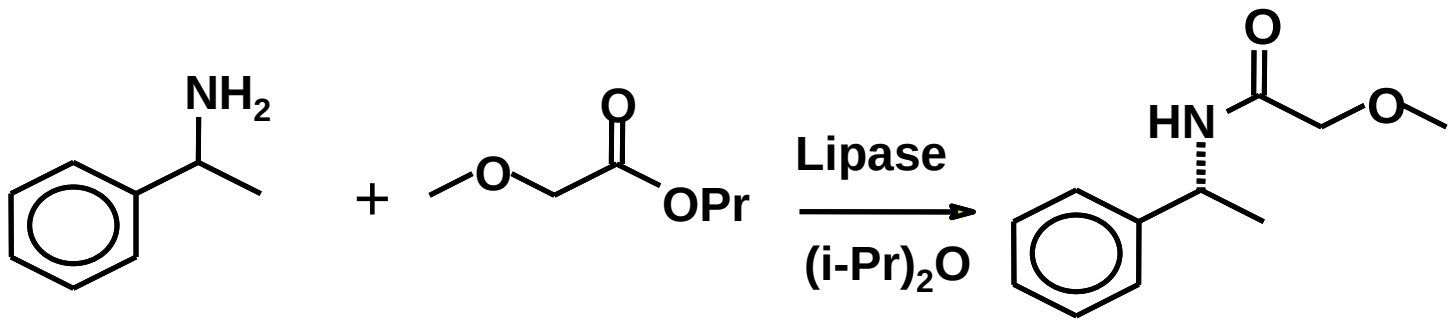


CaLB CLEA in a Fixed Bed Reactor

100 % activity after >300 h on stream

K. Robins, Lonza

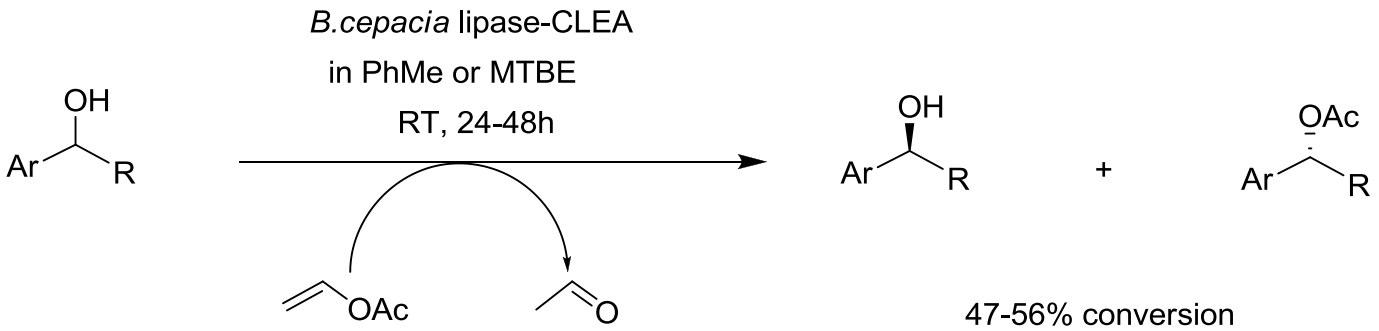
CaL B in Organic Medium



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

AM = aqueous media ***OM = organic media***

Lipase CLEAs

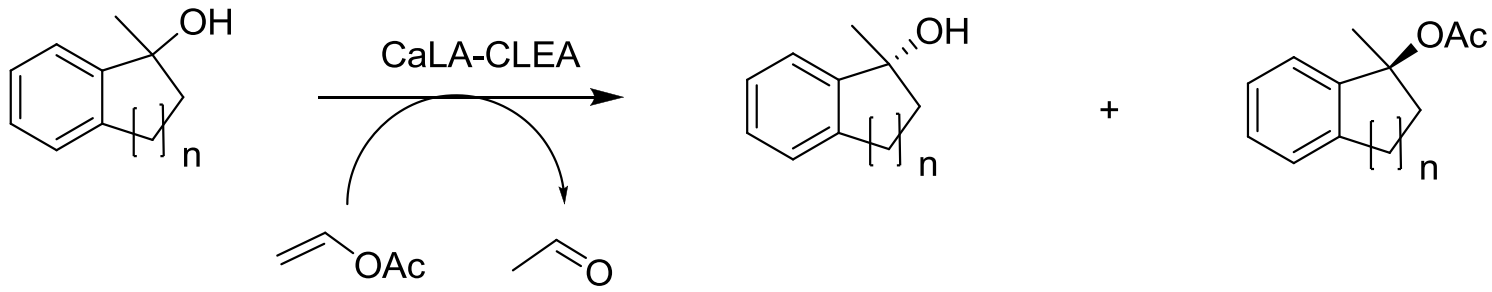


47-56% conversion

E = 35 - >200

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

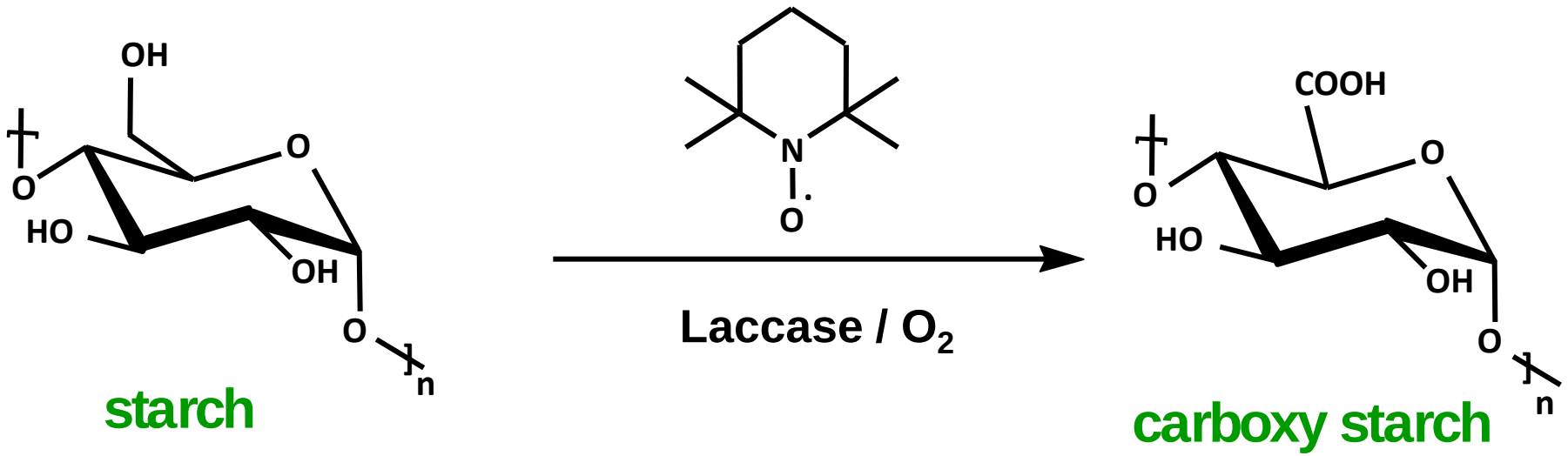
P. Hara, U. Hanefeld, L. T. Kanerva, J. Mol. Catal. B: Enzymatic 2008, 50, 80.



n = 1 or 2

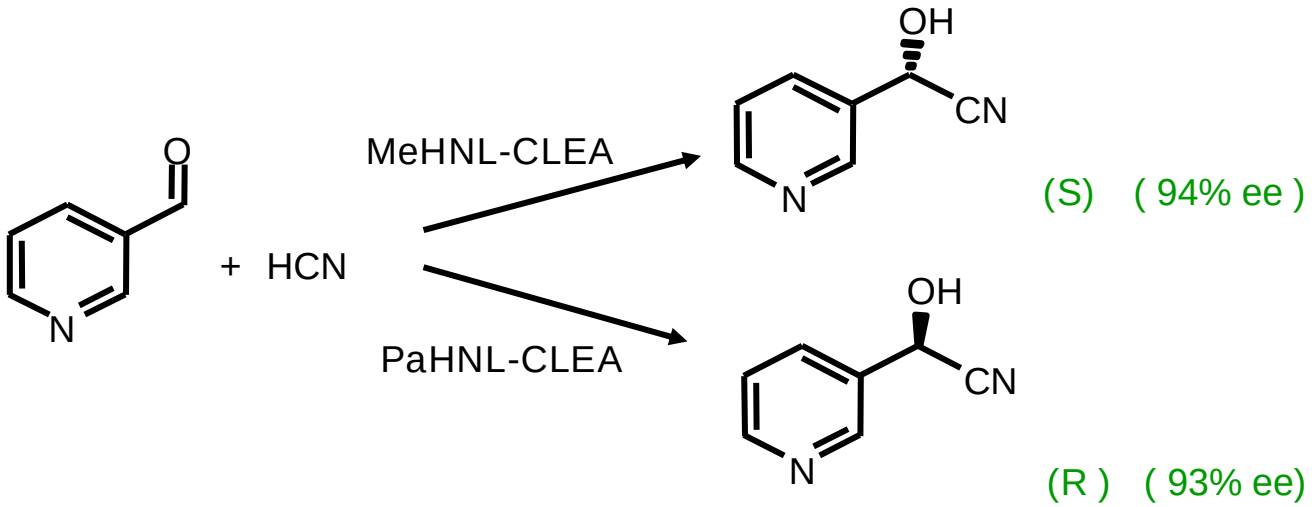
D. Özdemirhan, S. Sezer, Y. Sönmez, *Tetrahedron Asymm*. **2008**, *19*, 2717.

Oxidoreductase CLEAs: Laccase



- Biodegradable alternative to polyacrylates (SNAP)
- TEMPO/NaOCl environmentally unfriendly
- Laccase / TEMPO / O_2 : Enzyme costs too high owing to suicide inactivation
- Increase operational stability with a laccase CLEA
- Also with cellulose to carboxycellulose

Hydroxynitrile Lyase CLEA

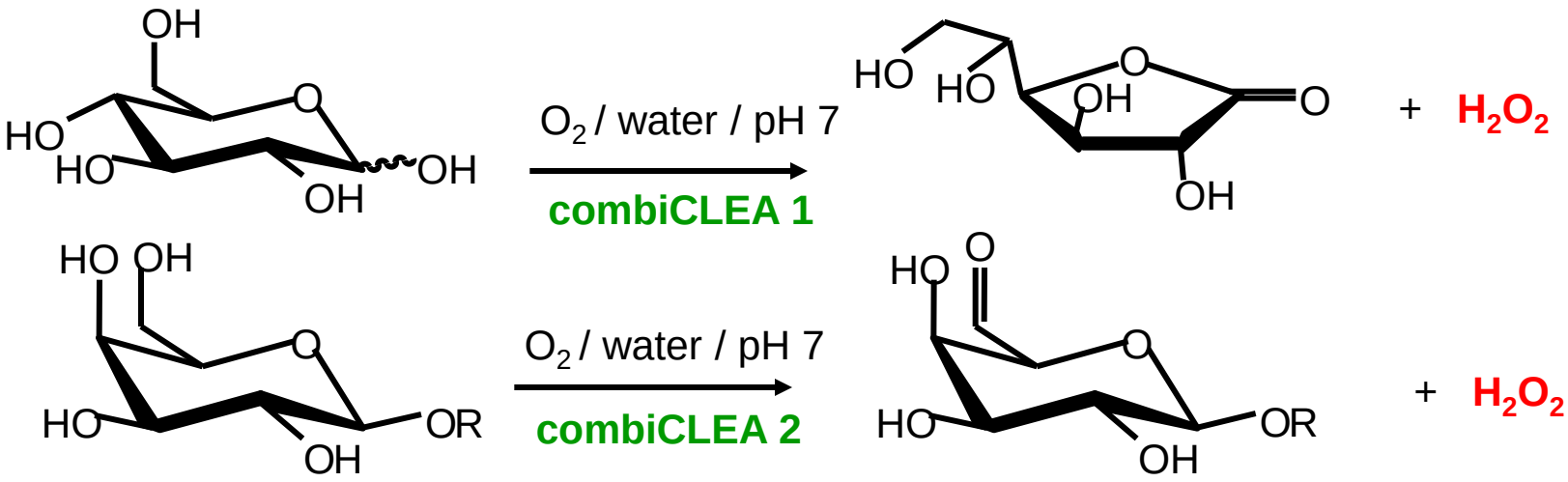


- Low reaction temp. (5^{o}C)
- Microaqueous environment (0.18% H_2O in DCM)
- Immobilization as a CLEA

”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 (2007), 48(8), 1473-1477.

Combi CLEAs

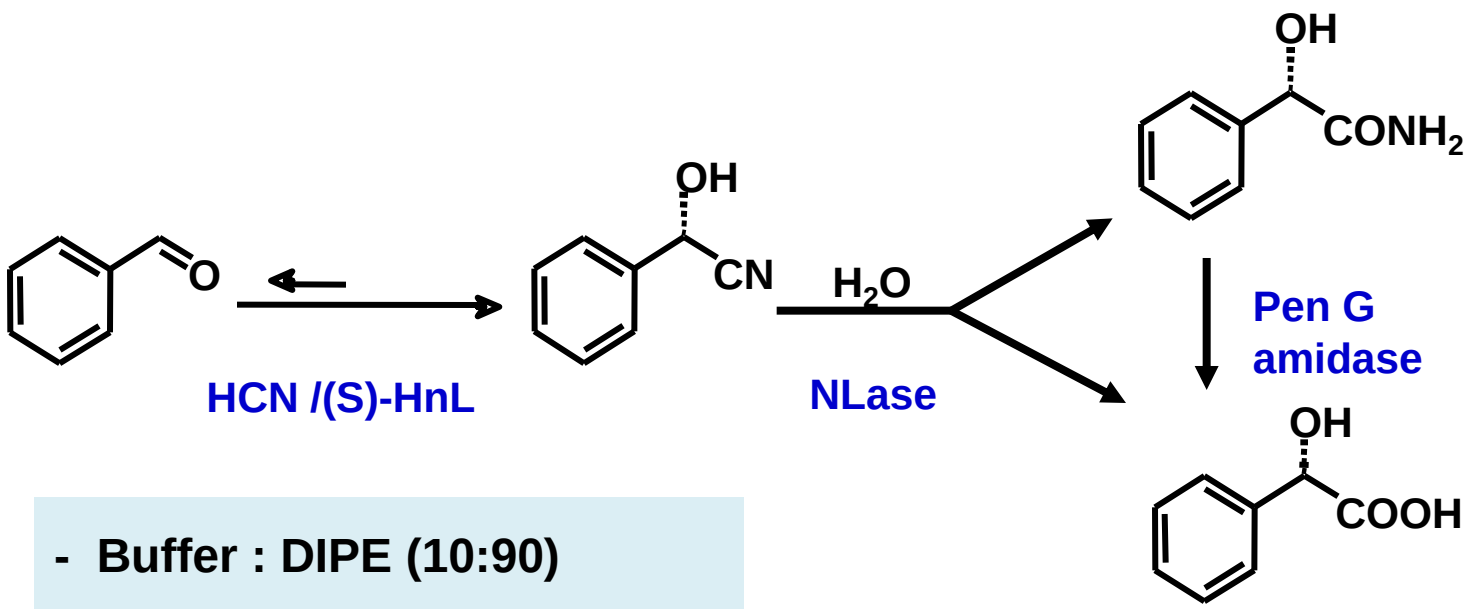


Activity	Free enzyme	CLEA
1st use	100%	100%
2nd use	-	100%

combiCLEA 1 = Glucose oxidase / catalase

combiCLEA 2 = Galactose oxidase / catalase

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



- **Buffer : DIPE (10:90)**
- **pH 5.5 / RT / < 5h**
- **HnL/ NLase / Pen.acylase combi-CLEA**

Conv. 96% / ee >99%

A. Chmura

Reactor Concepts

- **Stirred tank + filtration or centrifugation**
- **Microchannel reactors for sceening**
- **Fixed bed (oils & fats conversion / biodiesel) (particle size/pressure drop) mix with e.g celite**
- **Fluidized bed (density of particles important)**
- **Magnetic CLEAs in magnetically stabilized fluidized bed**
- **CLEA suspension in a filter slurry reactor**

Process Intensification: Microchannel Reactors

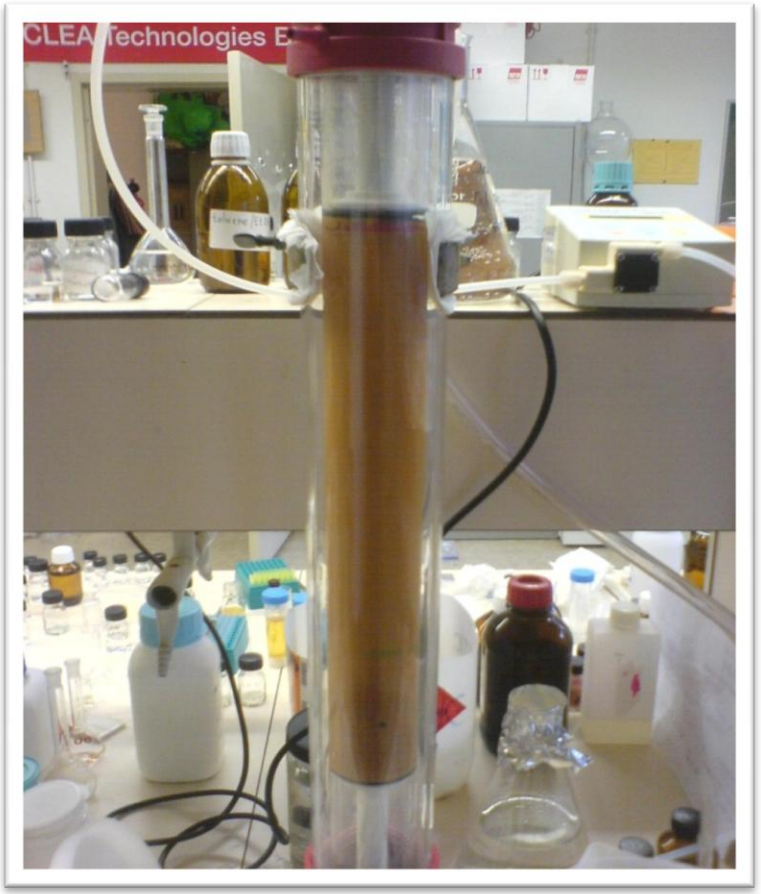
- Numbering up vs scaling up
- 10^2 x larger surface/volume ratio
- Efficient mass & heat transfer
- For rapid screening of enzyme scope

CLEAs in microchannel reactors (gamma-lactamase)
- 100% Activity retention

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

Reactor Configuration

Pen Acylase in Filter Slurry Reactor (FSR)



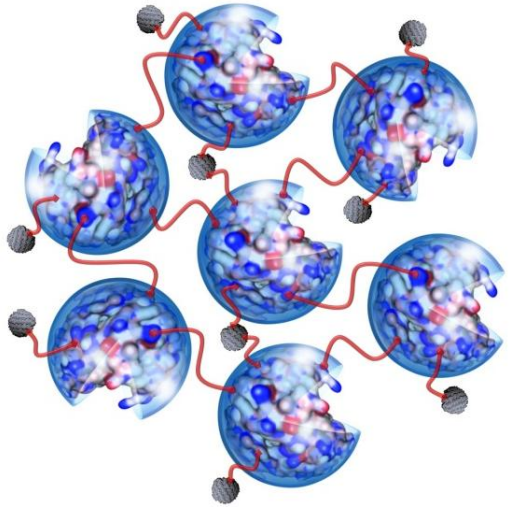
Alcalase® CLEA in fluidized bed

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

Magnetic CLEAs

homogeneous three-dimensional network of cross-linked aggregated enzyme particles and magnetic nanoparticles.

- High enzyme loading
- NO enzyme leaching
- Mechanically robust
- Separation by magnetic decantation
- Tunable magnetic strength



- mCLEA can be produced from any enzyme– currently with 2 lipases and catalase
- Potential applications in the pharmaceutical, food and feed industries, and diagnostics
- Composition of matter patent pending

CLEA Technologies

Magnetic CLEAs

Synthesis of mCLEA

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

mCLEA in magnetically stabilized fluidized bed

CLEA Technologies

Magnetic CLEAs

Candida antarctica lipase A mCLEA (not optimized)

E : NP (% w/w)	AR mCLEA* (%)	Activity of mCLEA compared to the normal CLEA (%)	Immobilisation efficiency ** (%)	Leaching*** (%)	Magnetic strength (emu/g)
95 : 5	45	136	100	0	4.9
85 : 15	32	97	100	0	11
60 : 40	35	106	100	0	26
10 : 90	41	124	100	0	53.5

* AR - Activity recovery: % of activity of the mCLEA compared to the free enzyme

** Determined upon the activity found in the supernatant and wash

*** Leaching: (%) of activity compared to the activity of the mCLEA
– leaching test performed in water for 70 h.

CLEA Technologies

CLEAs : Summary

- Easy, broadly applicable and cost-effective
- Applicable to partially purified enzymes
- No leaching of the enzyme in aqueous media
- High catalyst productivity and improved stability
- Rapid optimization using parallel experimentation
- Various options for reactor configuration
- Combi CLEAs for catalytic cascade processes
- Smart CLEAs (e.g. magnetic CLEAs)

- **The best is yet to come**

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Thank you. Any questions?