ALMAC

Partnering to Advance Human Health

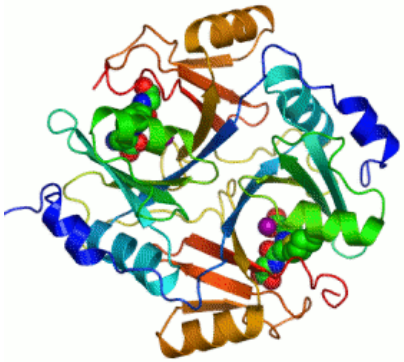
Biocatalysis and bioprocessing -

Genes to GMP

Prof. Tom Moody

www.almacgroup.com

Bioprocessing



An integrated group of biologists and chemists who:

Discover. New enzyme discovery platforms

Screen. Screen for and utilize enzymes in processes

Evolve. Improve properties of enzymes (process efficiency, economics)

Supply. Manufacture (immobilised) enzymes and enzyme-derived products

Key Expertise:

- Enzyme discovery (metagenomics)
- Building panels of enzymes
- Active site modelling and enzyme design
- Evolution tools (saturation mutagenesis, random mutagenesis)
- Fermentation development & scale-up
- Enzyme immobilization & supply
- Bioprocess development and manufacture of chemicals
- Metabolite synthesis

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Reaction Experience

Decades of process experience….

Reaction Experience		
Alkylation	Enzyme immobilisation	Nitrilase reactions
Aminoalkylation	Heck Reaction	Optical Resolution
Asymmetric Synthesis	Hydrogenation	Organolithium Chemistry
Biotransformations	Hydrolase bioresolutions	Organometallics
Base Catalysis	Grignard Reactions	Organosilicon Chemistry
Transfer Hydrogenation	KRED bioreductions	Oxidation
Condensations	Reductions	P450 Biooxidation
Dealkylation	Leuckart Reaction	Ritter Reaction
Dieckmann Condensation	Methylations	Stereoselective Reactions
Diels Alder Reaction	Michael Reaction	Suspension polymerisation
Enzymatic Resolution	Mannich Reaction	Transaminase bioreactions

…Coupled with biocatalysis manufacturing technologies

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Manufacturing

Ideal tank sizes to develop and manufacture Multi-Ton scale intermediates (biocatalytic processes & immobilisation)

Multi-purpose Pilot Plant

- 7 reactors from 100 to 1,000L
- Total capacity of 2,600L
- Glass-lined, Hastelloy and Stainless Steel

Manufacturing Plant

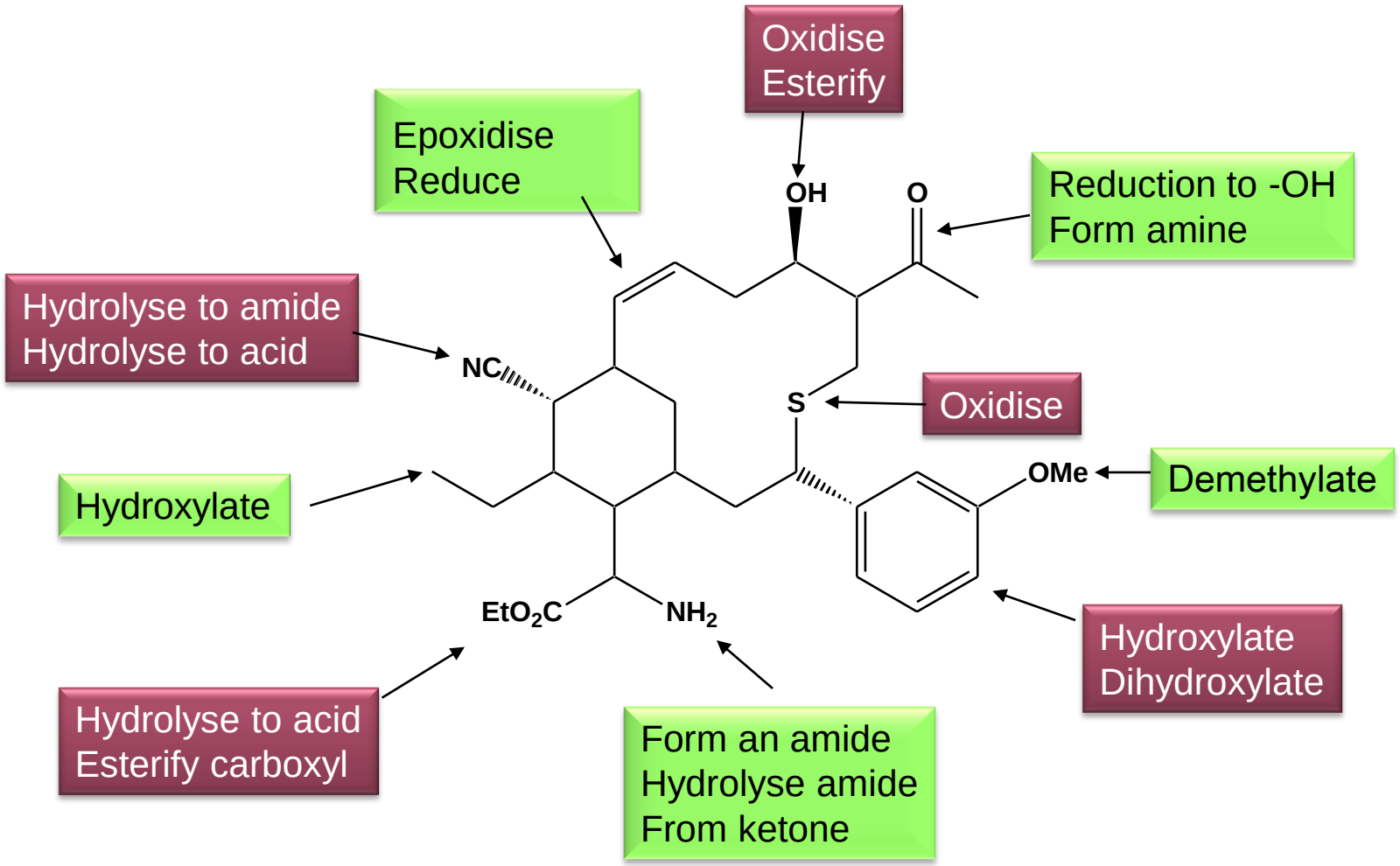
- 13 reactors from 2,200L to 6,300L
- Total capacity of 45m^3
- Glass-lined and Stainless Steel

Work-up

- Centrifugation from 1.0 to 2.5 tonne
- Driers: 0.5 to 0.6 tonne

arran
CHEMICAL COMPANY LTD
A Member of the Almac Group

Almac Technology



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The Good Bits

- ✓ Chemo & Regioselective
- ✓ High stereoselectivity
- ✓ Simple and easy to perform

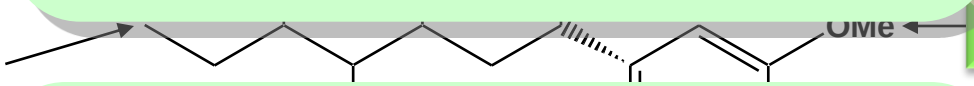
Green & Sustainable

- ✓ Water as the solvent
- ✓ Ambient temperature
- ✓ Atmospheric oxidant
- ✓ Non toxic waste

Hydrolyse to amid
Hydrolyse to acid

on to -OH
mine

Hydroxylate



Demethylate

oxylate
droxylate

Hydrolyse to a
Esterify carbo

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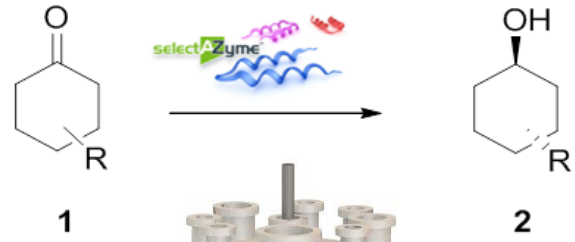
kg to tonne manufacture



Fermentation Development

Fermentation Scale-up

Bulk Enzyme supply

Scale-up
- Raw material sourcing
- Quality

Almac Process Dev
- Project Siting
- Almac site
- Partner Selection
- Customer site

Successful POC
- Use test of Demo batch sample

Enzyme Screening

Enzyme build and selection

Enzyme Evolution

Bioprocess Scale-up

Isolation

Product Extraction Purification *via*
- Distillation
- Crystallisation
- Re-slurry
- Lipase mediated biopolish
- Solvent extraction

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Specific Enzyme Panel Building

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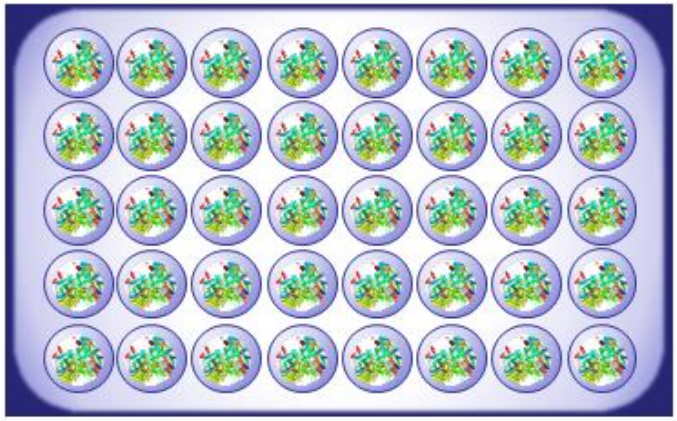
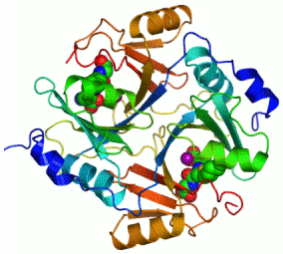
Enzyme panel building

Bespoke enzyme panel





- Bespoke enzyme panels can be developed within weeks using Almac bioinformatics GIDS platform
- GIDS – gene informed database searching
- Tailormade panel of enzymes expressed in 96 well plate format

- 1 enzyme only possible; typical is 25, 50 or 96
- 1g to tonne supply of enzyme available

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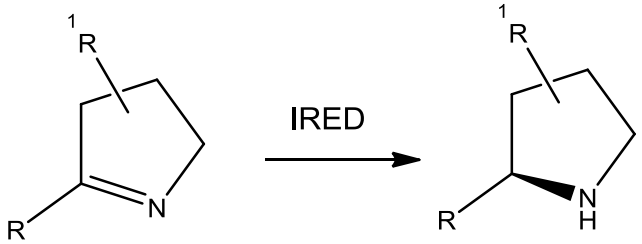
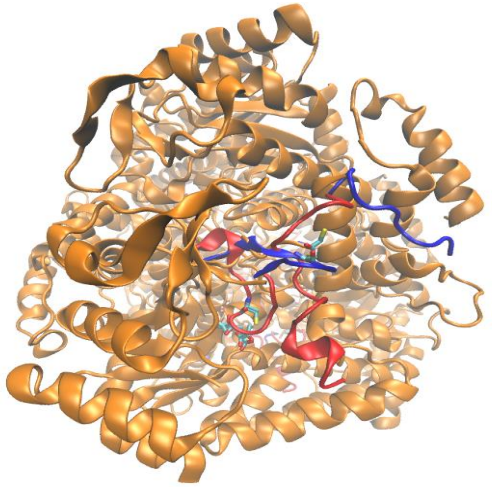
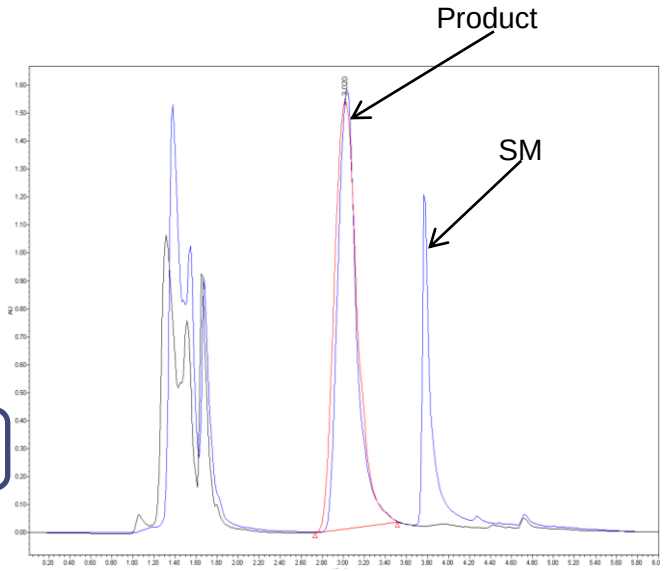
IRED





- *In silico* design of 50 IRED enzyme library
- Gene synthesised, cloned and expressed
- Screened against customer substrate – hits found

IRED no.	% HPLC peak area @ 260 nm		% HPLC peak area of product enantiomers		ee
	Product	SM	(S)	(R)	
3	9	91	54	46	8
5	2	98	0	100	100
8	77	23	89	11	78
9	2	98	13	87	74
12	3	97	100	0	100
16	3	97	77	23	54
20	100	0	100	0	100
26	1	99	89	11	78
27	31	69	92	8	84
28	8	92	0	100	100



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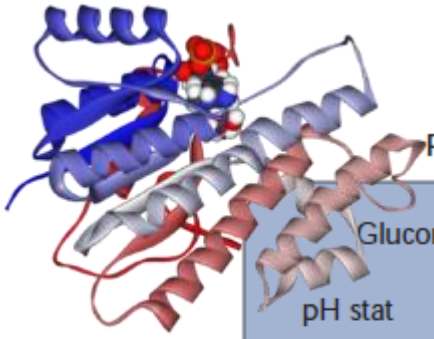
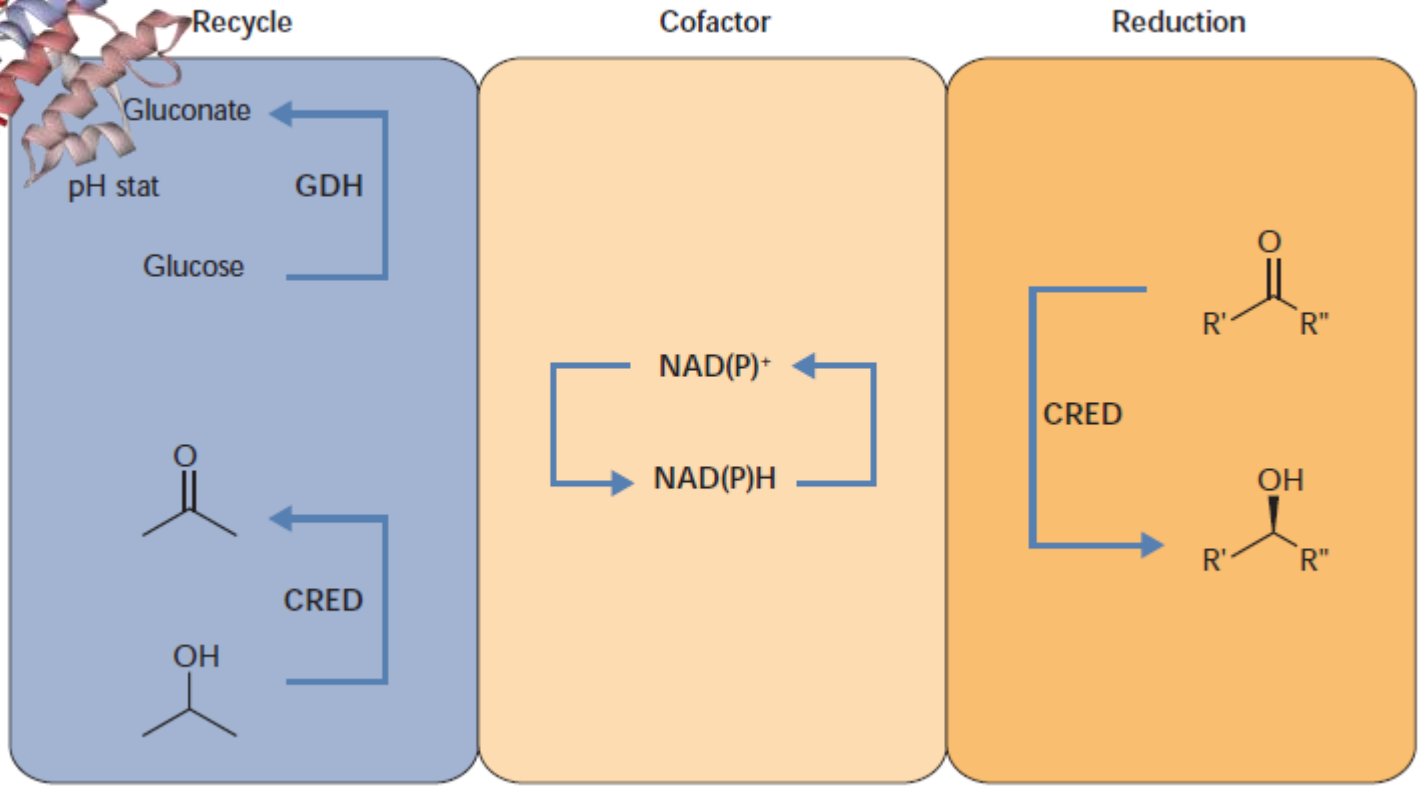
CRED enzymes

CREDs

Reaction mechanism





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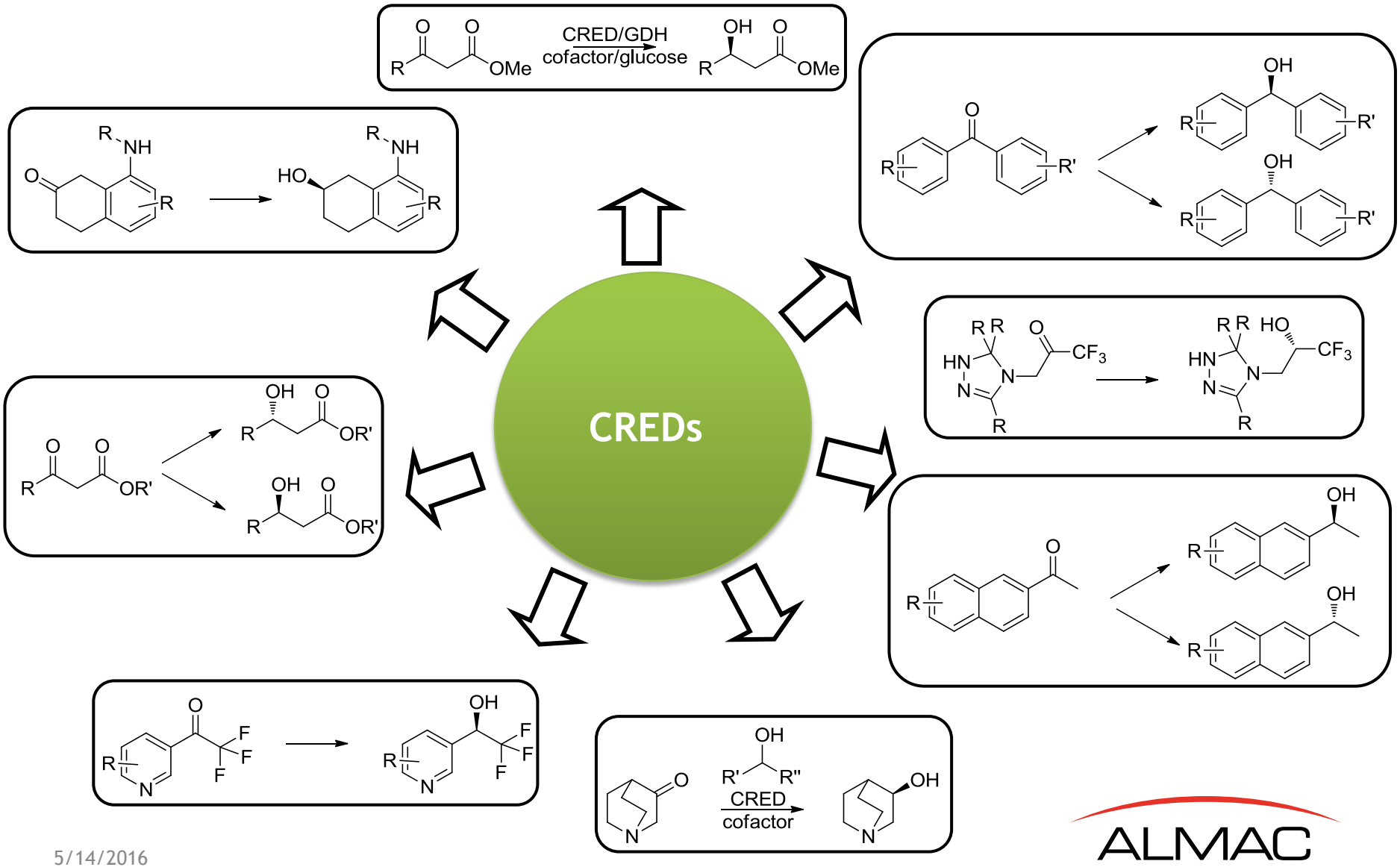
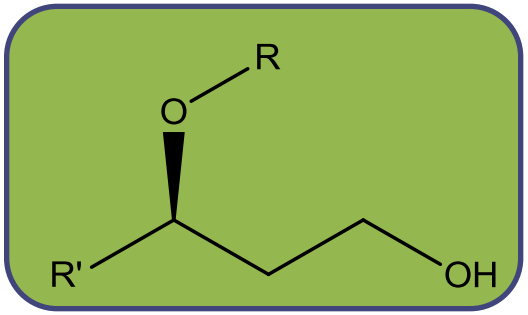
CREDs



Bioreduction



Chemocatalysis failed to deliver new specification of >99.2%ee

- Project for a Japanese Customer
- Building block for Phase III API
- Specification: >99% (GC), max. 0.1% for unknown imps, up to 0.5% named imps; >99% ee
- No scaleable biosynthetic route to intermediate
- Fermentation scale-up to 1000s of litres
- Ultimately tonne process required
- <<$1000 / kg

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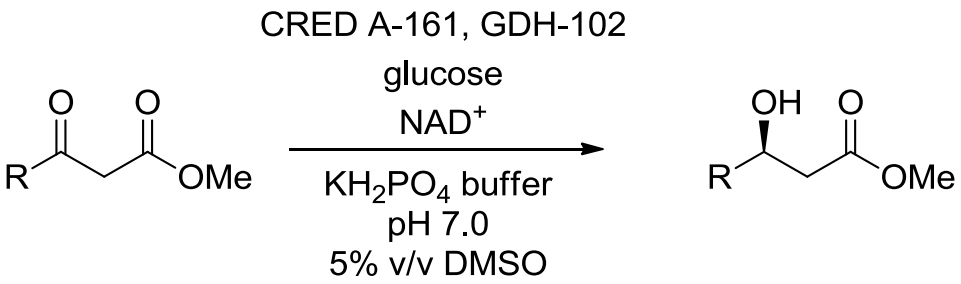
Bioreduction



CRED Screening:

- A161 identified as hit with >99.5% ee
- Enzyme is NADH-dependent
- GDH/glucose used for co-factor recycle

Process Optimisation:

- 5% DMSO co-solvent, pH 7.0, 30 deg C
- 0.1% w/w of lyo cell free extract CRED/GDH sufficient
- 180 g/L substrate >99.8% converted within 12 hours
- Workup by extraction with MtBE; crude product telescoped into next step

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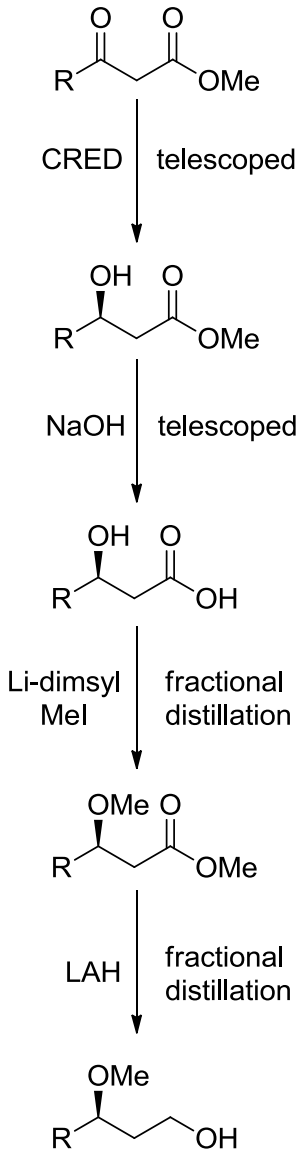
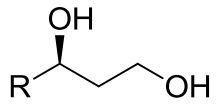
Bioreduction



Impurity control

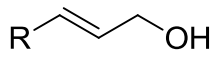
Control of Diol-impurity starts here.
High reaction completion target
of > 99.8% conversion.



Diol-impurity

Control of alk-3-enol impurity starts here.

High reaction completion targets.
Choice of base for deprotonation.
Unstabilised THF used for methylation.
Fractional distillation in both final steps.



vinyl-impurity

Achieved:
99.3% ee; diol <0.2%; alk-2-enol <0.15%
BHT and Me-BHT <0.2%, all other <0.1%

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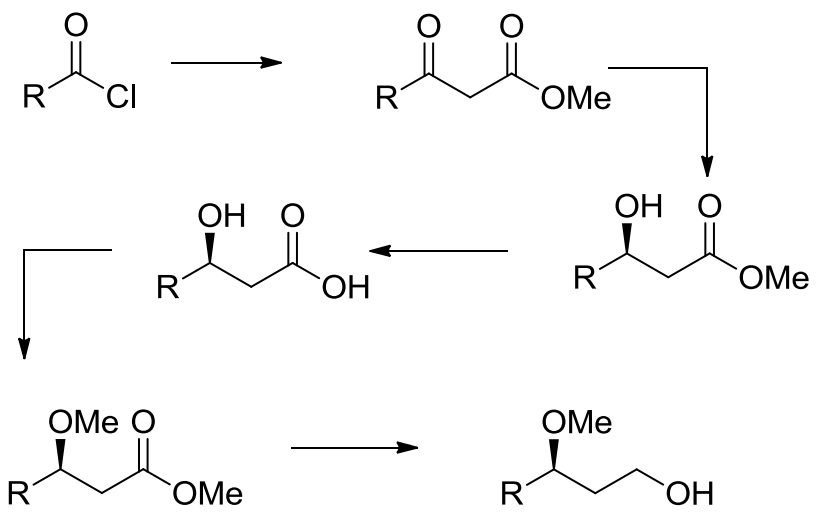
Bioreduction

from POC sample to commercial output

5 Step Process Yields
(%POC / %pilot batch / %final)

Step 1: 75% / 50% / 85%
Step 2: 91% / 90% / 95%
Step 3: 95% / 95% / 98%
Step 4: 80% / 70% / 85%
Step 5: 70% / 70% / 75%



Process modifications after pilot batch experience:
Step 1: switched from Meldrum's acid to methyl potassium malonate SM
Step 4: switched from n-BuLi to n-HexLi, relaxed specification
Step 5: improved hydrolysis protocol

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ERED enzymes

Adv Syn Cat – 2016

Aromatisation Using Ene Reductase Enzymes

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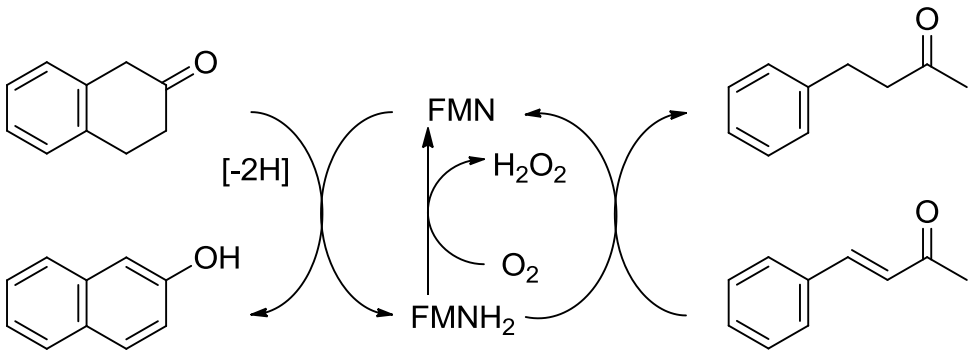
[a]**Almac**, Department of Biocatalysis Isotope Chemistry, 20 Seagoe Industrial Estate, Craigavon, BT63 5QD, Northern Ireland, United Kingdom, Phone: +44 (0) 28 3833 2200; e-mail: paul.kelly@almacgroup.com

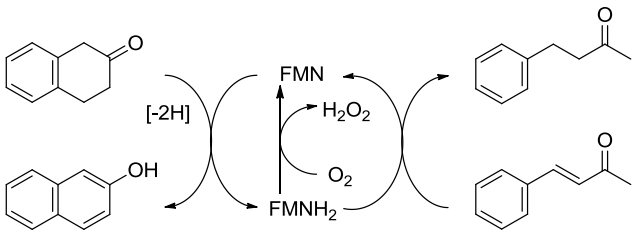
[b]**Merck & Co., Inc.,** Department of Process Research, Merck Research Laboratories, P.O. Box 2000, Rahway, New Jersey 07065, USA

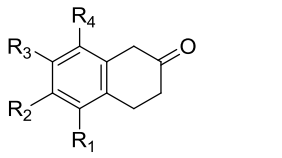


EREDs

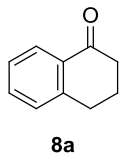
EREDs for Phenol/Naphthols (*Adv. Synth. Catal.*)

- Conversions of tetralones to naphthols in absence of reduction target (**9a**) confirmed up to >99% in some cases

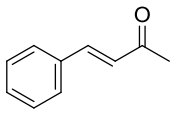




	1a	2a	3a	4a	5a	6a	7a
R_1:	H	MeO	H	H	H	H	H
R_2:	H	H	MeO	Cl	Br	H	H
R_3:	H	H	H	H	H	MeO	H
R_4:	H	H	H	H	H	H	MeO

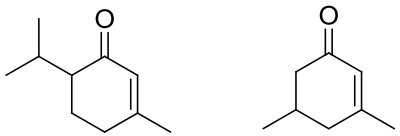


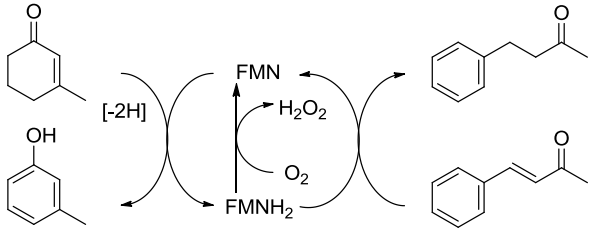
8a



9a

- ERED 69 and 7-MeO-Tetralone selected for optimisation and scale up to ~1g to follow
- Additional examples of cyclohexenones to phenols to be included





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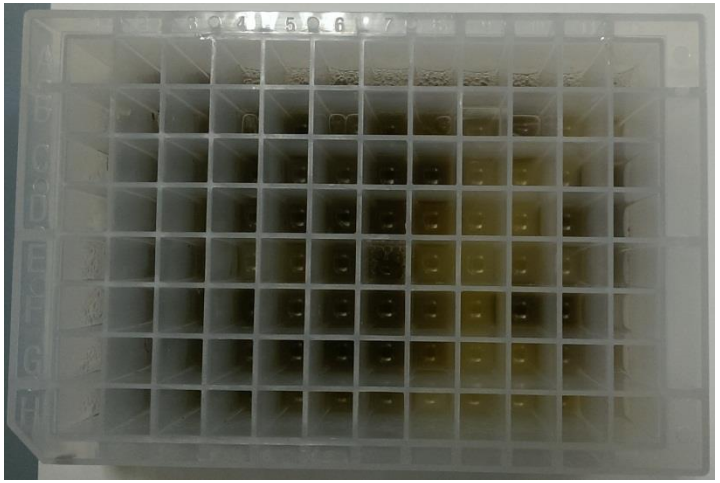
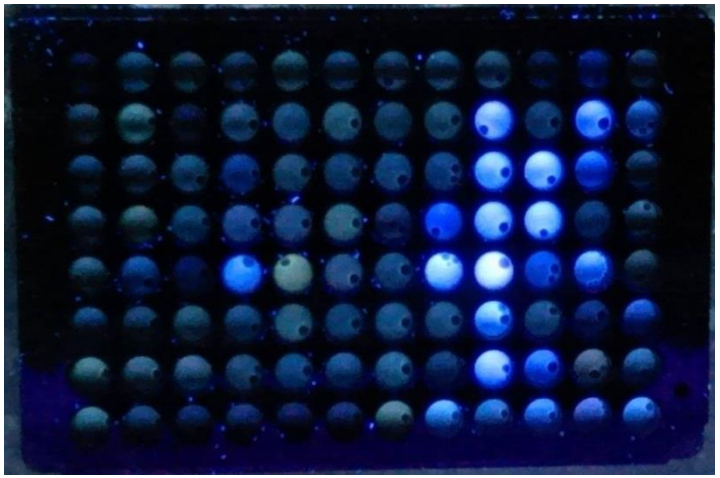
EREDs

Analysis by Fluorescence

- Red-orange precipitate in many wells
- Others clear/yellow



- 200 µl of sample taken
- 22 µl of 1M NaOH added
- Observed under UV (365nm)
- Read by fluorescence plate reader
  - λex = 355 nm, λem = 460 nm
  - Gain set to 82.5% for 5 mM 2-Naphthol
- Reads baselined to empty vector control



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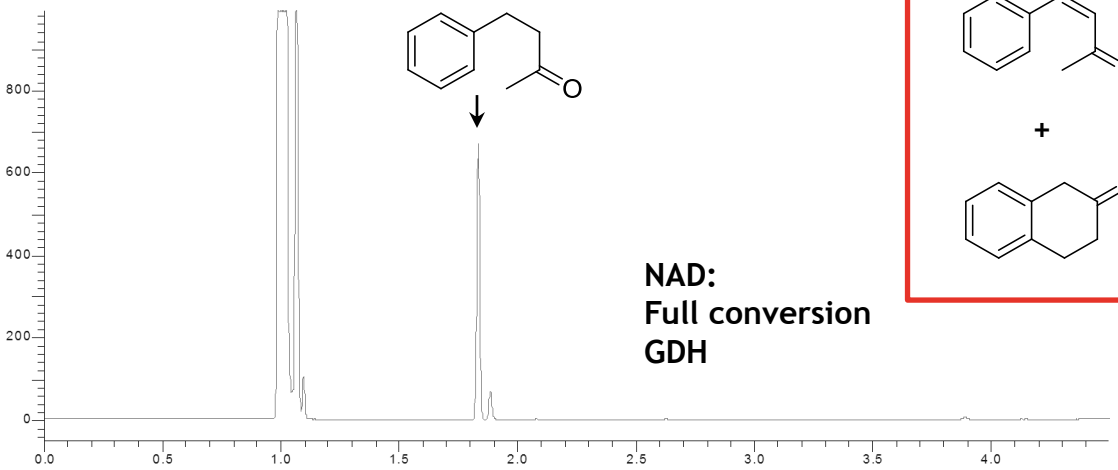
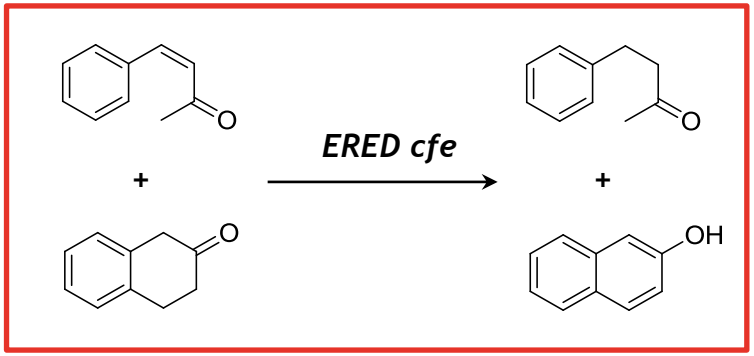
EREDs – Phenolic preparation

ERED - E9



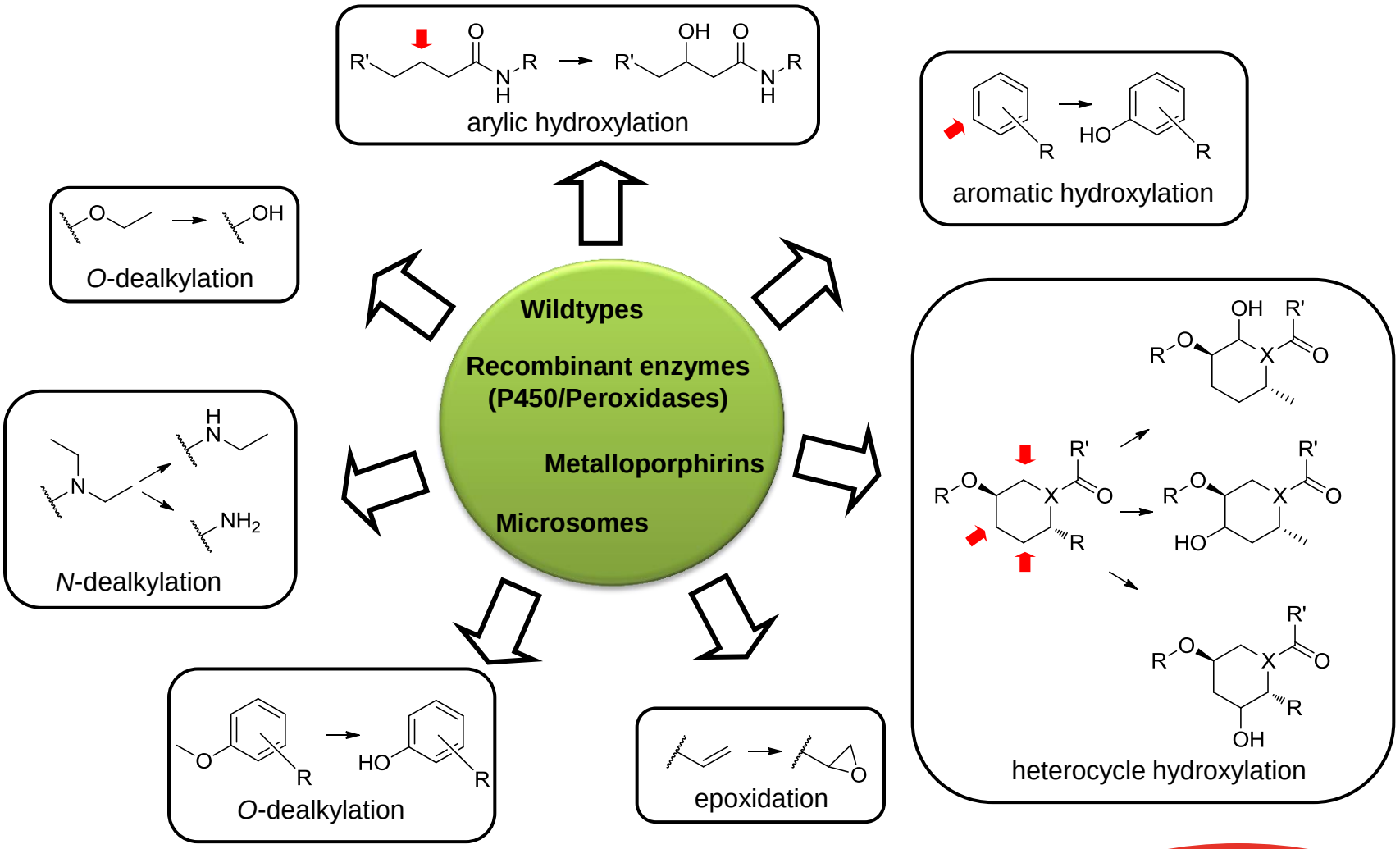
NAD:
Full conversion
GDH



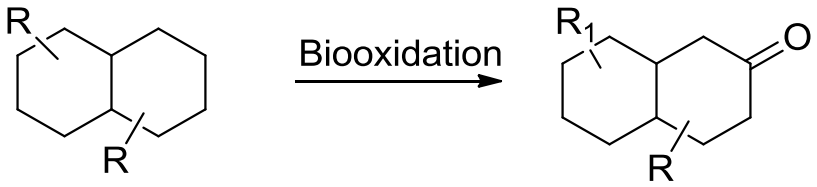
B-tetralone:
Good conversion

P450 enzymes

Biooxidation

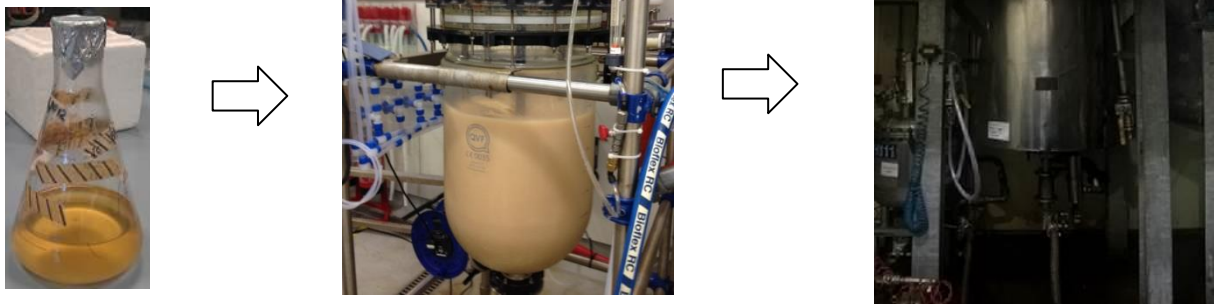


Biooxidation



Customer project

- P450 project
- Enzyme screening and hit ID, enzyme engineering
- Reaction optimisation and scale up from 100 mL to 100 L to 1m^3
- Reaction engineering



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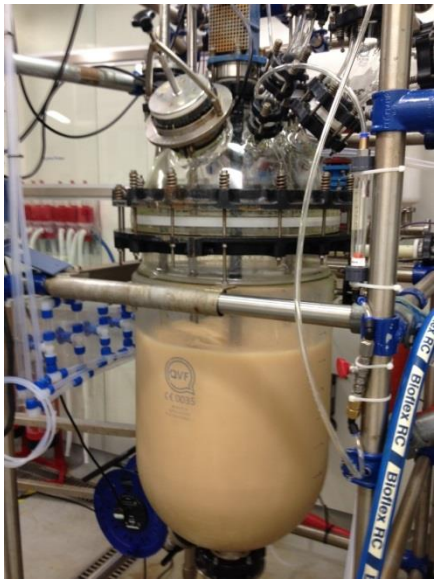
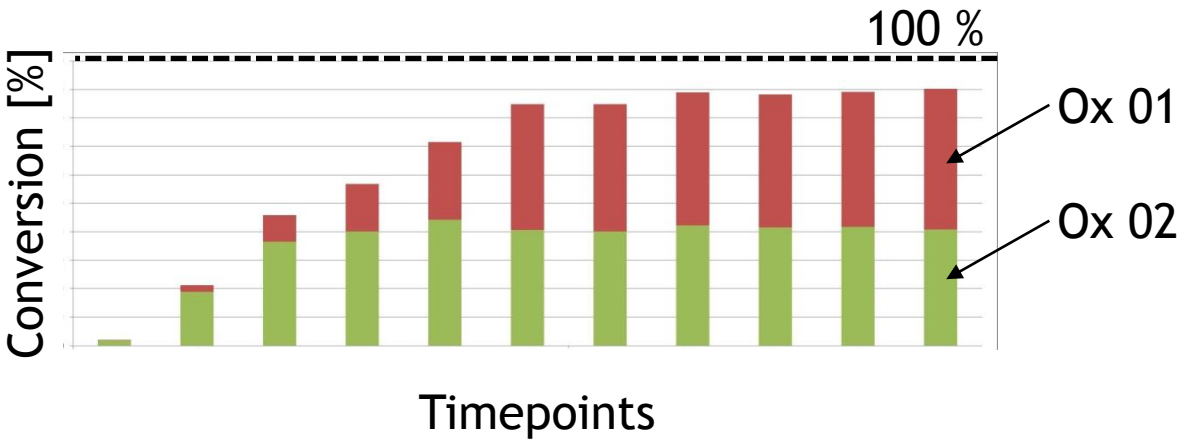
Biooxidation

Two Step P450 Oxidation

Terpenoid → Oxidation Product 1 → Oxidation product 2





- P450 reaction scaled up to 100 L
- Isolation of ~ 100g desired oxidation products
- NADPH recycling system used
- 1 m3 scale-up successful
- Next scale 6m3 in a chemical reactor

- Fermentation for catalyst supply – 15m3

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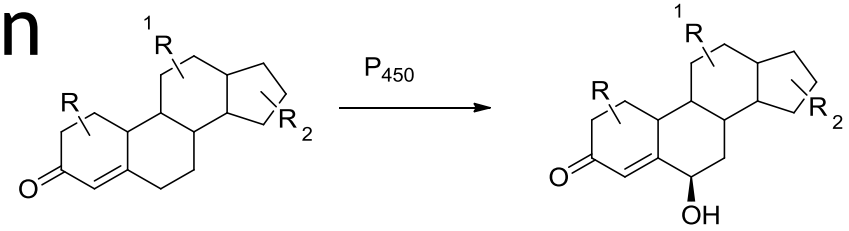
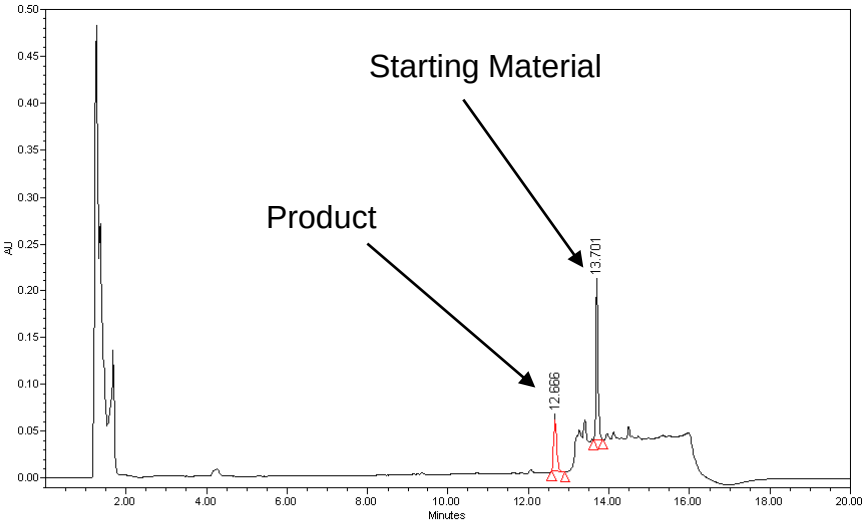
Biooxidation

P450 Oxidation



Modular Project Deliverables

- P_{450} and Streptomet™ screen
- Active P_{450} identified
- P_{450} ALM-CYP18 identified
- Evolution underway
- **Biooxidation scaled to 5 L in a fermenter**



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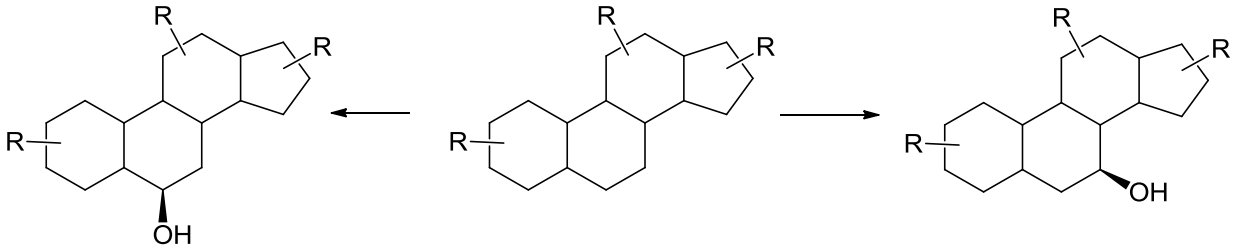
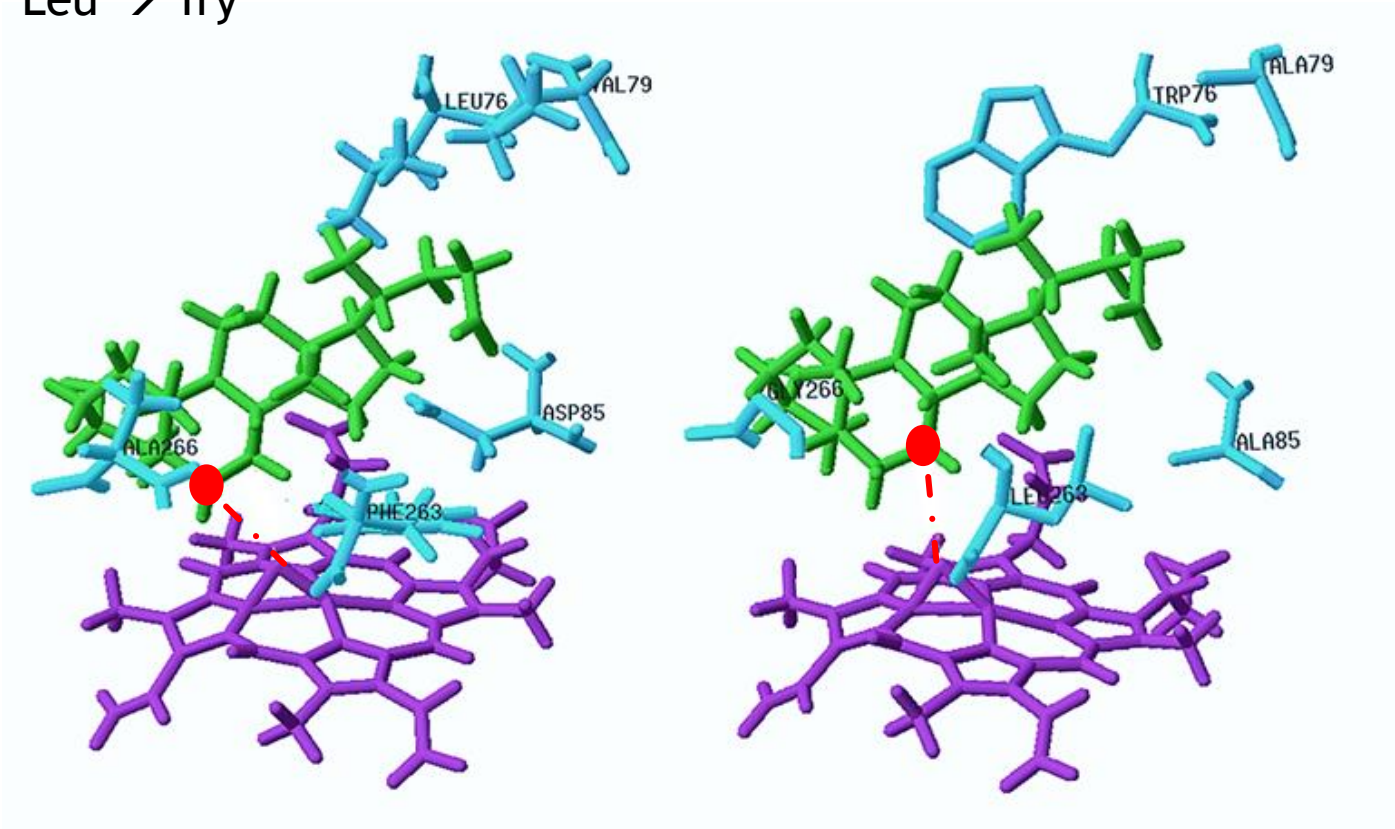
Biooxidation

P450 Oxidation – Molecular modelling



Leu → Try



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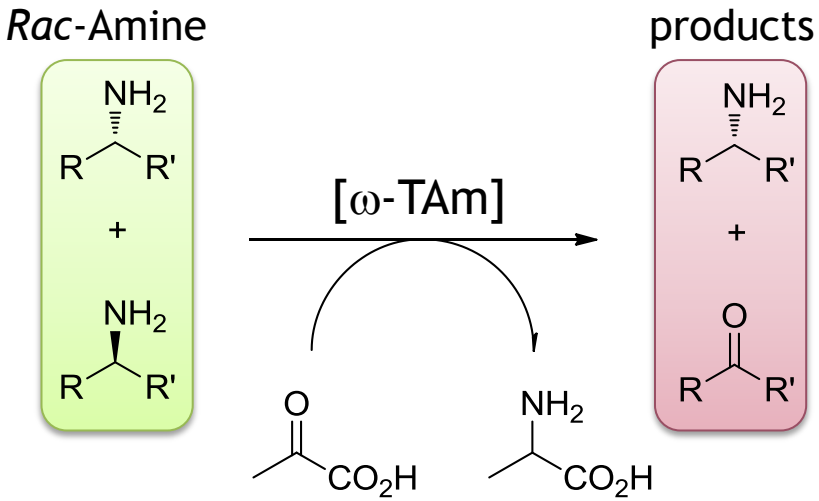
TAm enzymes

Transaminase

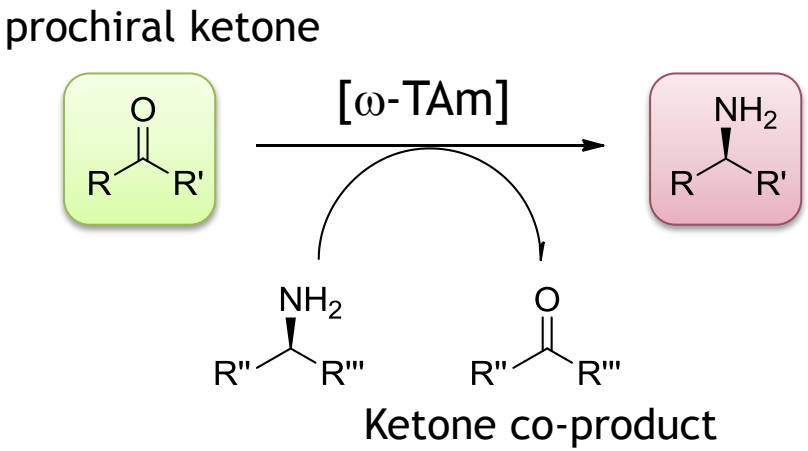
Preparation of enantio-enriched α-chiral primary amines

a) Kinetic resolution



a) Kinetic resolution starting with racemic amines (limited by 50% yield)

b) Asymmetric synthesis



b) Asymmetric synthesis of prochiral ketones (theoretically 100% yield possible)

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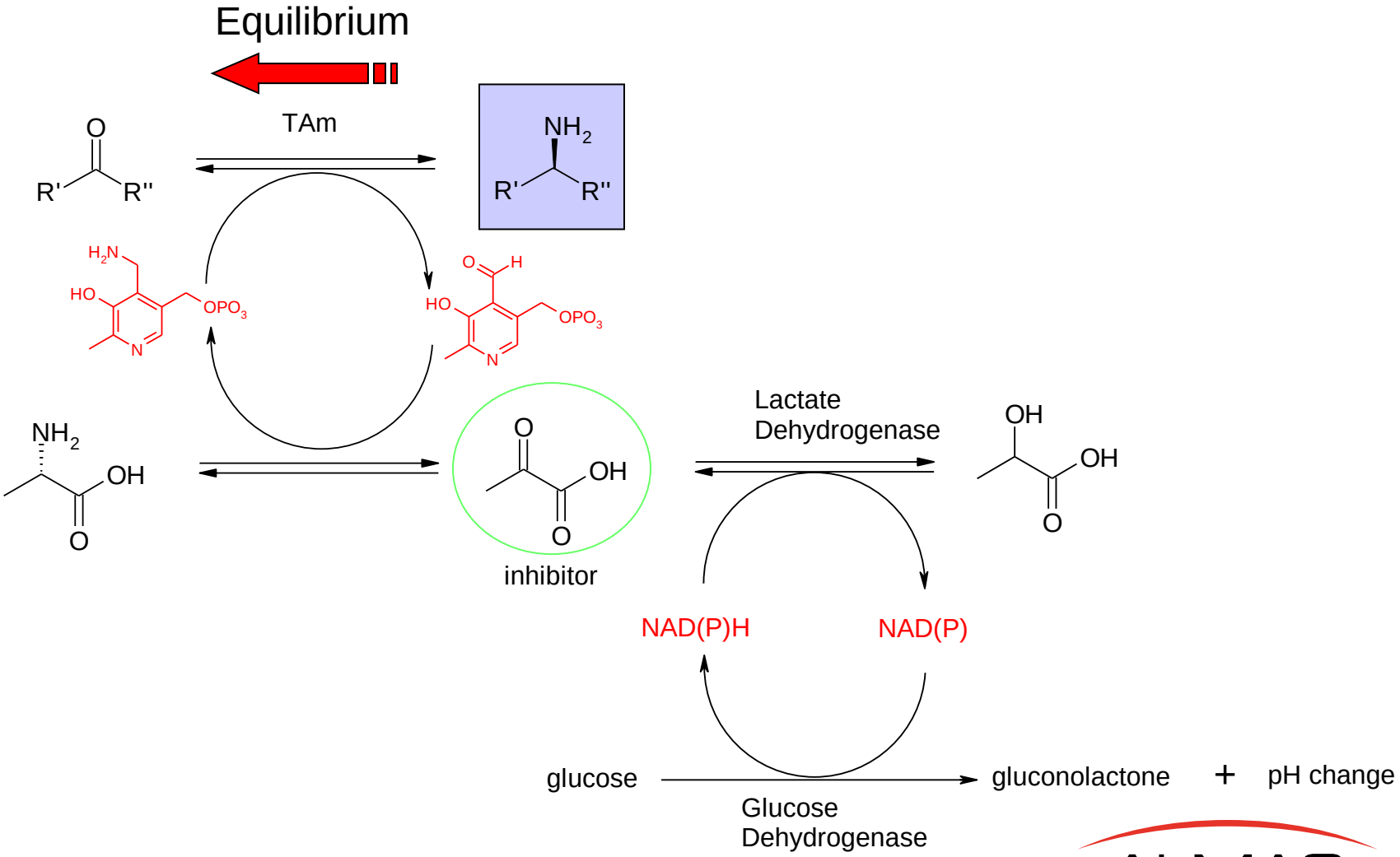
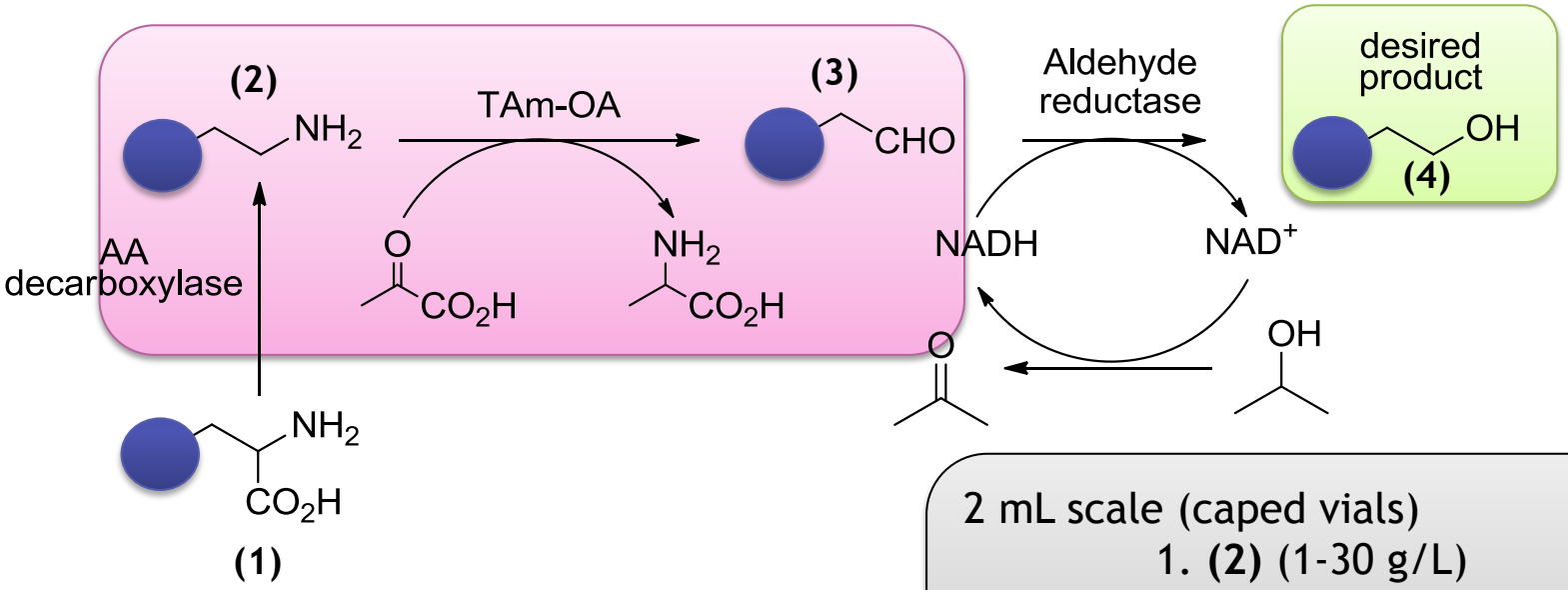
Transaminase

Strategy used at Almac to remove inhibitory side products



Transaminase - Cascade



2 mL scale (caped vials)

1. **(2)** (1-30 g/L)
2. **(3)** (0-15 g/L)
3. L-Ala (1-50 g/L)
4. pyruvic acid (5-200 mM)

pH 7.4 (adjusted before reaction)

Conclusion

- TAm is active with substrate **(2)** (5-30 g/L)
- Inhibition with L-alanine (70 % residual activity at 10 g/L), and pyruvic acid (75 % residual activity at 50 mM)
- TAm – maximum apparent specific activity 0.35 U/mg_{CFE} (at 30 g/L substrate **(2)**), and 0.1 U/mg_{CFE} (at 7 g/L substrate **(2)**)

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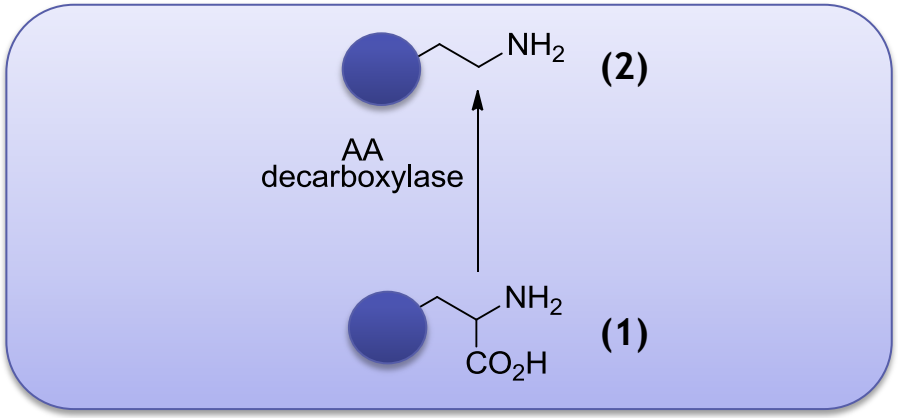
Transaminase - Cascade

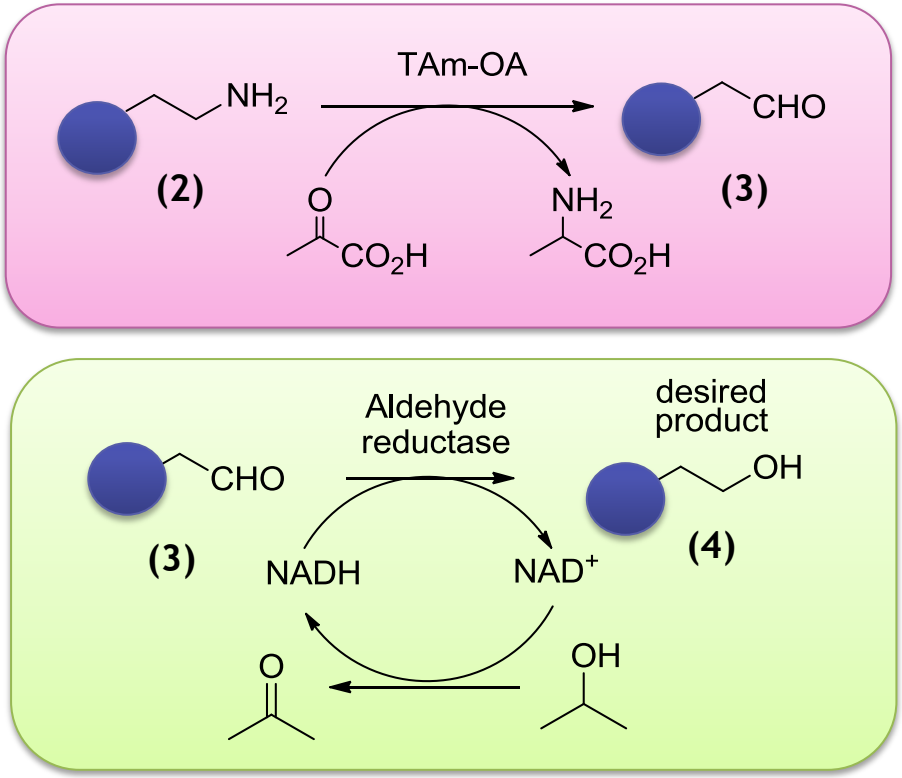
Step 1



5 g scale, 25 g/L L-AA
open vessel with pH control
10 % enz, 0.04 % PLP, pH 7.4, 63% conversion in 42 h

5 g scale, 25 g/L L-AA
Closed vessel, no pH control
20 % enz, 0.04% PLP,
pH 7.14 (initial), pH 7.54 (final)
97% conversion by HPLC in 18 h

Step 2 and 3



Drop-wise addition of **(2)** and pyruvic acid with pH control and
(3) – 5 g reaction completed in 16 h

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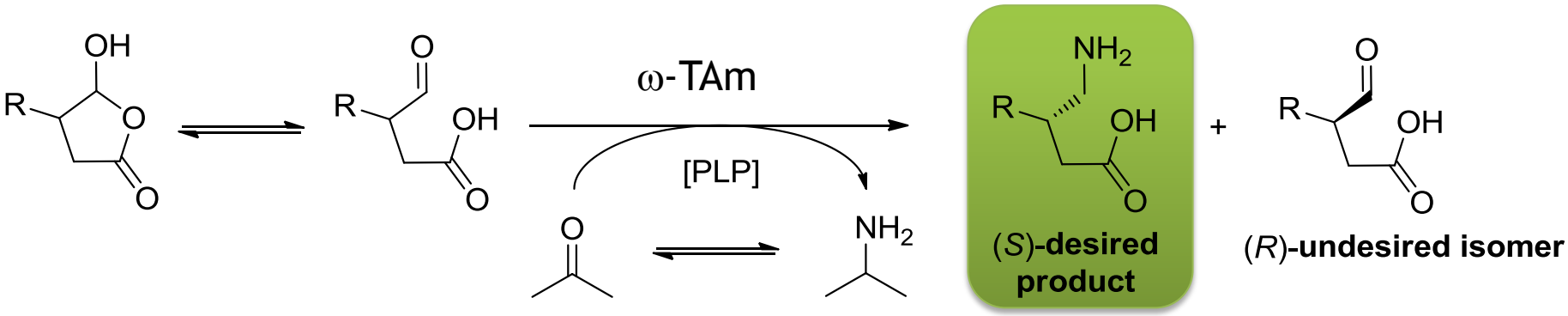
Transaminase - DKTAm



- Project Aim:

- process optimisation of the transamination to afford quantitative yield while maintaining ee of >95%

- Development of a DKTam

- TAm immobilised

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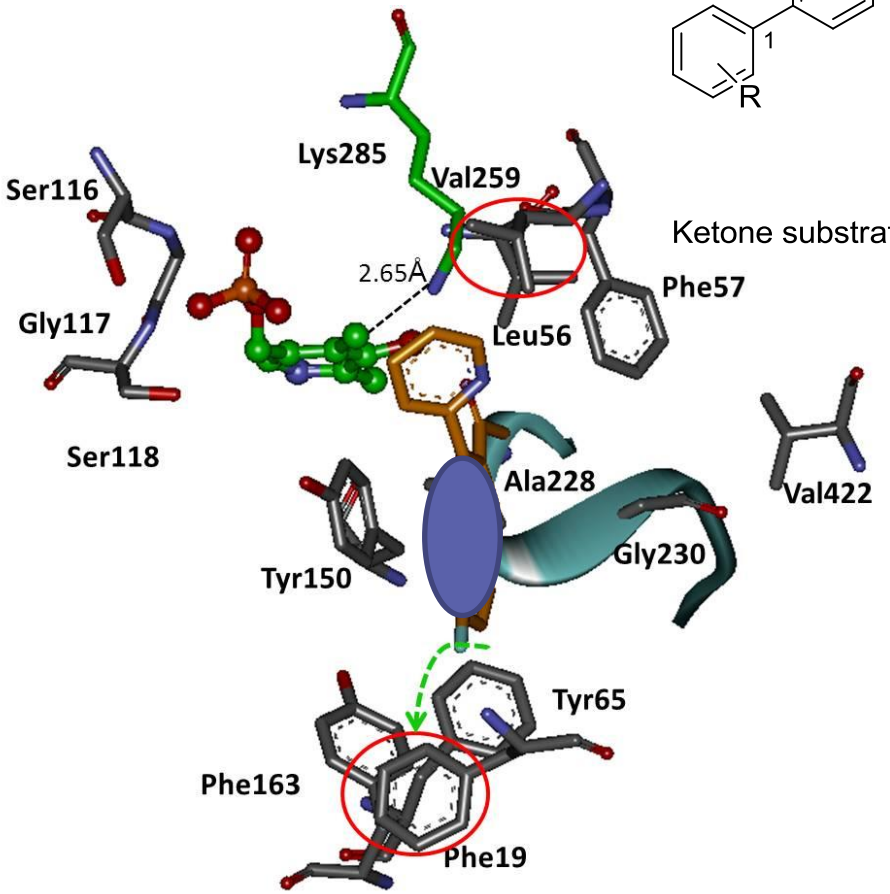
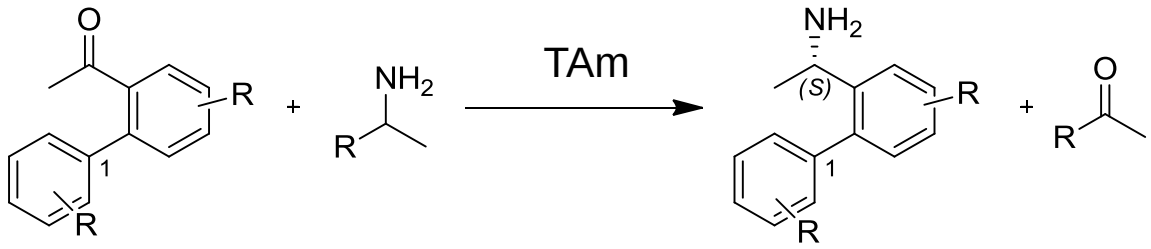
Directed Evolution

Substrate specific mutagenesis by protein modelling





Ketone substrate amine donor Amine product (*S*) side product

- **Selected residues will be mutated**
- **Reduced screening effort**
- **Increased chance of success**
- **“Smart libraries”**

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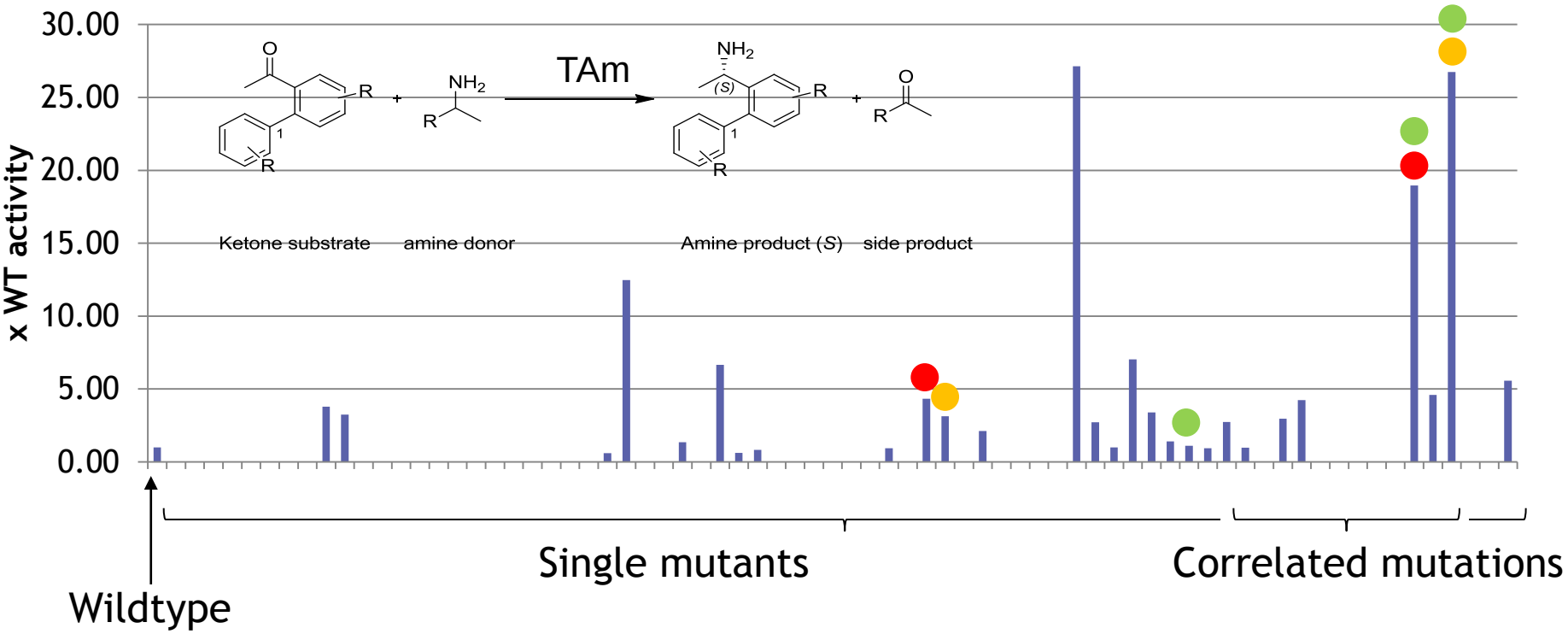
Directed Evolution

Combination of techniques



⇨ 72 Mutants screened => first round improvement: 25 x wildtype reaction

ChemComm

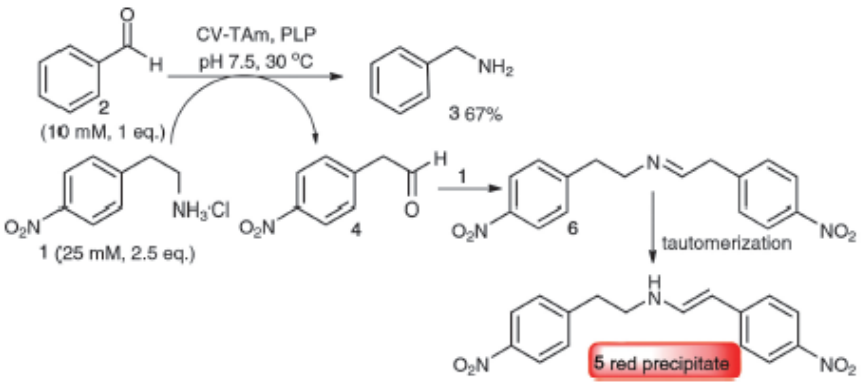
COMMUNICATION

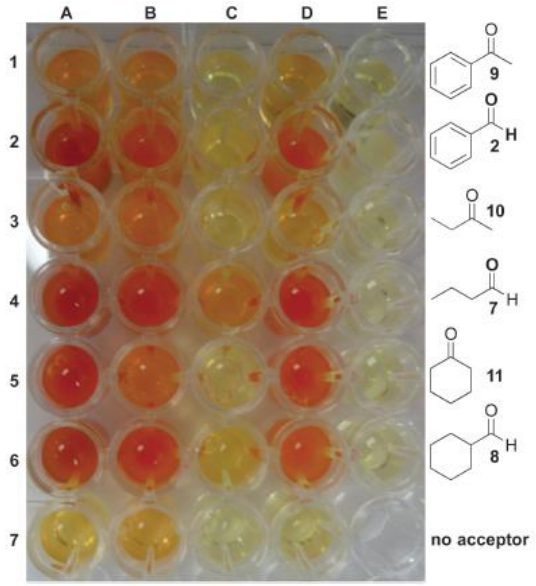
A rapid, sensitive colorimetric assay for the high-throughput screening of transaminases in liquid or solid-phase†

D. Baud,[a] N. Ladkau,[a] T. S. Moody,[b] J. M. Ward[c] and H. C. Hailes*[a]

Cite this: *Chem. Commun.*, 2015, 51, 17225

Received 13th August 2015,
Accepted 23rd September 2015





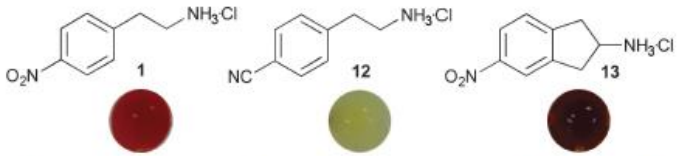
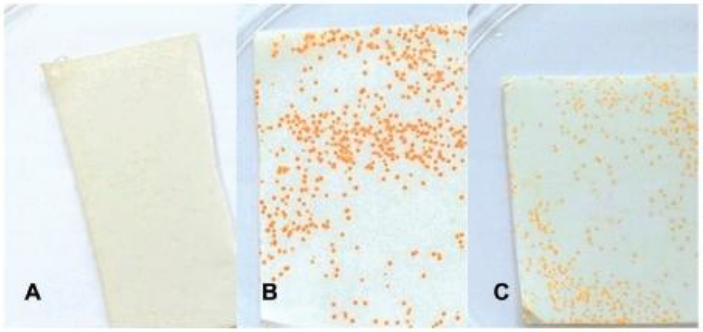


Fig. 2 Assay coloration when using amino donors **1**, **12** and **13** with CV-TAm and acceptor **7**.



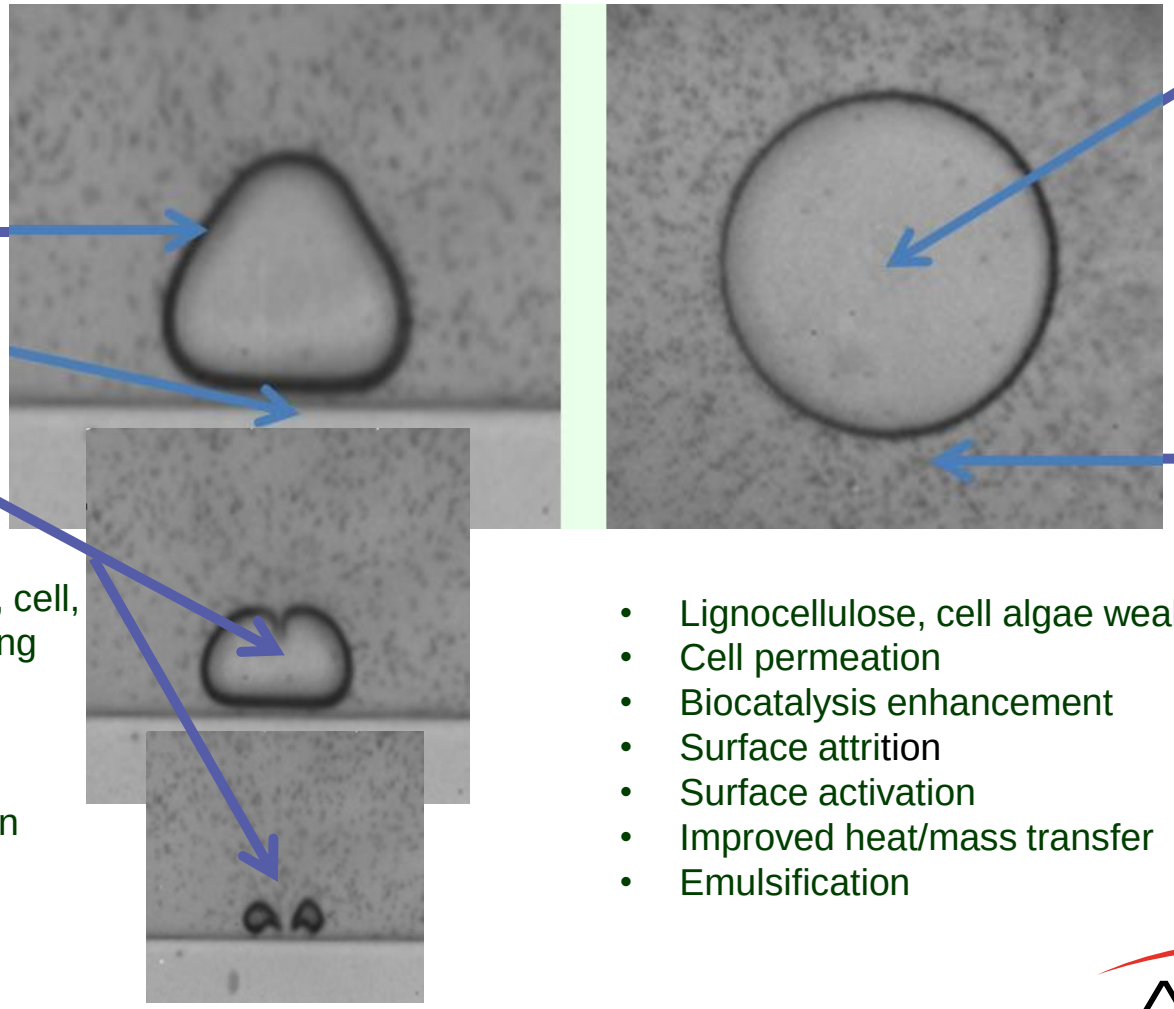
US process intensification

Ultrasound

Celbius

Acknowledgement Prof. T Mason, Uni. of Coventry

UNSYMMETRIC COLLAPSE
Inrush of liquid from one side of the collapsing bubble produces powerful jet of liquid targeted at surface



IN THE CAVITY extreme conditions on collapse 5000°C and 2000 atmospheres

IN THE BULK MEDIA intense shear forces

- Lignocellulose, cell, algae weakening
- (Bio)polymer degradation
- Crystallization
- Cell permeation
- Emulsification

- Lignocellulose, cell algae weakening
- Cell permeation
- Biocatalysis enhancement
- Surface attrition
- Surface activation
- Improved heat/mass transfer
- Emulsification

ALMAC

Ultrasound

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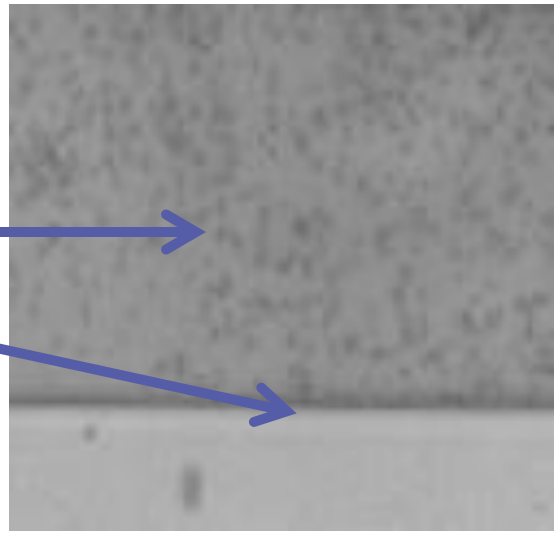

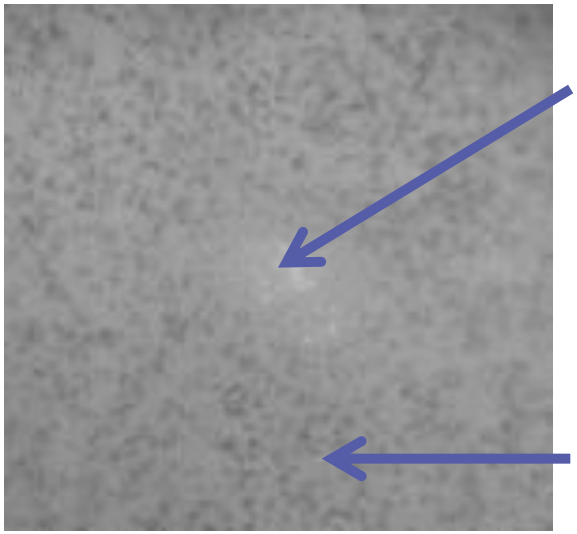

IN THE CAVITY extreme conditions on collapse 5000°C and 2000 atmospheres

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- Lignocellulose weakening
- Cell permeation
- Biocatalysis enhancement
- Surface attrition
- Surface activation
- Improved heat/mass transfer

Video courtesy of University of Twente, Netherlands.
and Shimadzu Europa GmbH, Duisburg, Germany

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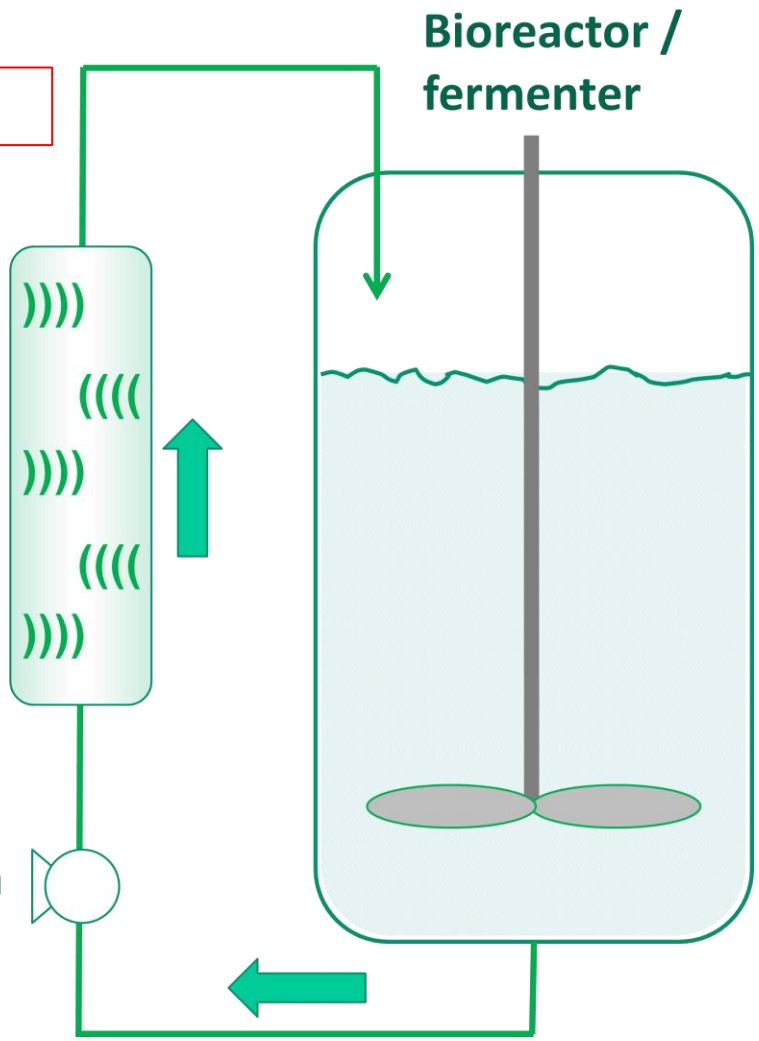
Process Operation

Temperature 0 to 70°C

50W - ~ 5 kW Depending on main reactor vol. & power reqd.

Recirculation 10 - 100 L/min



Reactor 50 - 5000 L

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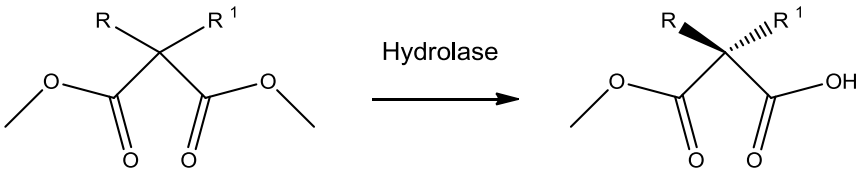
Process intensification



- Process being developed for multi-tonne, Step 2 of a 5 step process
- Process scale-up resulted in mixing problems
- Poor performance for reaction time
- **Options?** Enzyme evolution or Application of US or both

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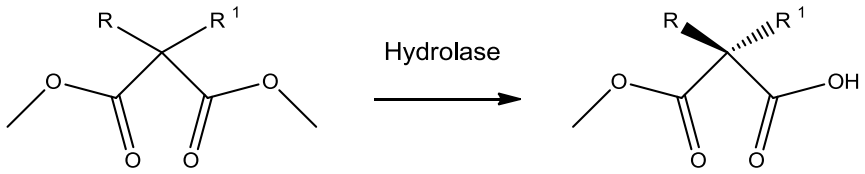
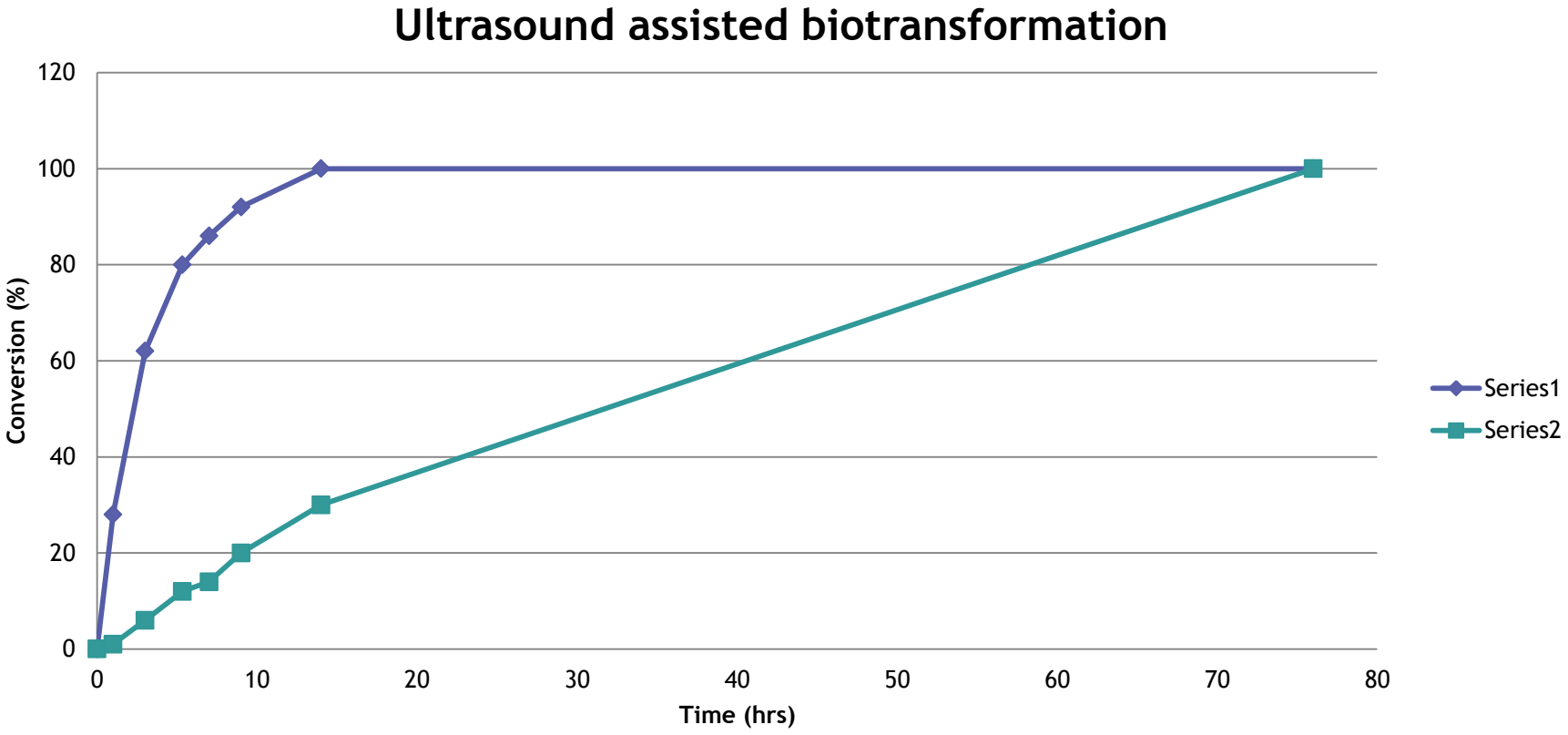
Process intensification





Current technology
Configured in reactor / recirculation systems

Pictures courtesy of Celbius

Medium out

Flanged cylindrical pipe

Transducers bonded to deliver ultrasound

Medium in

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Immobilised enzymes

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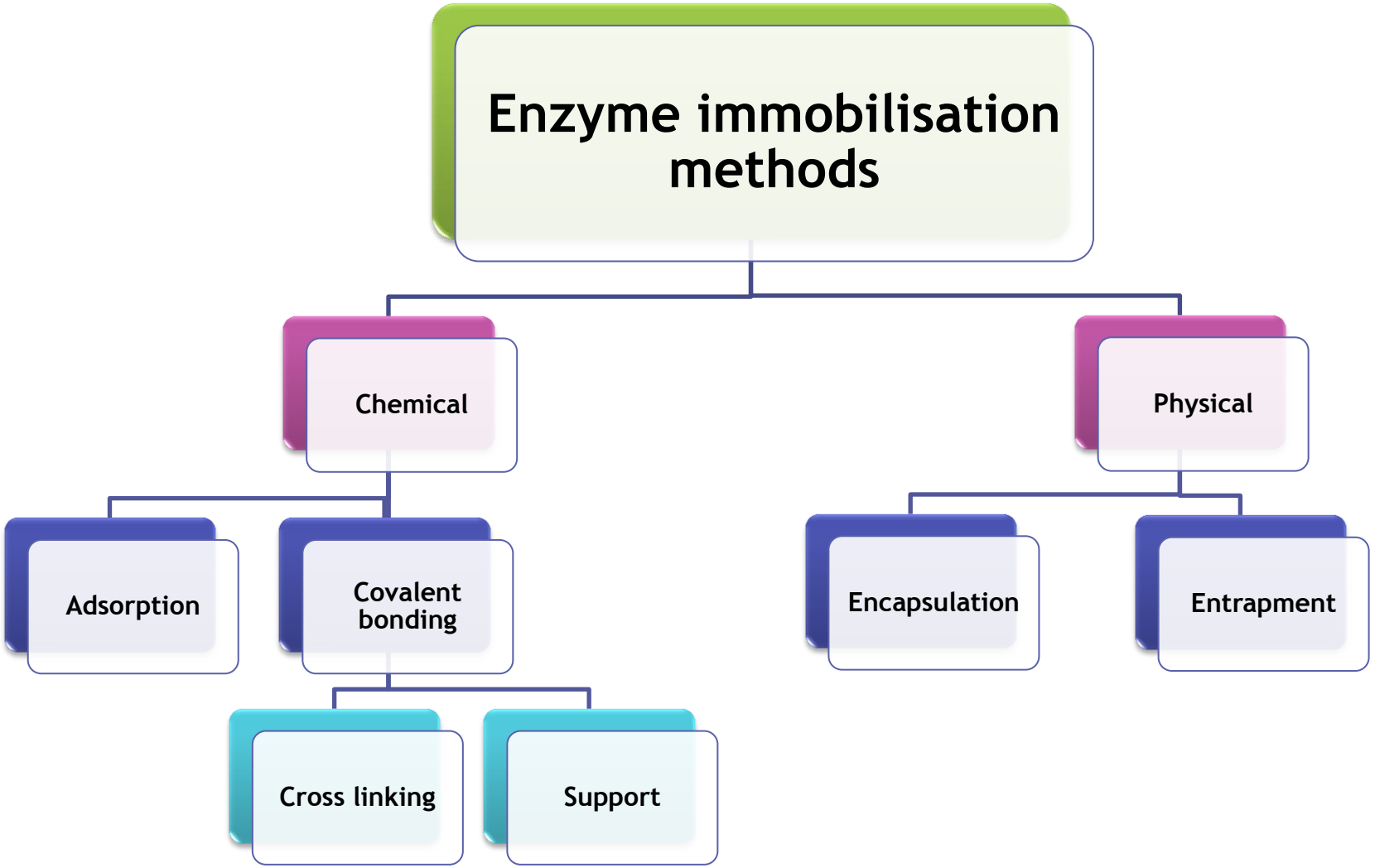
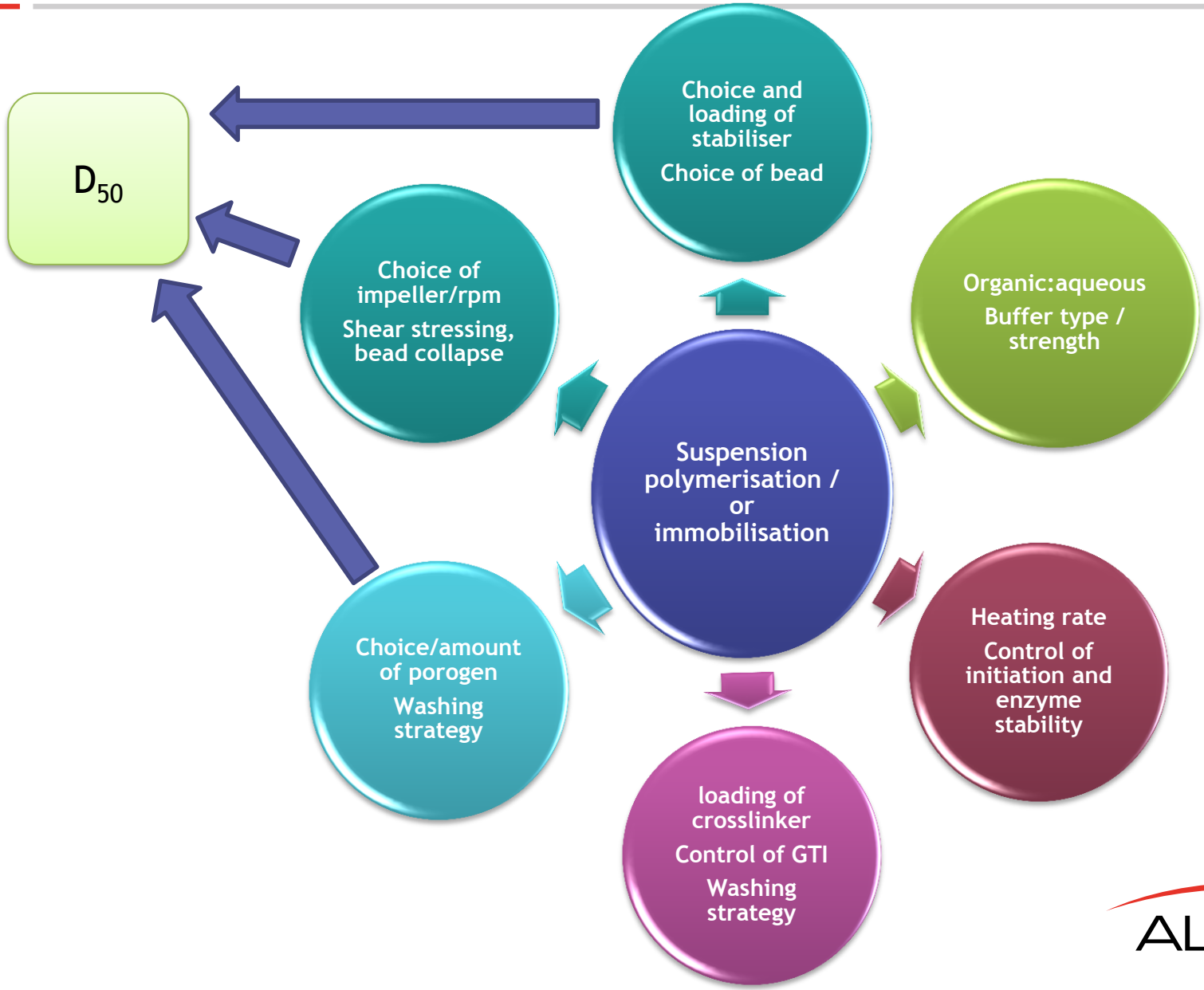
Immobilisation



Immobilisation



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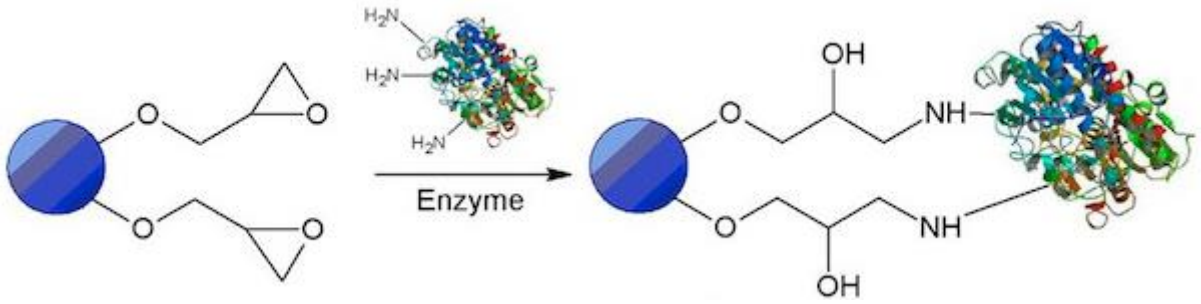
Immobilisation



- Optimal for working in biphasic systems or aqueous systems
- Resins are mechanically stable
- Flow systems or CSTR
- Multipoint covalent binding
- Form very stable covalent linkages with different protein groups

- **Enzyme classes immobilised**

Transaminase | **Carbonyl reductase** | **Hydrolase**

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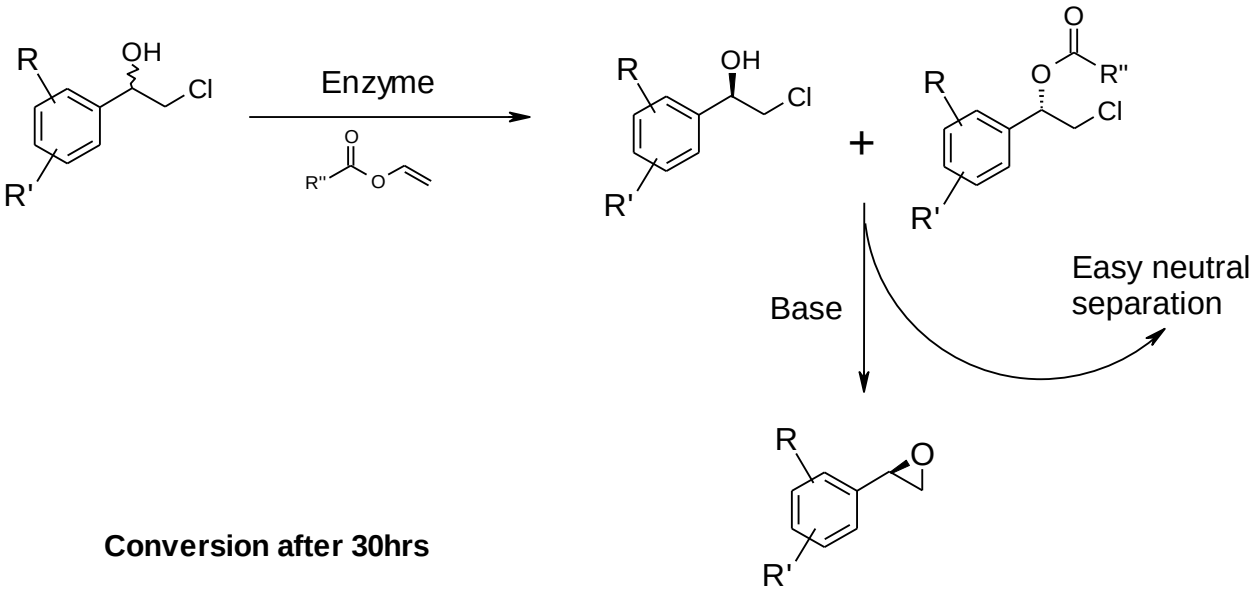
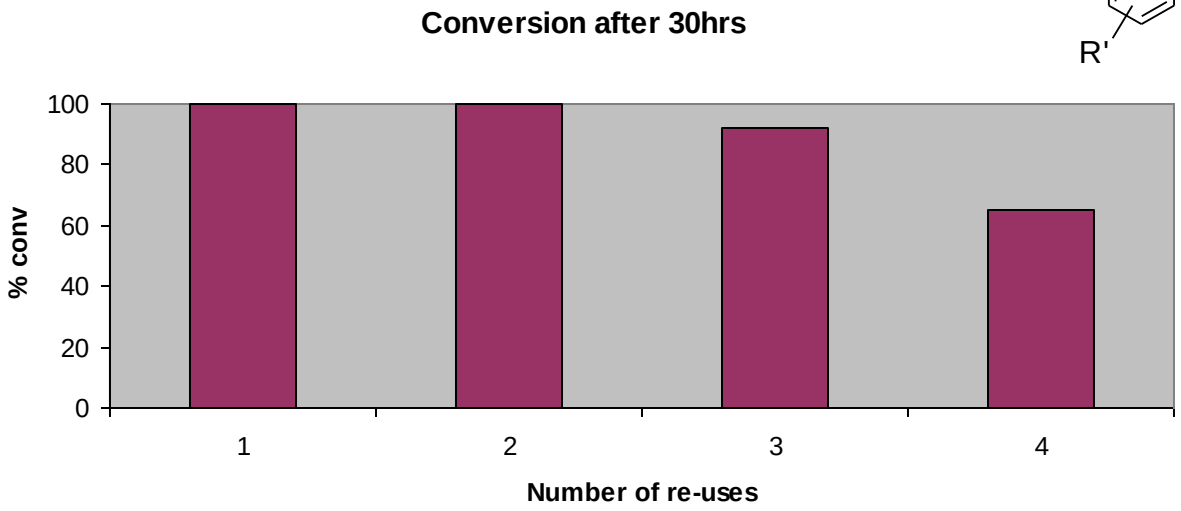
Immobilisation



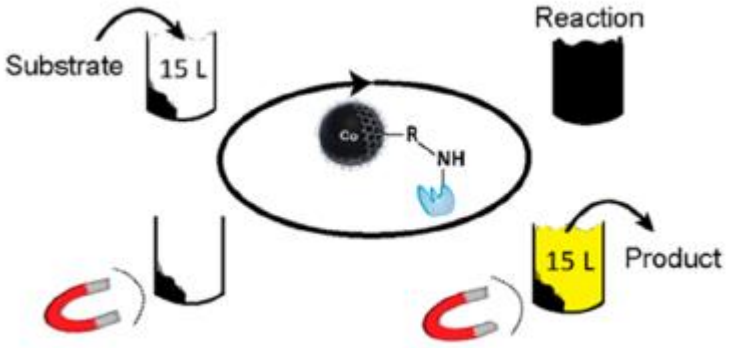


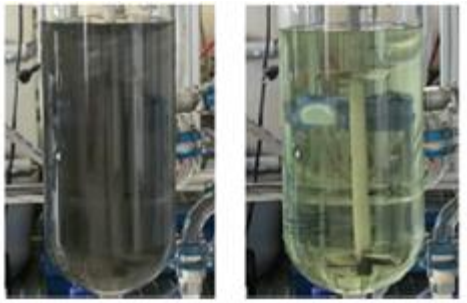
Development:
>99 % ee
Scale: 450 Kg
Manufacture

Immobilisation – Turbo Beads

- Enzymes immobilised on carbon coated nanoparticles
  - Metal coated with outer layer
  - Functionalised
  - Enzyme attached

  Ease and **quick** recovery of immobilised catalyst by use of magnet





- **Enzyme classes immobilised**

Hydrolase

Bioconjugate Chem. 2014, 25, 677−684

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API Testing – Enzyme Removal & Detection

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ORGANIC PROCESS RESEARCH & DEVELOPMENT
OPR&D
Review
pubs.acs.org/OPRD

Case Studies Illustrating a Science and Risk-Based Approach to Ensuring Drug Quality When Using Enzymes in the Manufacture of Active Pharmaceuticals Ingredients for Oral Dosage Form

Andrew S. Wells,*,† John W. Wong,‖ Peter C. Michels,¶ David A. Entwistle,‡ Keith Fandrick,■ Gregory L. Finch,⊥ Animesh Goswami,# Heewon Lee,■ Stefan Mix,● Thomas S. Moody,● Long Pang,‡ Robert K. Sato,‡ Nicholas J. Turner,□ and Timothy J. Watson▽

●Almac, Department of Biocatalysis and Isotope Chemistry, 20 Seagoe Industrial Estate, Craigavon BT63 5QD, Northern Ireland United Kingdom

Assay	Range	Comment
NanoOrange®	10 ng/mL to 10 µg/mL	Low protein-to-protein signal variability Detection not influenced by reducing agents or nucleic acids
BCA	0.5 µg/mL to 1.5 mg/mL	Samples must be read within 10 minutes Not compatible with reducing agents
Bradford	1 µg/mL to 1.5 mg/mL	Proteins precipitate over time High protein-to-protein signal variability Not compatible with detergents
Lowry	1 µg/mL to 1.5 mg/mL	Lengthy, multistep procedure Not compatible with detergents, carbohydrates or reducing agents
Absorbance at 280 nm	50 µg/mL to 2 mg/mL	High protein-to-protein signal variability Detection influenced by nucleic acids and other residues

DOI: 10.1021/acs.oprd.5b00369
Org. Process Res. Dev. 2016, 20, 594−601

	Example 3: API 3	Example 4: API 4	Example 5: API5	Example 6: API 6
Enzymatic step	Pre RSM, C-C bond formation	Pre RSM, ketone reduction	Post RSM, Alcohol oxidation	Pre RSM Ketone Reduction
Stage in synthesis	Several steps before API	Several steps before API	Penultimate step	Several steps before API
Enzyme type	Enzyme (Cells from E. coli fermentation)	KRED (liquid formulation from in E. coli fermentation)	KRED (whole cell formulation produced in Gluconobacter oxydans)	KRED (Dry powder produced from E. coli fermentation)
Control strategy	Test for total proteins, DNA, endotoxins and microbiological residues; demonstrate fate and purge; no enzyme residue specifications for API	Test for total proteins; no enzyme residue specifications for API	Test for total proteins, DNA, endotoxins and microbiological residues; demonstrate fate and purge; no enzyme residue specifications for API	Test for total proteins; in-process testing; demonstrate fate and purge; no enzyme residue specifications for API

Biocatalysis and more

Project Management

Enzyme Discovery

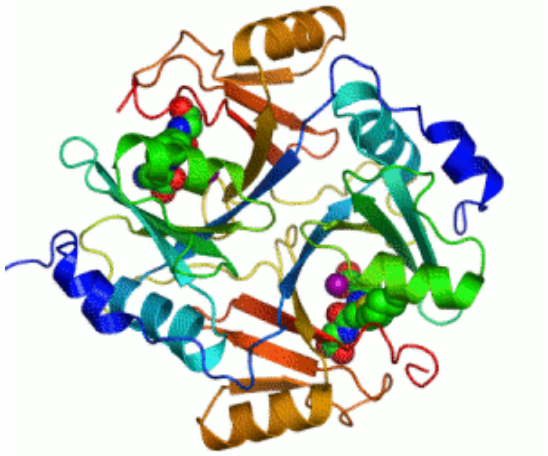
Enzyme Evolution

ALMAC Biocatalysis

Quality Assurance

Early & late phase chemical development

Analytical Expertise



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Prof. Tom Moody

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