selectAZyme^{TM} Biotransformation and Scale up

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Team Leader Biocatalysis, Almac

April 2013

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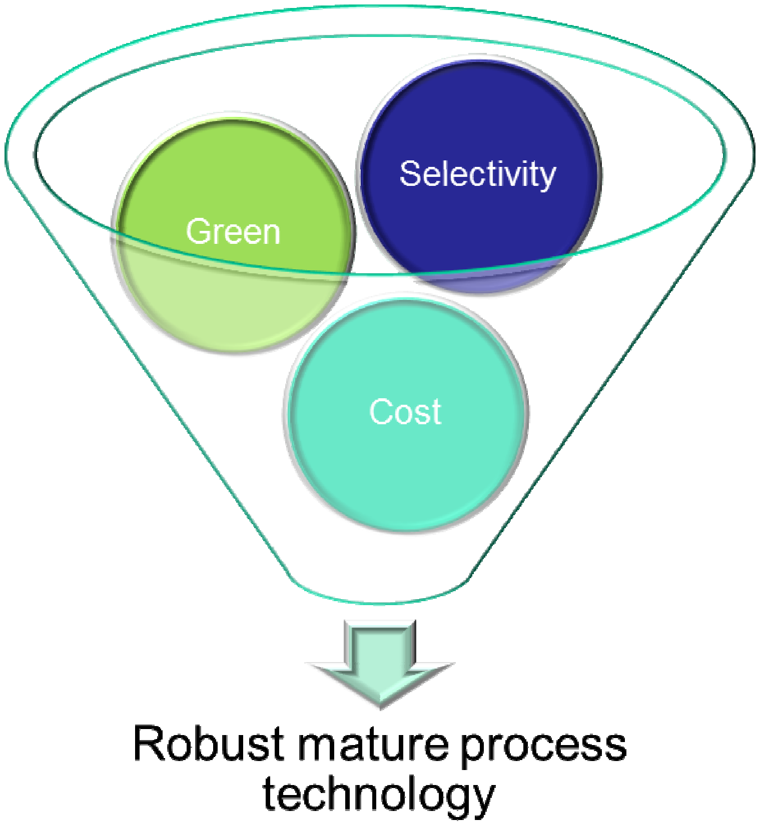
Contents



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Almac Group Global presence

- EU headquarters, UK
- US headquarter, Pennsylvania USA
- Asia (Japan and Singapore)
- 48 Distribution Depots in over 41 countries worldwide
- Partnership in Asia
- Financially stable and privately owned
- 30 years experience serving 600+ clients
- Employs approx. 3400 staff worldwide
- Asset investment in excess of $740M

History

1968	Galen founded by Sir Allen McClay
1997	Galen floated on stock market
2000	Galen acquired by large pharma company
2001	Sir Allen McClay retires
2002 2003	Sir Allen McClay comes out of retirement and acquires Galen service divisions

2004 2006	Galen's service divisions are integrated
2006	Almac Group globally launched
2008	Almac Group grows rapidly
2009	Almac Group achieves foundation status McClay FOUNDATION
2010	North American HQ opened

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UKHQ

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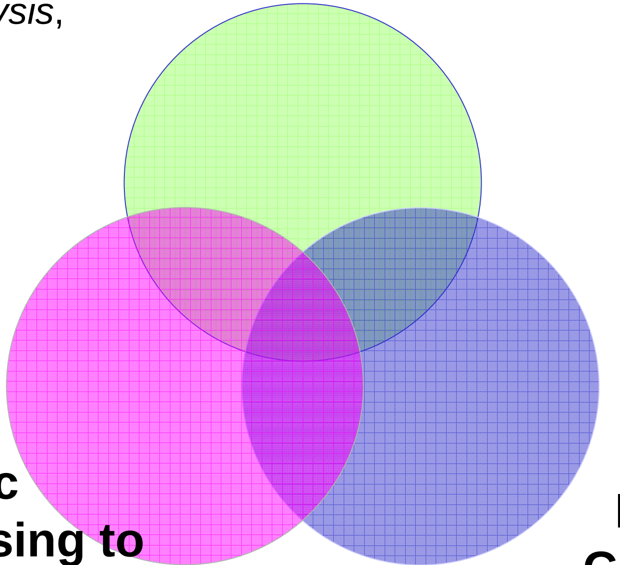
Group

Biocatalysis

***Moody, T.S.**, et al.;*
Adv. Syn. Catalysis,
2012



Academic
Molecular sensing to
Colorimetric assay design

***Moody, T.S.** et al.;*
Photochem. Photobiol. Sci.
2012, **11**

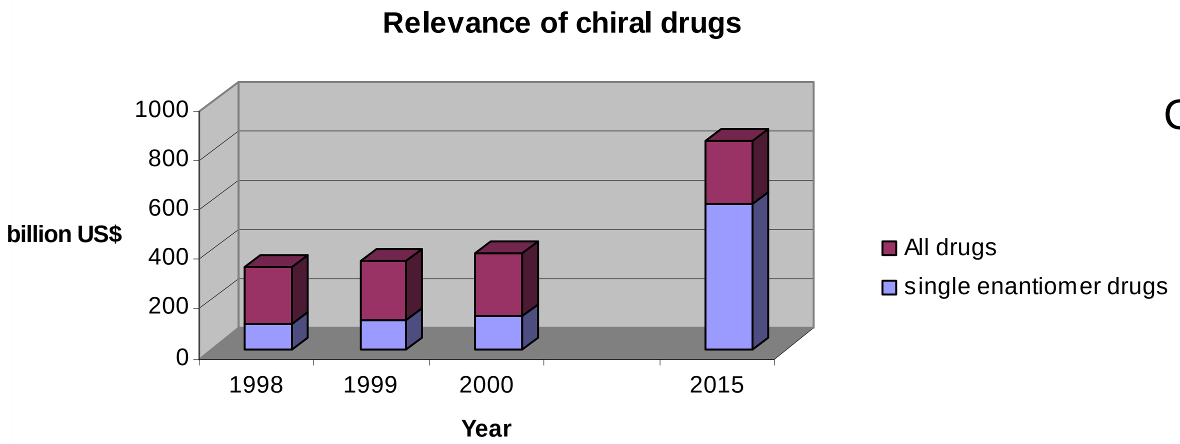
Isotope
Chemistry

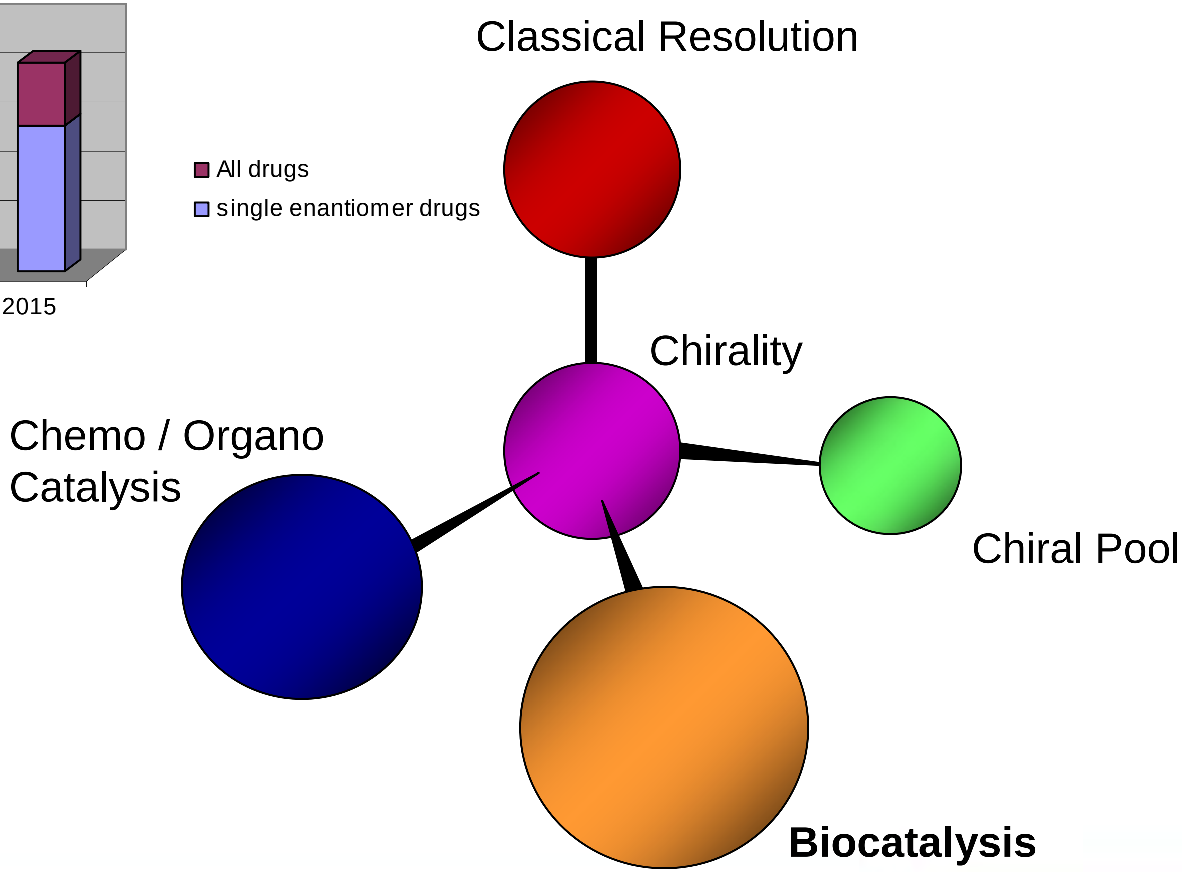
***Moody, T.S**. et al.; Journal of Labelled Compounds and Radiopharmaceuticals*, **2011**, 54(7), 387-398

Dedicated group of 28 people

Molecular
Microbiology
Chemisty
Enzymology
Isotope chemistry
Chemical engineering
Analytical

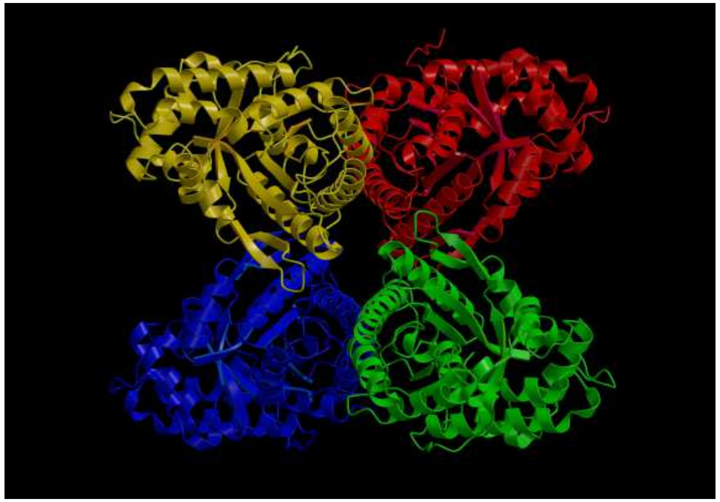
Exploring Manufacturing Routes To Chiral Building Blocks:





The trend for new chiral pharmaceutical reagents is continuing. In 2000, 35% of intermediates were chiral and this number is expected to increase to 80% by 2015

What is a Biocatalyst??



A 'biocatalyst' is an enzyme or micro-organism that can be used as a catalyst for the synthesis of fine chemicals or pharmaceuticals

The active constituent of a biocatalyst is always an enzyme

Enzymes are polypeptides that typically have MW's 20KD-300 KD.

• Enzymes can be stable aqueous buffers, organic solvents and at elevated temperatures.

•Enzymes activity is defined in Units (U) per milligram=U/mg.

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Biocatalysis pros and cons

- Chemo- and regio-selectivity not seen with chemical reagents
- Non-hazardous reagents which work in water
- Unique activities
- Leave no harmful residues in effluent streams
- Reduce the number of synthetic steps

- Cheap!

Unfamiliar reaction conditions for traditional organic chemists

MAJOR HURDLE!!

Modern Biocatalysis → Mainstream Technology??

- Biocatalysis must be a first-line option, not an alternative!!
- Must not be tried after everything else has failed.
- Considered even at discovery end!!

Four trends are helping:

Greener Processes

Wide Availability of Robust Enzymes

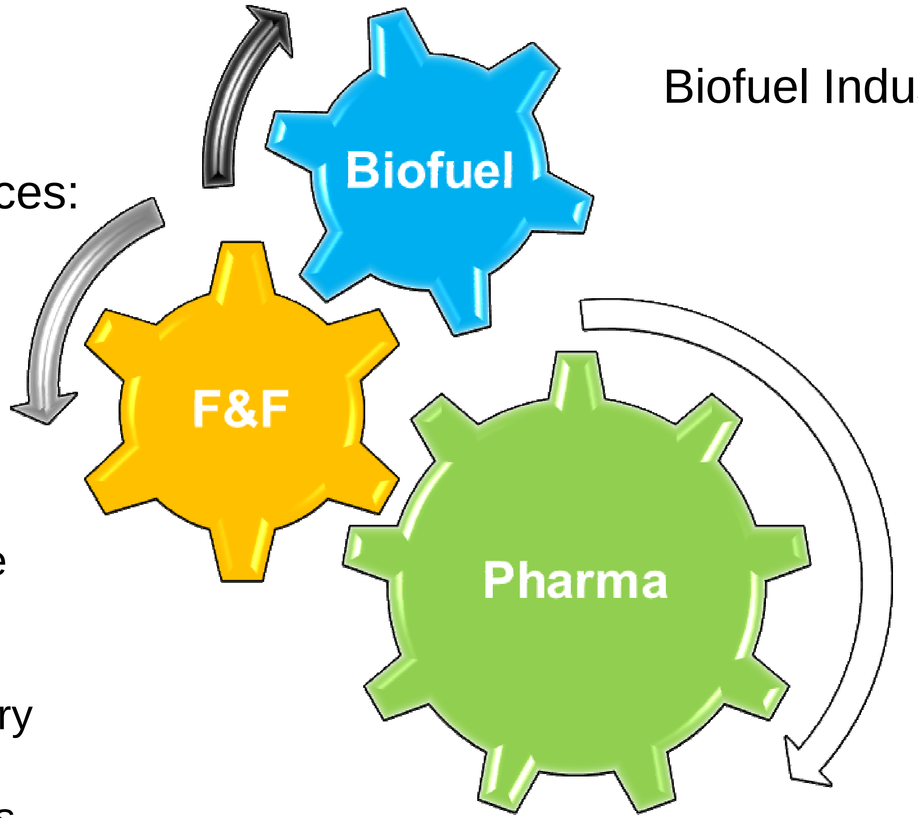
Process Intensification, Reduction in Costs

Nothing helps like SUCCESS!!

Technology

Biofuel Industry: Biodiesel

Flavour and fragrances:
Woody acetate



High ee – Typical >99%ee
Chemo- & Regioselective
Minimise PG's chemistry
No expensive metal recovery
Mild conditions
Excellent space-time yields

Green and Renewable

Pharma industry:
Med Chem → Manufacturing

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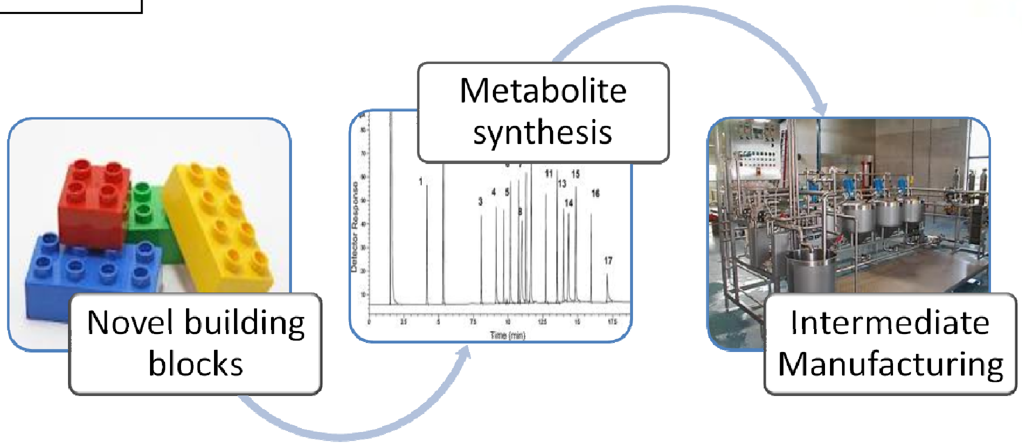
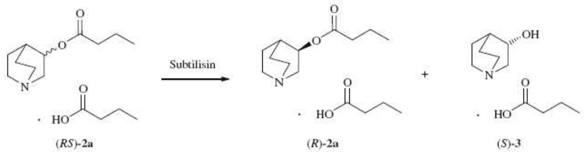
Technology



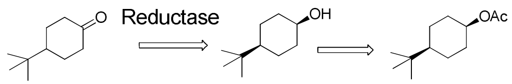
Pharma: Bioresolution

- 300 g/L



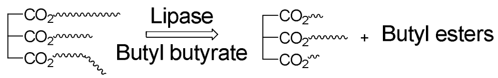
F&F: woody acetate

- 500 g/L



Biofuels: biodiesel

- 700 g/L



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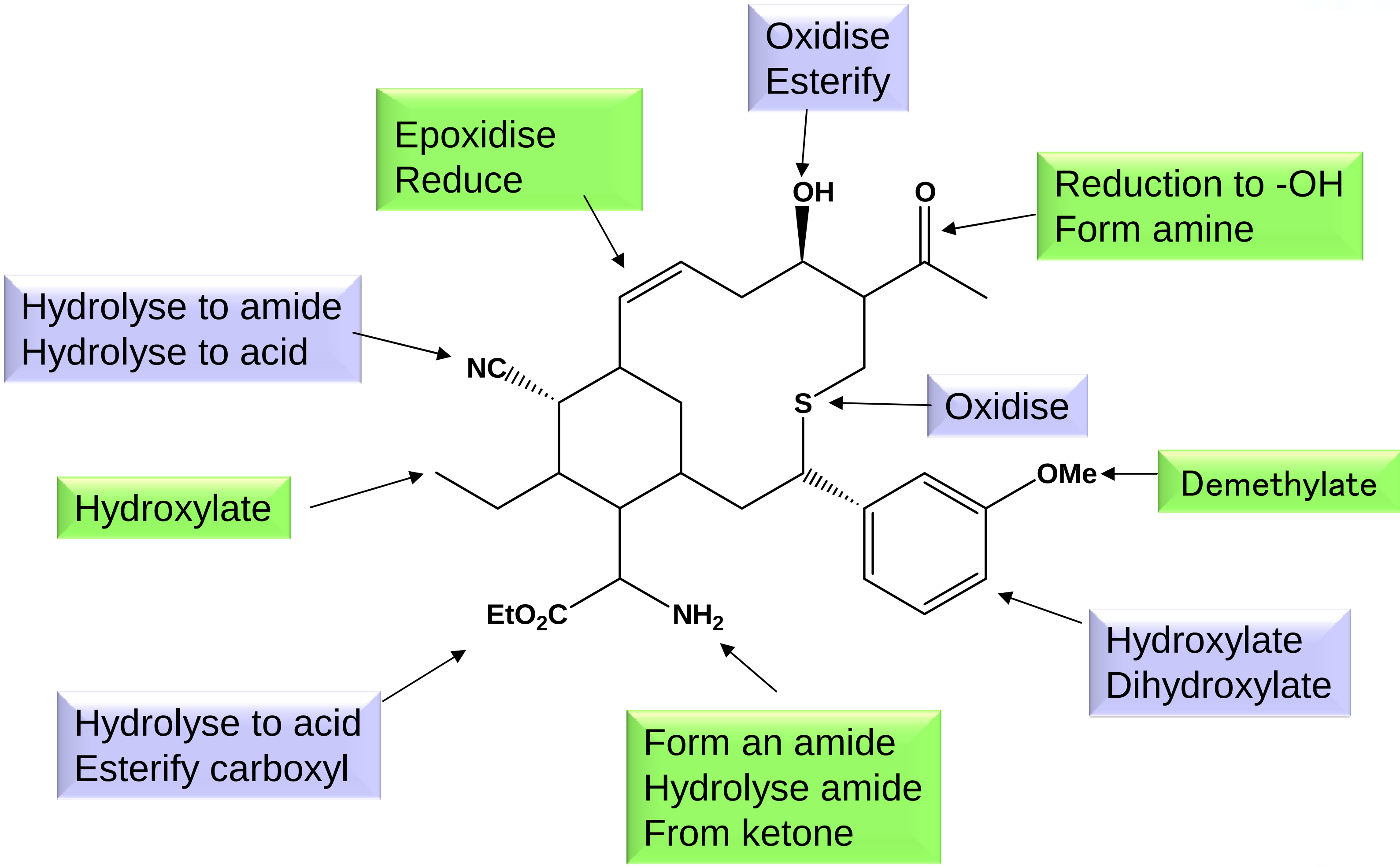
Technology



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Technology

Enzyme Platforms	Product classes
Aldolases	Alcohols, Diols, Amino alcohols
Proteases	Peptides, Amines, Carboxyesters
Lipases and Esterases	Alcohols, Esters, Carboxylic acids
Ammonia lyases	Amino acids
Hydantoinases, Carbamoylases, Racemases	Amino acids
Amidases	Amino acids, Amides
Acylases	Amino acids, N-Acetyl-Amino acids
Hydroxynitrile lyases	Cyanohydrins
Omega-Transaminases	Amines
Oxidases	Alcohols, Aldehydes, Carboxylic acids
Dehydrogenases (alcohol & amino acid)	Alcohols and Amino acids
Nitrilases	Carboxylic acids, Nitriles
Nitrile hydratases	Amides, Nitriles
Monooxygenases (P450, Baeyer-Villiger)	Alcohols, Sulfoxides
Epoxide hydrolases	Epoxides, Diols

Enzyme Partner with

DSM
BRIGHT SCIENCE. BRIGHTER LIVING.

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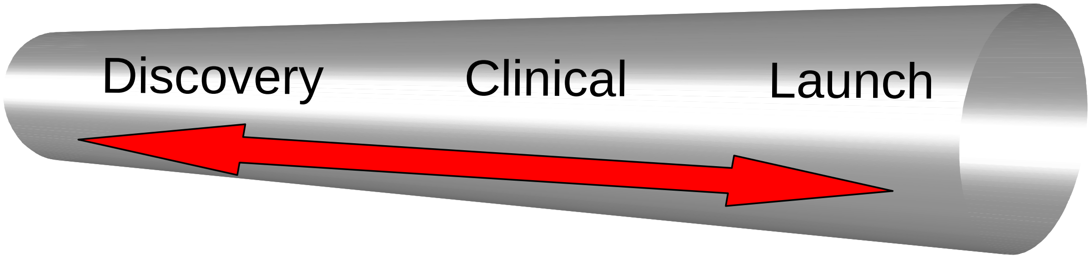
Technology



mg-10 g | 100 g-1 kg | 1 kg-100 kg | >100 kg

"Off-the-Shelf" biocatalysts

Process Dev

Speed

COST

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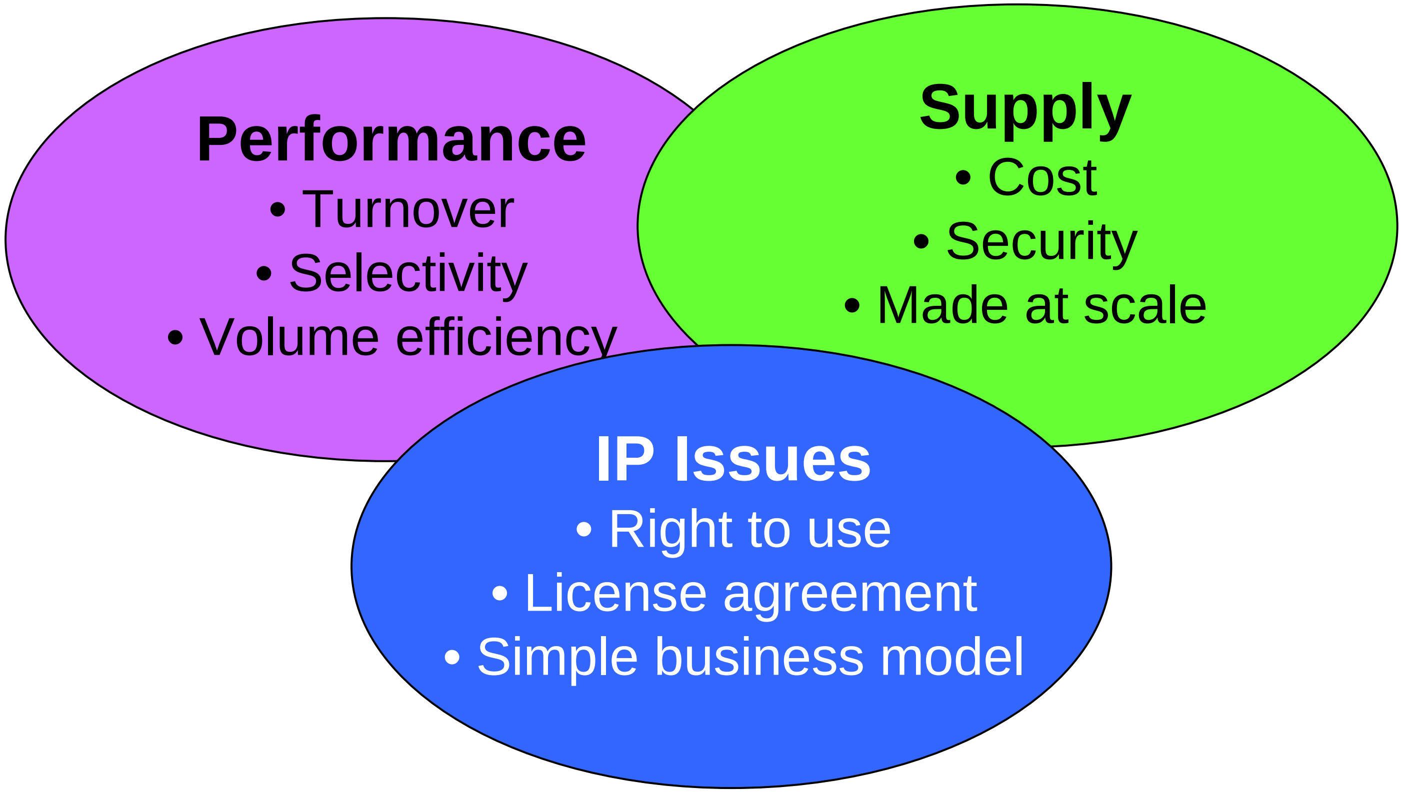
A ***commercially viable*** biocatalyst is defined by…..



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CASE STUDIES

Hydrolase Enzymes

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Protic ionic liquids

Tetrahedron: *Asymmetry* 20 (2009) 2112–2116

Contents lists available at ScienceDirect

Tetrahedron: *Asymmetry*

ELSEVIER

journal homepage: www.elsevier.com/locate/tetasy

Simple one-pot process for the bioresolution of tertiary amino ester protic ionic liquids using subtilisin

Maude Brossat *, Thomas S. Moody *, Stephen J. C. Taylor, Jonathan W. Wiffen

Biocatalysis Group, Almac Sciences, David Keir Building, Stanmillis Road, Belfast BT9 5AG, Northern Ireland

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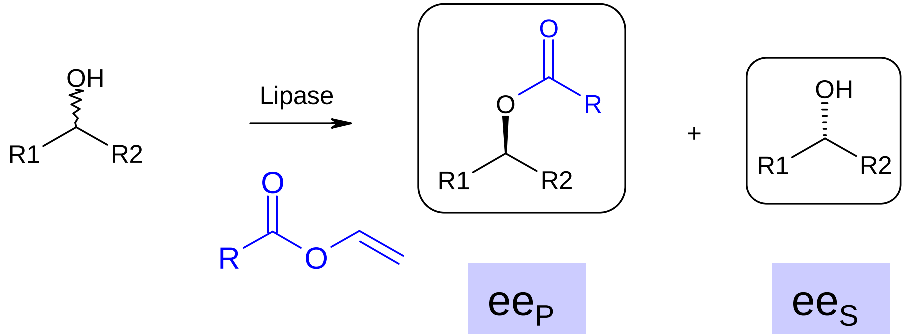
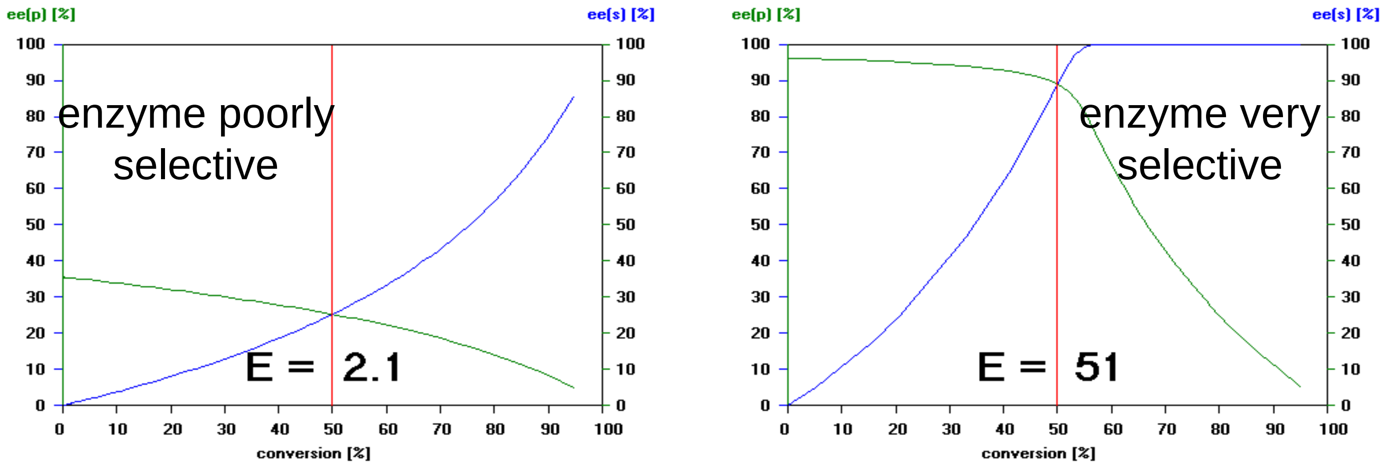
Kinetic Resolution



Two important concepts: **ee** and ***E*** (enantiomeric ratio)

$$E = \frac{\ln\left(\dfrac{1-ee_S}{1+(ee_S/ee_P)}\right)}{\ln\left(\dfrac{1+ee_S}{1+(ee_S/ee_P)}\right)}$$



For a viable synthetic process: *E* > 30

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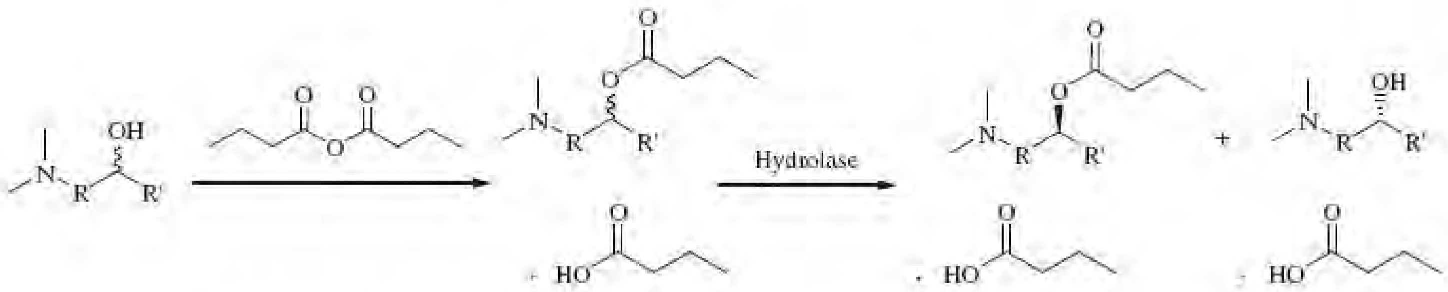
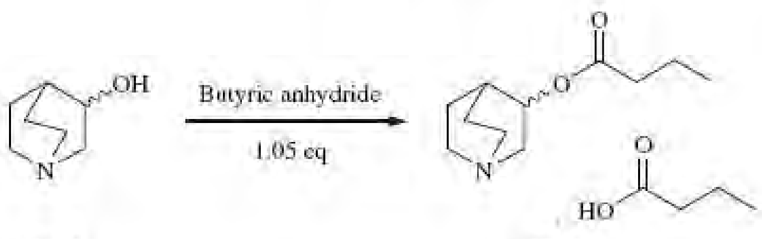
Protic ionic liquids



Scheme 1. Simple one pot process for the bioresolution of tertiary amino ester protic ionic liquids.



(*RS*)-**1** (*RS*)-**2a**

Scheme 2. Preparation of protic ionic liquid (*RS*)-**2a** from (*RS*)-**1**.

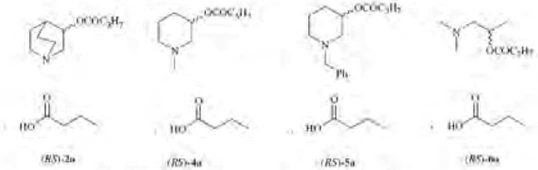


Figure 1. Tertiary amino ester protic ionic liquids (*RS*)-**2a**, (*RS*)-**4a** to (*RS*)-**6a**.

Table 1
Subtilisin-catalyzed hydrolysis of (*RS*)-**2a**, (*RS*)-**4a** to (*RS*)-**6a**

Entry	Substrate	ee substrate[a] (%)	ee product[b] (%)	Conv.[c] (%)	*E*[c]
1	(*RS*)-**2a**	97.0	90.4	52	79
2	(*RS*)-**4a**	28.9	89.2	25	23
3	(*RS*)-**5a**	48.3	3.7	92	1.5
4	(*RS*)-**6a**	40.7	73.8	36	10

[a] Ee determined by extraction of the ester with hexane and basic hydrolysis in MeOH using 2 M aqueous NaOH followed by HPLC using CHIRALCEL AD-H column.
[b] Ee determined by HPLC using CHIRALCEL AD-H column.
[c] Calculated from $ee_{alcohol}$ and ee_{ester} after 16 h reaction.

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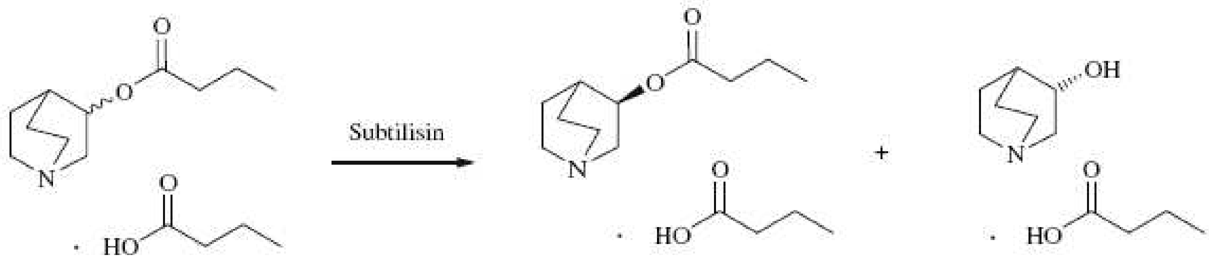
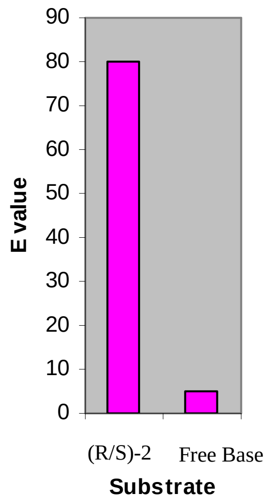
Protic ionic liquids



(RS)-2a (R)-2a (S)-3



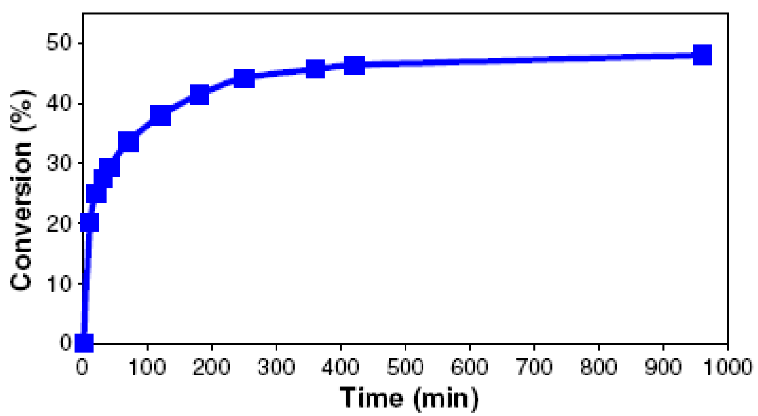
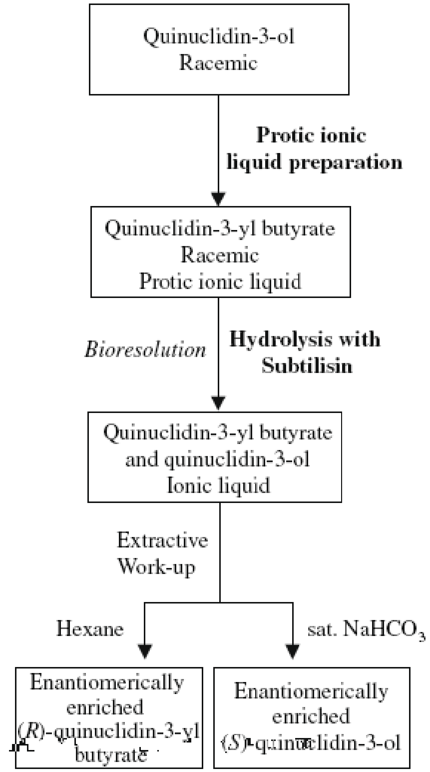


Figure 3. Reaction profile for the bioresolution of (*RS*)-**2a**.



Moody, T.S., *et al.*, *Tetrahedron Asymm.*, 20, *18*, 2112-2116 **2009**

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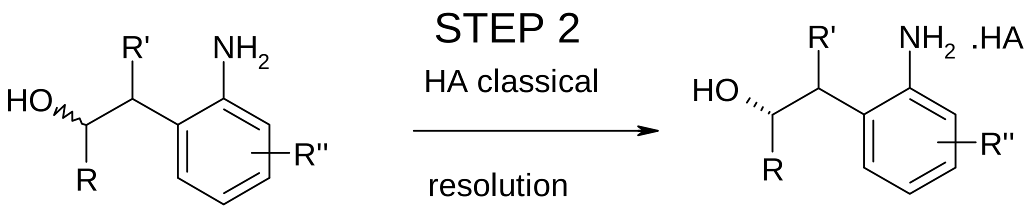
5 Step GMP process

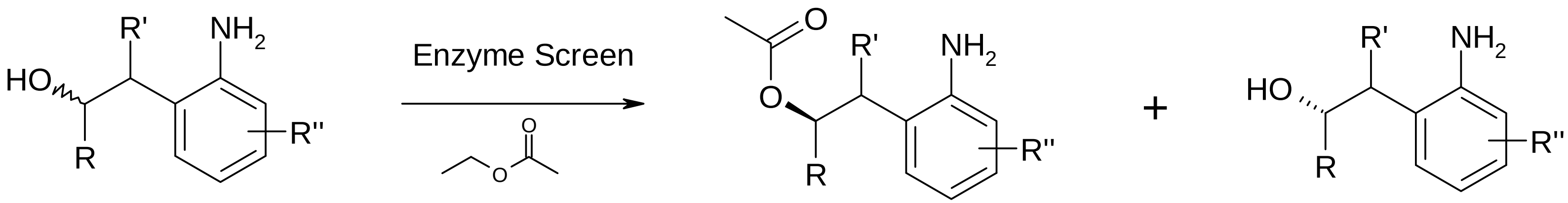


20% yield

Slow filtration

Isolation problems

Parallel Biocatalysis
Enzyme Selection (240 RXNs)



- Process fit to scale
- Increase yield to 80%
- Reduction in manufacture time for Step 2
- No change in manufacture deadlines

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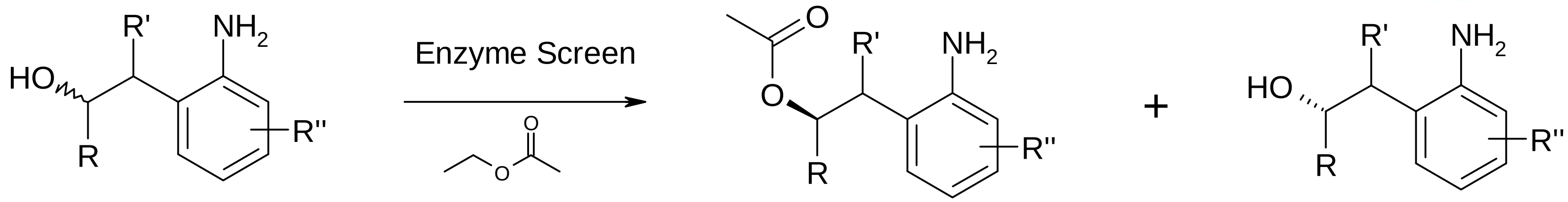
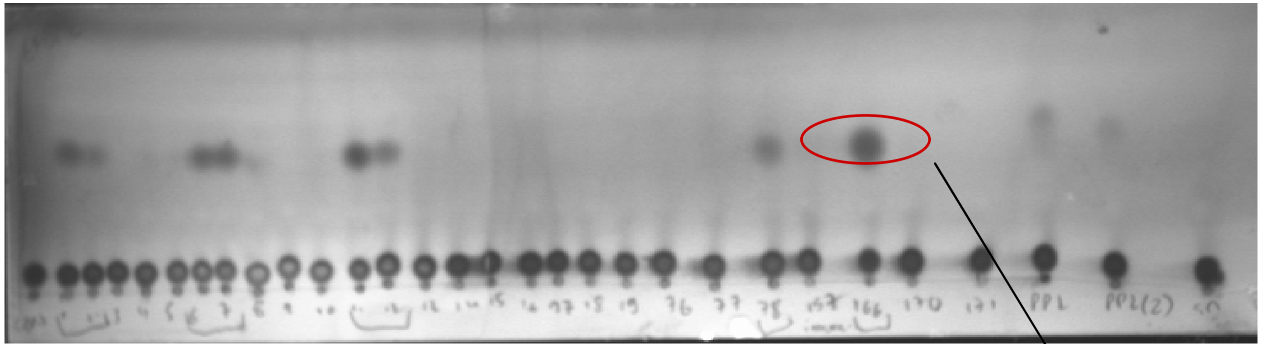
5 Step GMP process





Enzyme	*ee* (SM)	*ee* (product)	E value
1	16.6	91.5	28
2	3.9	76.3	7.6
6	15.4	88.9	19
7	16.2	91.8	28
11	23	92.6	34
12	7.6	80.4	9.7
78	6.6	46.4	2.9
166	91.8	96.7	>200
PPL1	1.3	44.6	2.7
PPL2	0.3	30.8	2.7

166 enzyme selected

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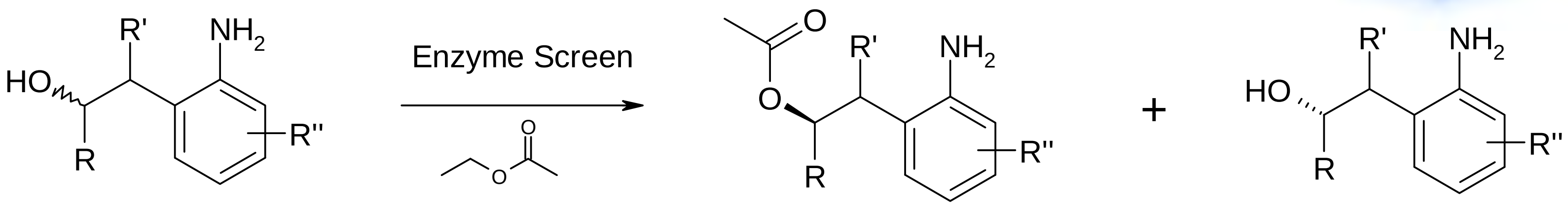
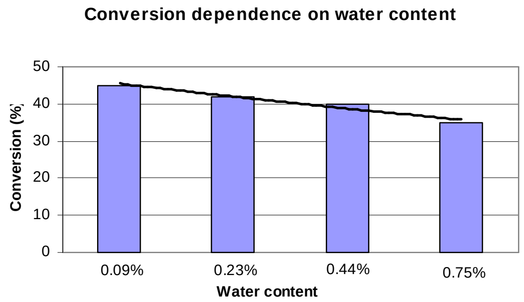
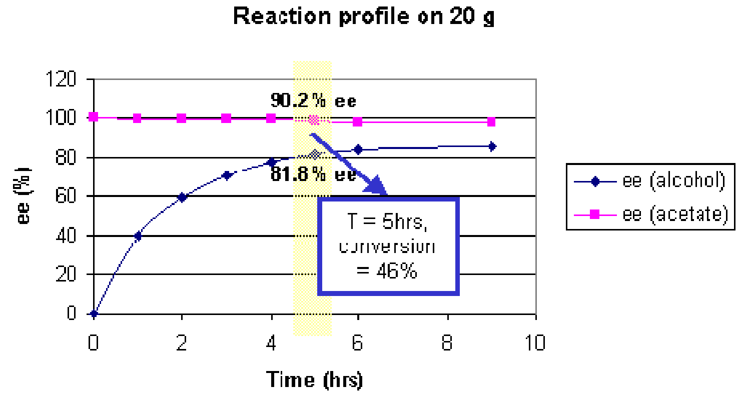
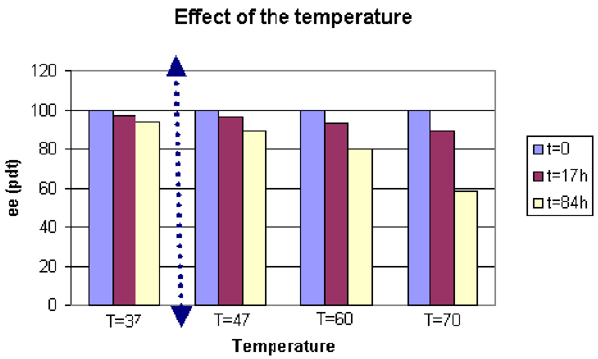
5 Step GMP process









Critical parameters

- Water content
- Enzyme loading
- Temperature
- Time studies
- Acyl donor choice
- Solvent choice
- Stirrer speeds

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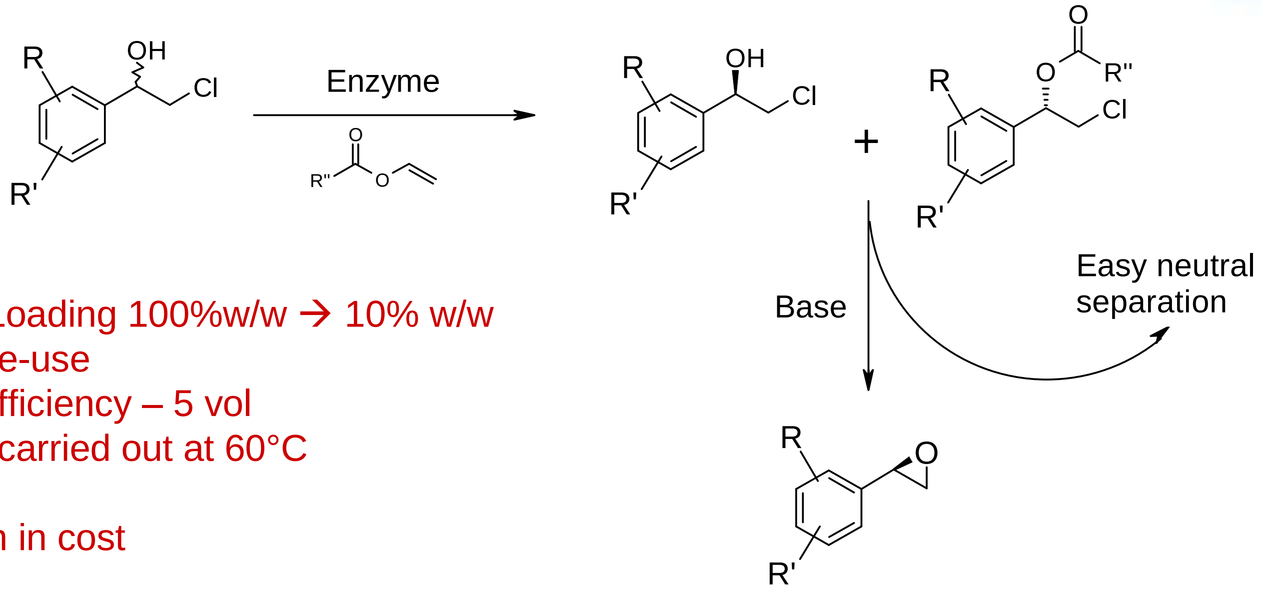
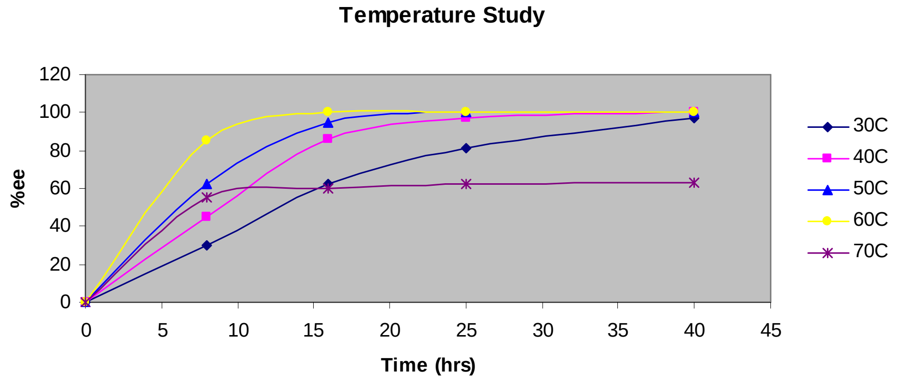
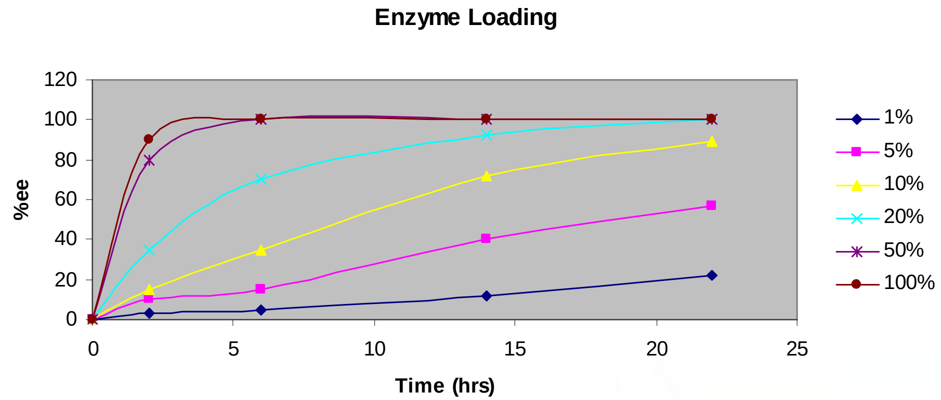
Tonne Manufacture



- Enzyme Loading 100%w/w → 10% w/w
- Enzyme re-use
- Volume efficiency – 5 vol
- Reaction carried out at 60°C

- Reduction in cost





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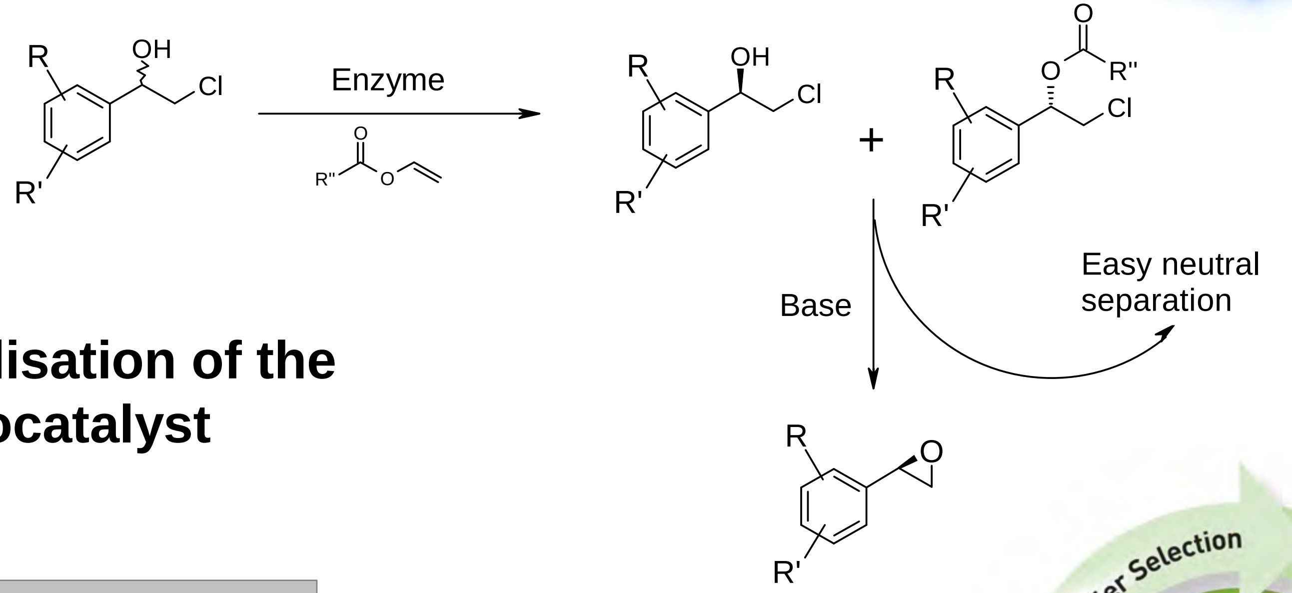
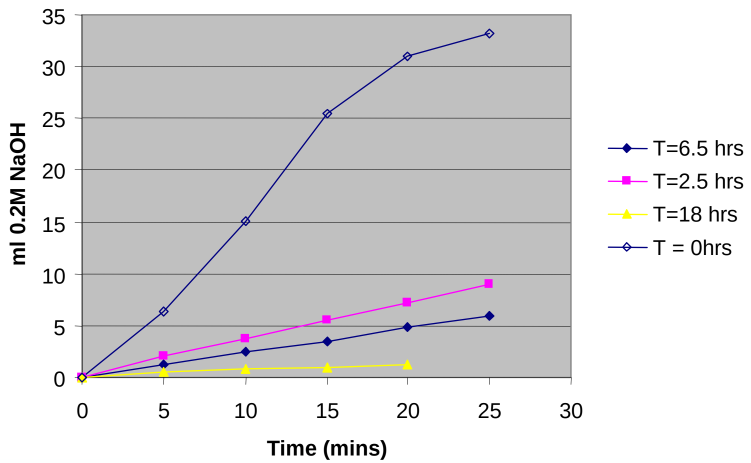
Immobilisation



Immobilisation of the Biocatalyst



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CASE STUDIES

Purification

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Organic Process Research & Development **2009**, *13*, 706–709

Development of an Acid-Washable Tag for the Separation of Enantiomers from Bioresolutions

Maude Brossat, Thomas S. Moody,* Florian de Nanteuil, Stephen J. C. Taylor, and Fatima Vaughan
Biocatalysis Group, Almac Sciences, Almac House, 20 Seagoe Industrial Estate, Craigavon BT63 5QD, Northern Ireland

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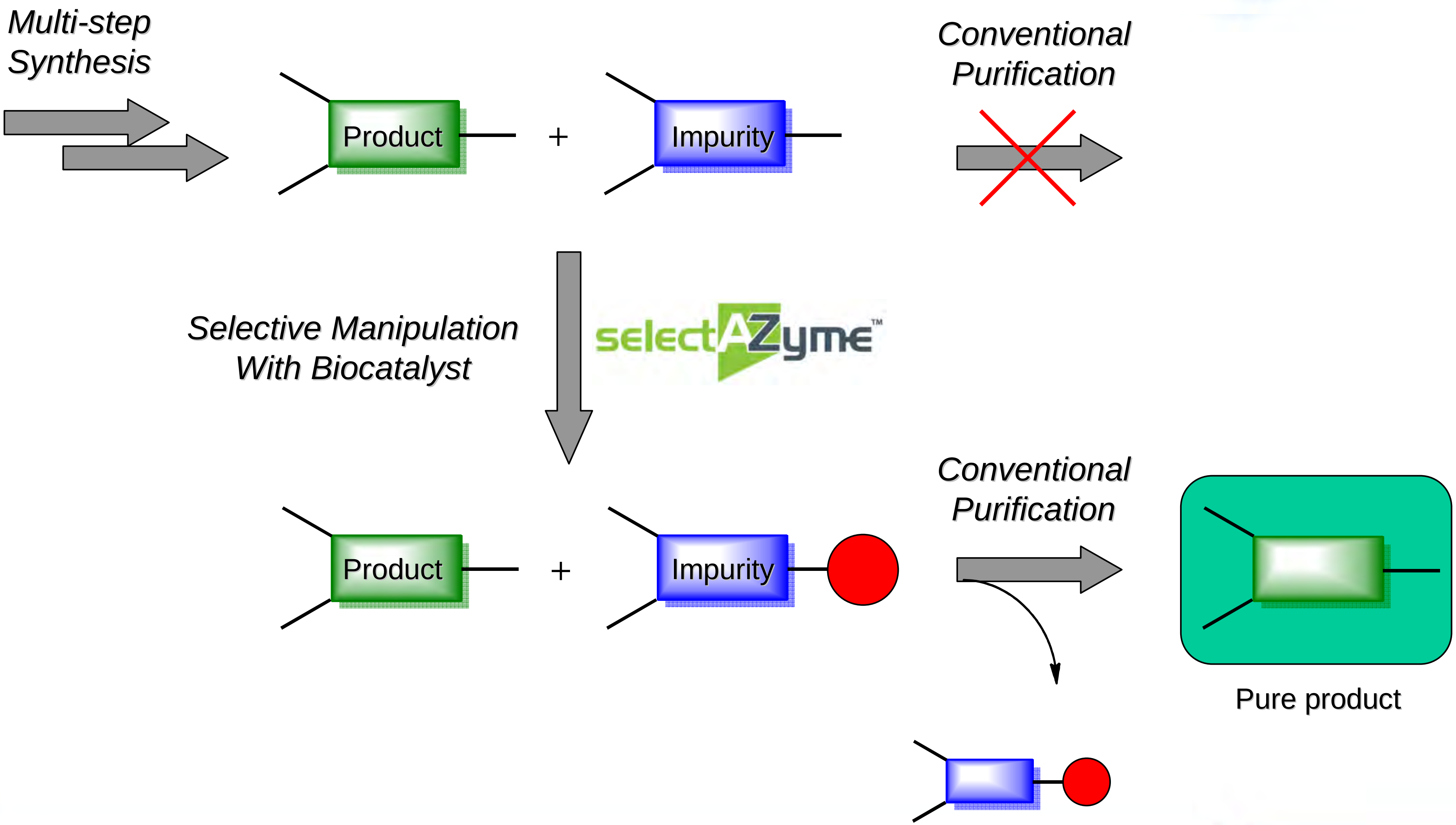
Impurity Removal



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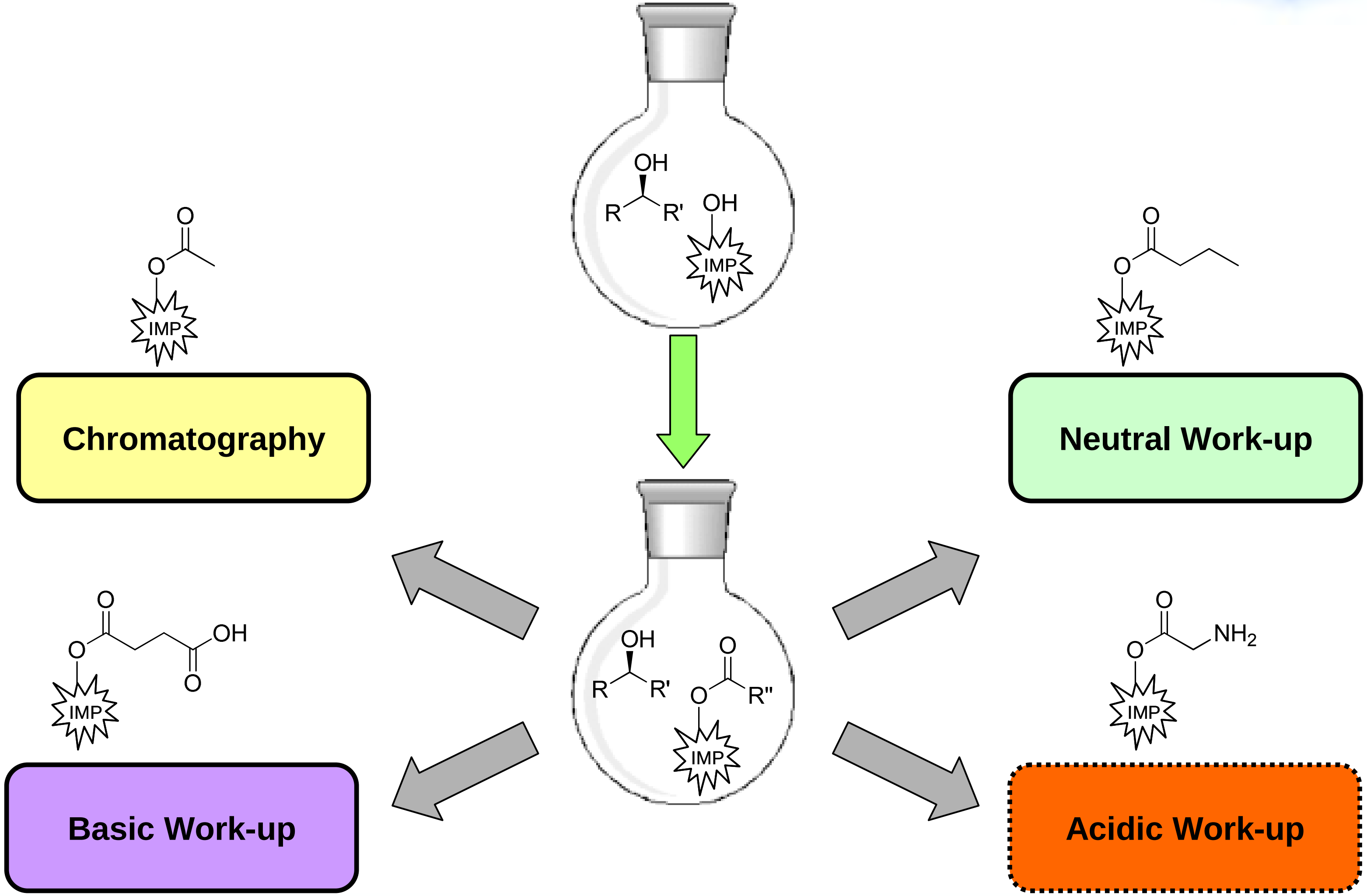
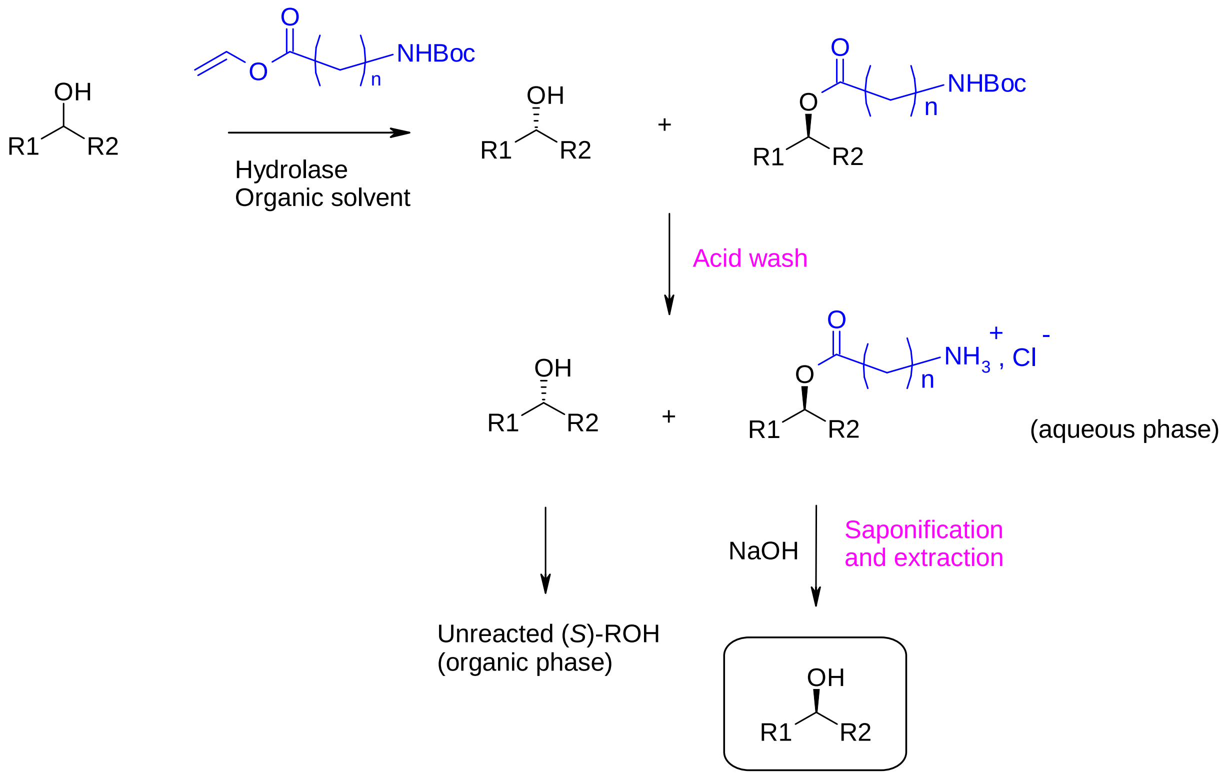
Purification



Purification



Brossat, M.; Moody, T. S.; de Nanteuil, F.; Taylor, S. J. C.; Vaughan, F. *Org. Process. Res. Dev.* ***2009****, Vol. 13, pg 706*

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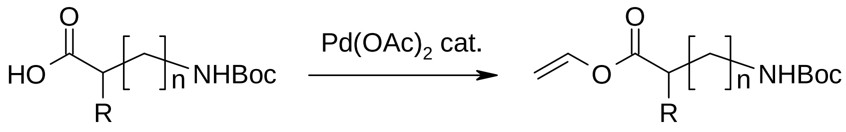
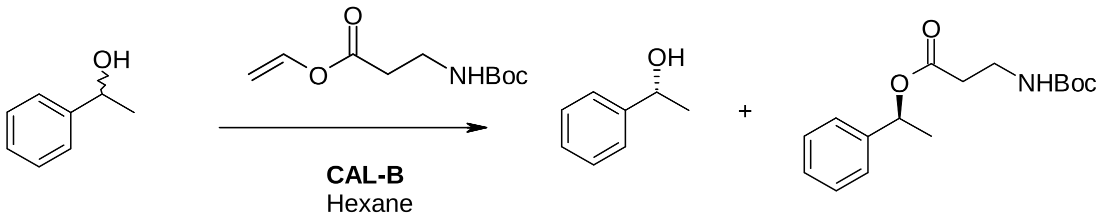
Purification



Synthesis of the vinyl ester amino acids

1, R=H, n=0
2, R=H, n=1
3, R=CH(CH_3)$_2$, n=0

4, R=H, n=0
5, R=H, n=1
6, R=CH(CH_3)$_2$, n=0



Bioresolution of phenylethanol using alanine and CALB

16 hrs   ee = 92.7%   ee = 96.7%   **E > 200**

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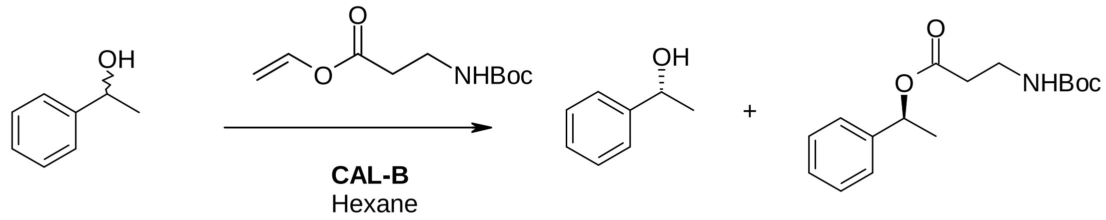
Purification

Solvent screen

Solvent	(S)-Alcohol (% ee)	(R)-Ester (% ee)	Conversion %	E
MTBE	98.6	93.6	51	170
Hexane	92.7	96.7	49	> 200
Heptane	100	94.3	52	> 200
THF	58.6	100	37	> 200
MeTHF	68.5	96.8	41	134
Toluene	99.9	84.4	54	90
Acetonitrile	98.8	100	5	> 200
Acetone	38.9	99	28	> 200
Cyclohexane	99.6	89.8	53	141



Bioresolution of phenylethanol using alanine and CALB

25%w/w CALB in hexane at 30°C

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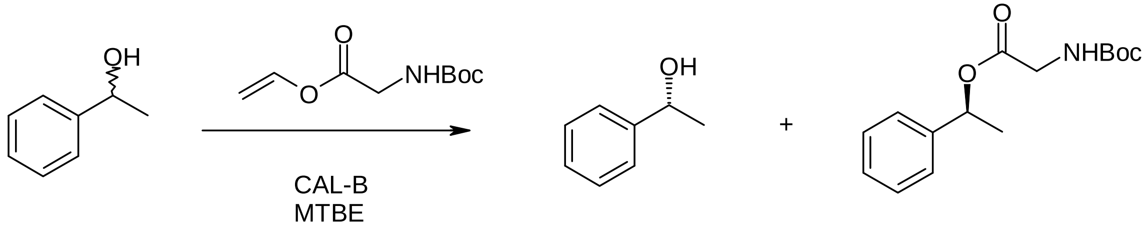
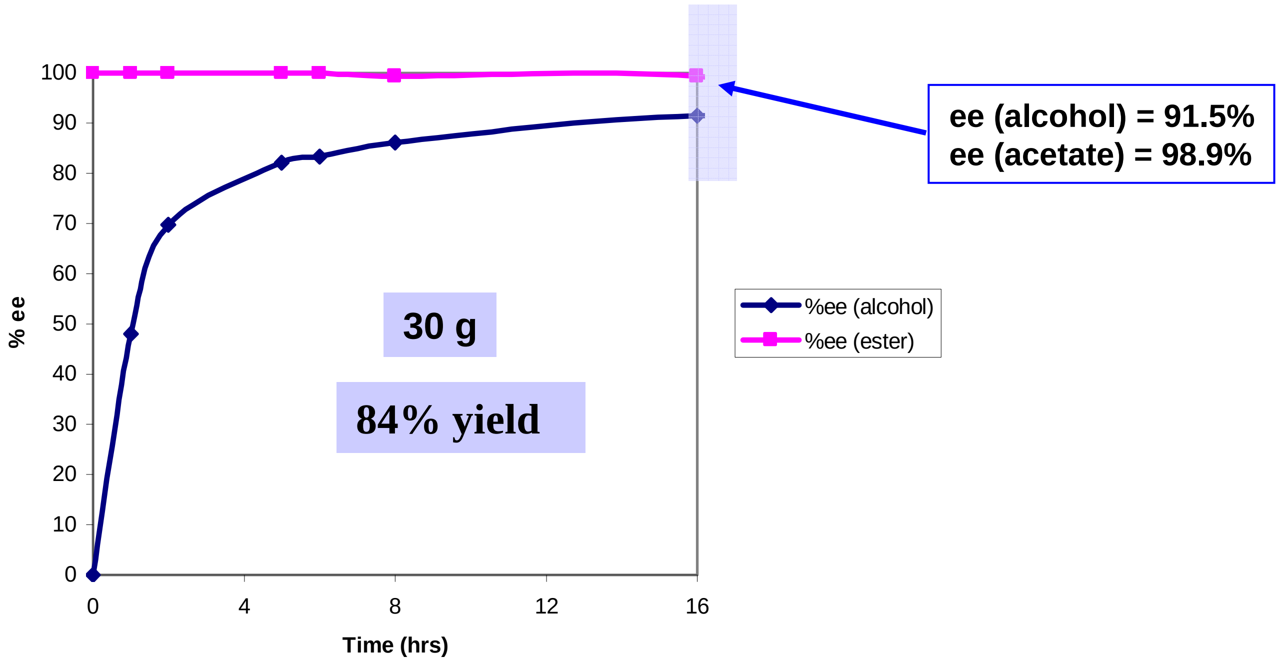
Purification





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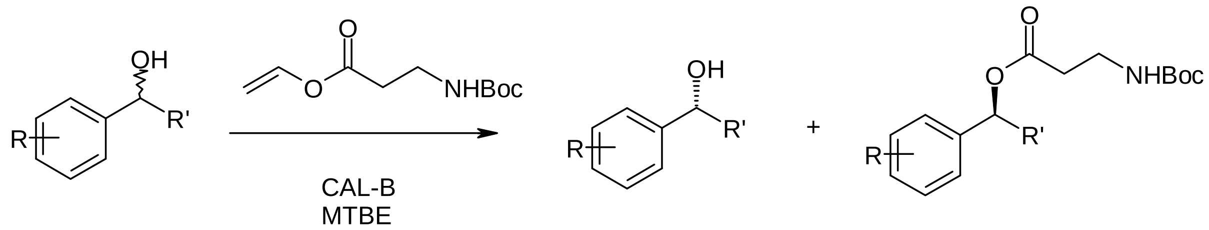
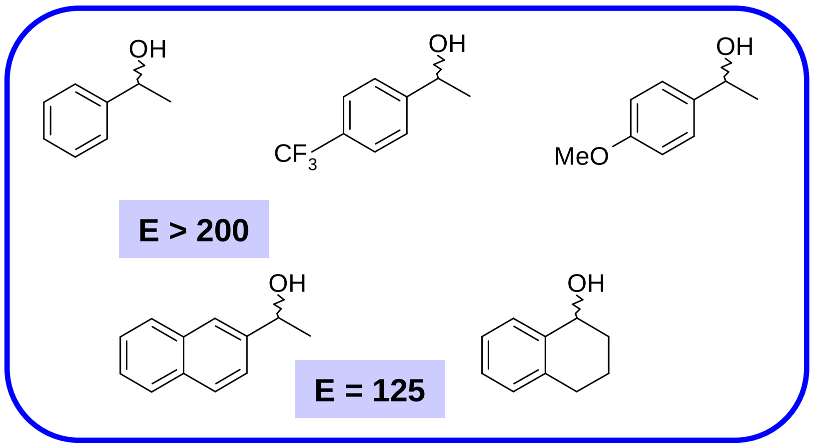
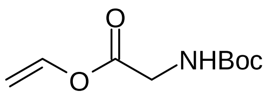
Purification

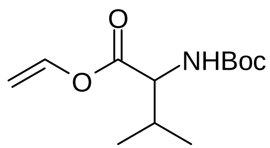
Amino acids screening:





Amino acids screening:





E = 105 **E > 200**

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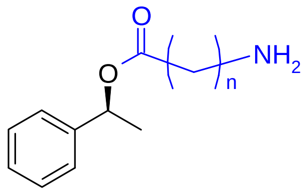
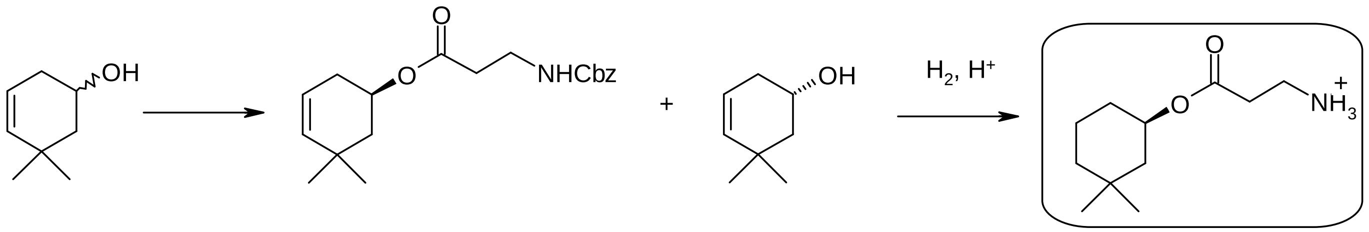
Purification

✓ Introduction of an amine tag:



→ Potential ee polish

✓ Tuning the vinyl donor to the chemistry: → Intelligent design



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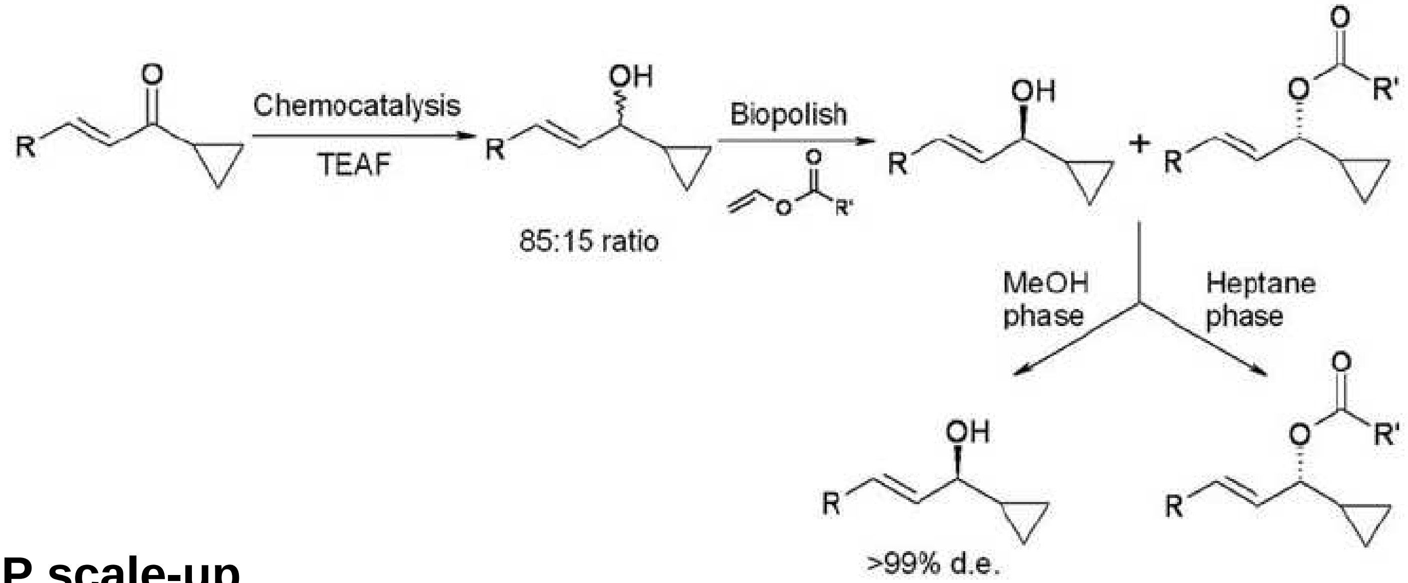
Greener, sustainable processes using efficient biocatalyst purification tools at Almac Sciences

Moody, T. S.; Pharma Chem, April/May edition, 36-38, **2010**. "Cleaner processes using biocatalyst purification tools at Almac Sciences".

Biopolish



GMP scale-up

Biocatalysis Technology to solve separation problem

>10 kg scale

Complex natural product (15 Steps)

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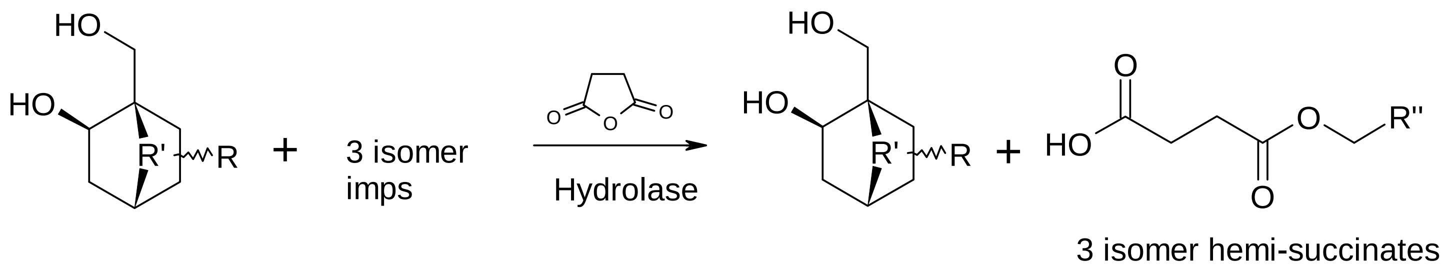
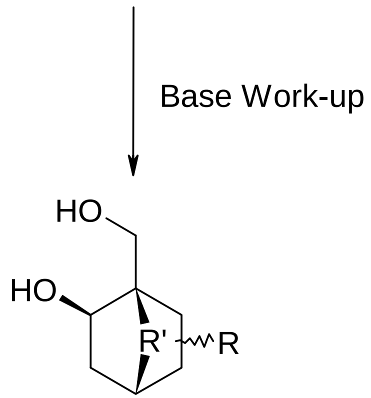
Biopolish in GMP Processing





PROBLEM

•Difficult purification of isomeric impurities

SOLUTION

- **Lipase selective acylation of impurities**

- Basic work-up - removal of imps
- Reaction carried out in THF (20vols)
- Reaction time 16 hrs

Delivery of material with purity >98% wt/wt

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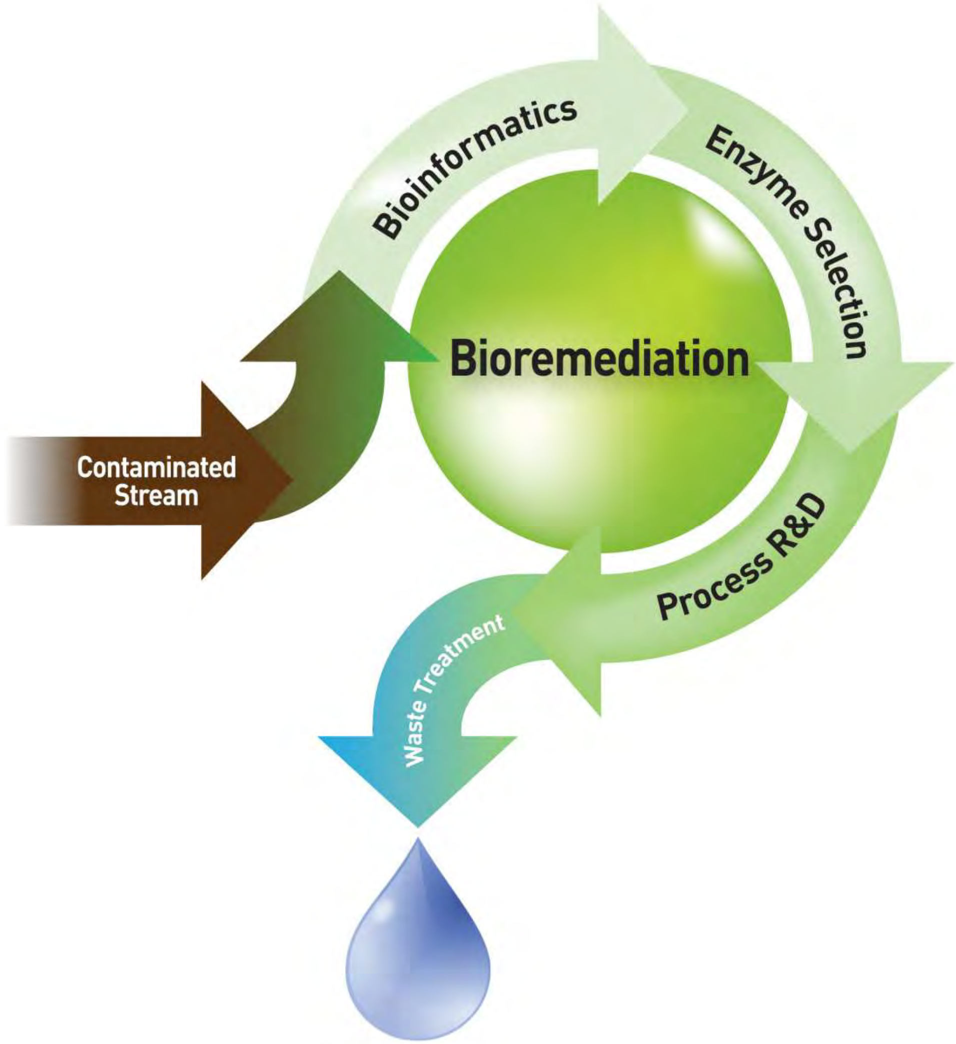
Cyanide Removal

Starting Point

- API process stream contained >1000ppm of cyanide.

Targets

- < 5 ppm of cyanide
- Avoidance of traditional chemical cyanide removal
- Reduction in process waste
- Treatment time < 6h
- Temperature limits and substrate concentations defined



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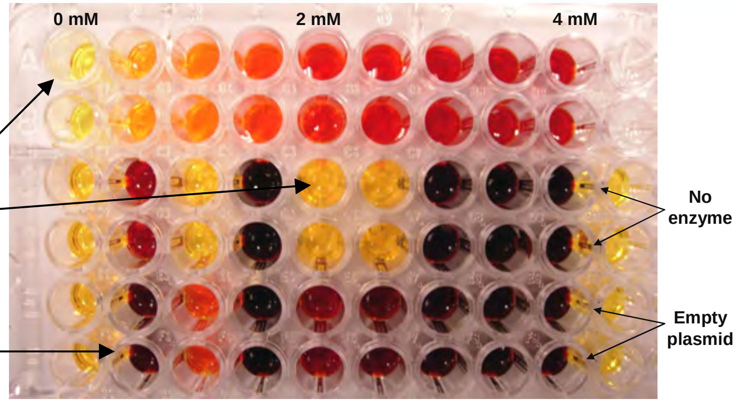
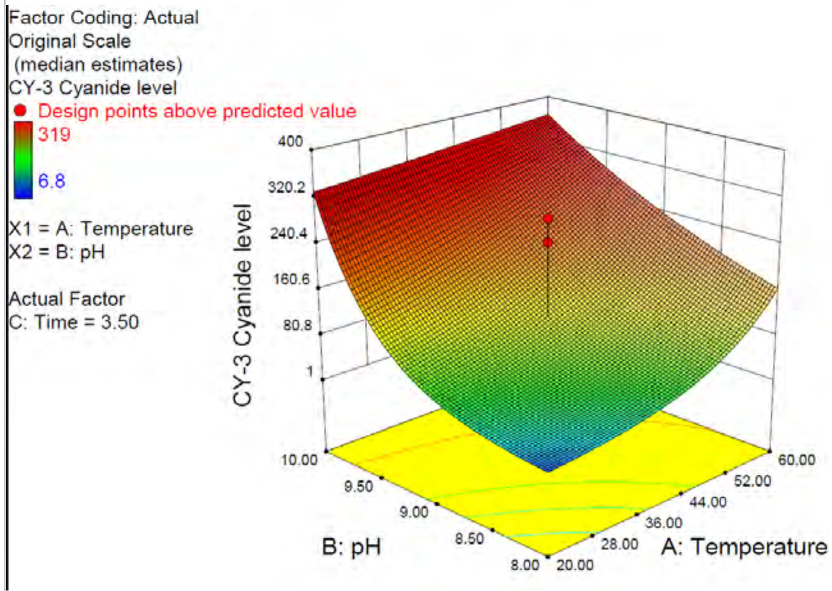
Cyanide Removal

Colorimetric assay for cyanide detection in presence of API





Project Progress

- Series of cyanide dihydratase enzymes identified using molecular biology.
- These were sequenced and expressed in a recombinant host
- Screened against cyanide containing feed stock
- Cyanide contamination lowered to <0.1ppm
- DoE experiments yielded optimum conditions of pH 8.0 and 30 ^{o}C with an enzyme loading of 0.5 mg / L of product

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CASE STUDIES

Oxidoreductase Enzymes

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CRED technology

Organic Process Research & Development

ARTICLE

pubs.acs.org/OPRD

Overcoming Equilibrium Issues with Carbonyl Reductase Enzymes

Susan J. Calvin,[†] David Mangan,[*,‡] Iain Miskelly,[‡] Thomas S. Moody,[‡] and Paul J. Stevenson[†]

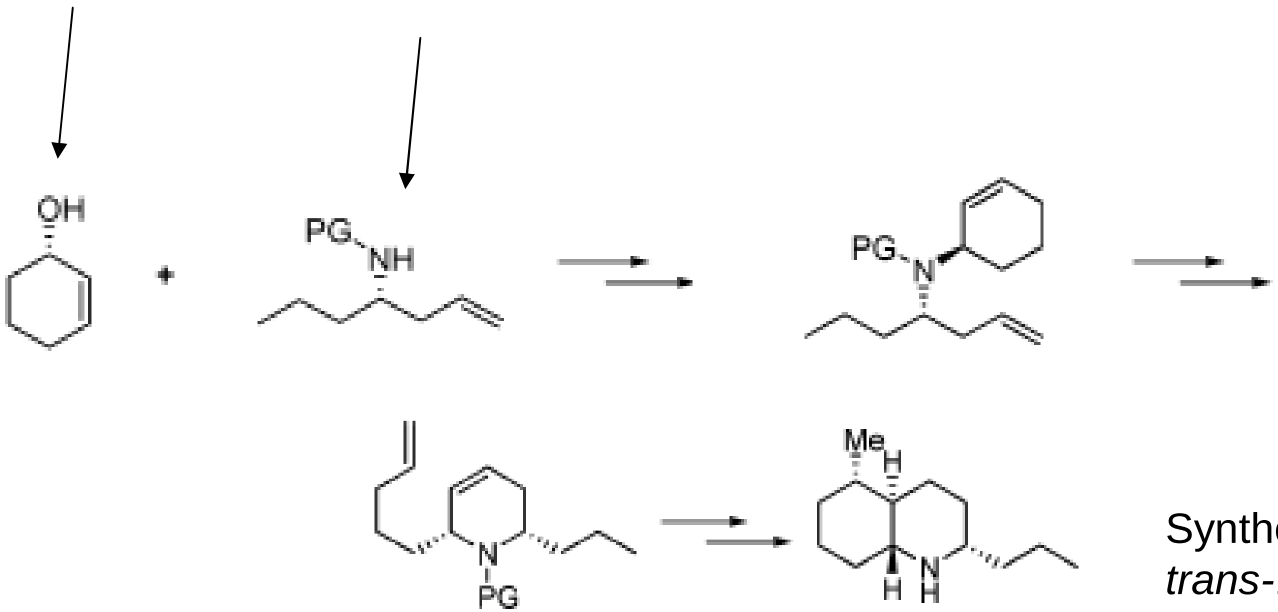
[†]School of Chemistry and Chemical Engineering, Queen's University Belfast, David Keir Building, Stranmillis Road, Belfast, Northern Ireland BT95AG.

[‡]Almac, Biocatalysis Group, David Keir Building, Stranmillis Road, Belfast, Northern Ireland BT95AG.

Driving Equilibrium

CRED

Transaminase



Synthesis of (+)-*trans*-195A

Moody, T.S., *et al.* OPRD, **2012**

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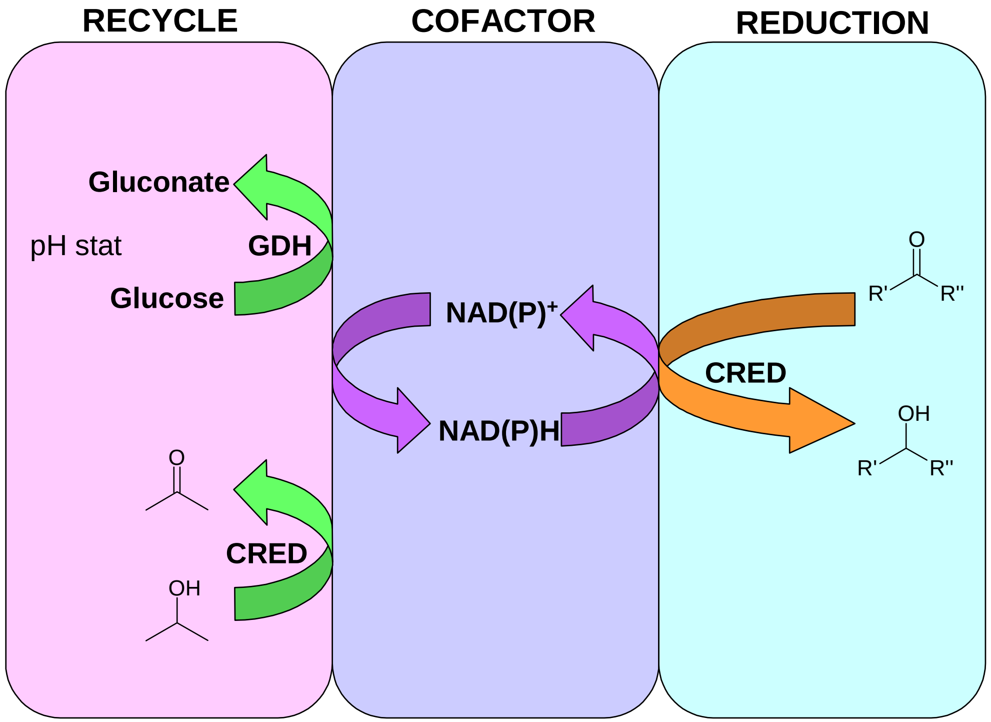
Driving Equilibrium



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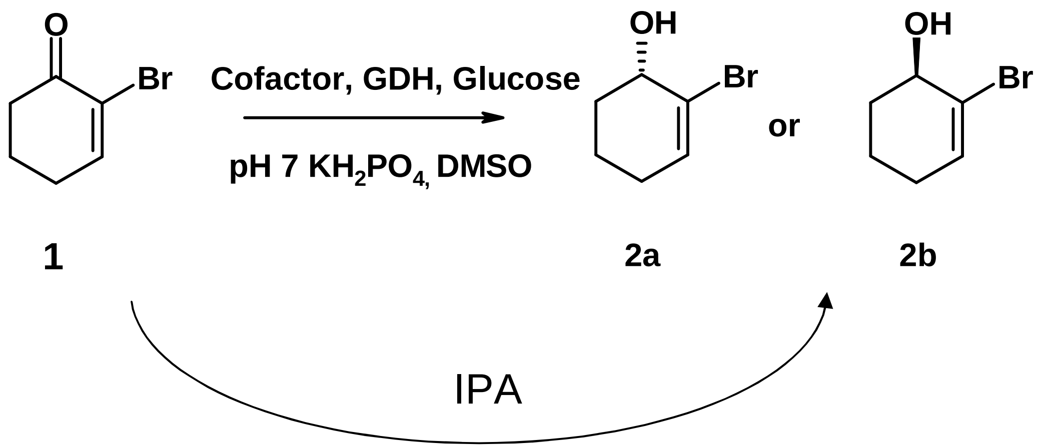
Driving Equilibrium



Equilibrium problems using IPA

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Driving Equilibrium

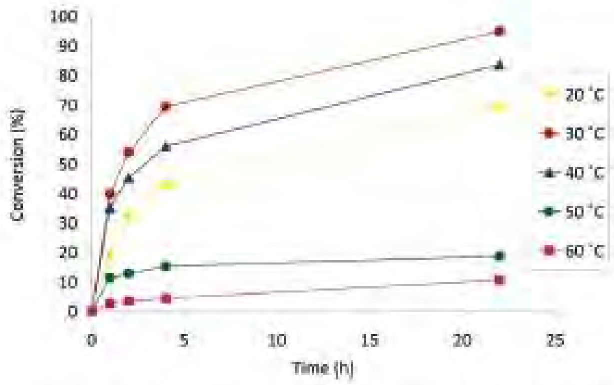

Figure 2. Thermostability assessment for A-601 in the conversion of ketone 1 to alcohol (*S*)-3.

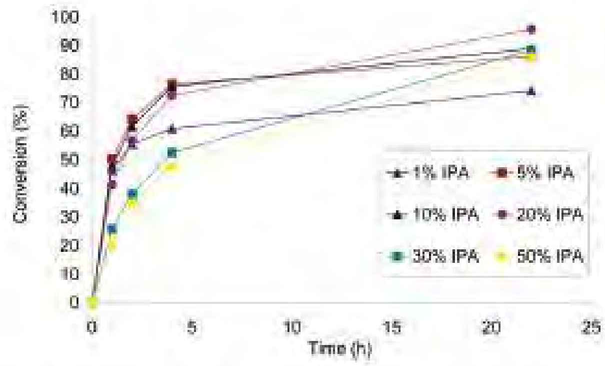

Figure 4. Effect of IPA loading on the conversion of ketone 1 to alcohol (*S*)-3.

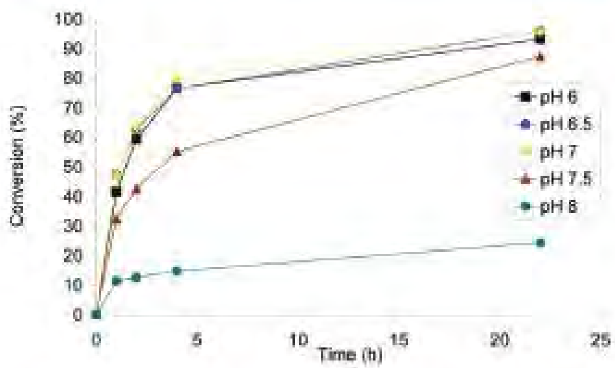

Figure 3. Effect of buffer pH in the A-601-mediated conversion of ketone 1 to alcohol (*S*)-3.

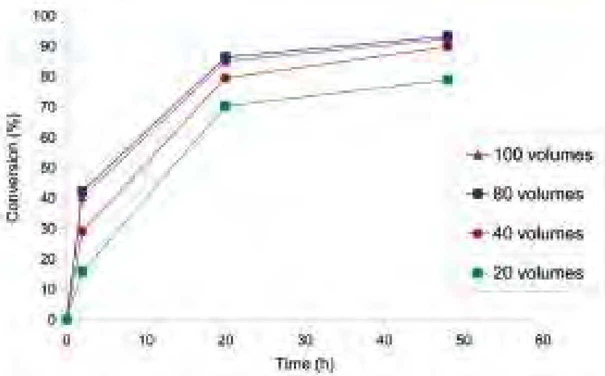

Figure 5. Effect of concentration on the A-601 reduction of ketone 1 to afford alcohol (*S*)-3.

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Driving Equilibrium

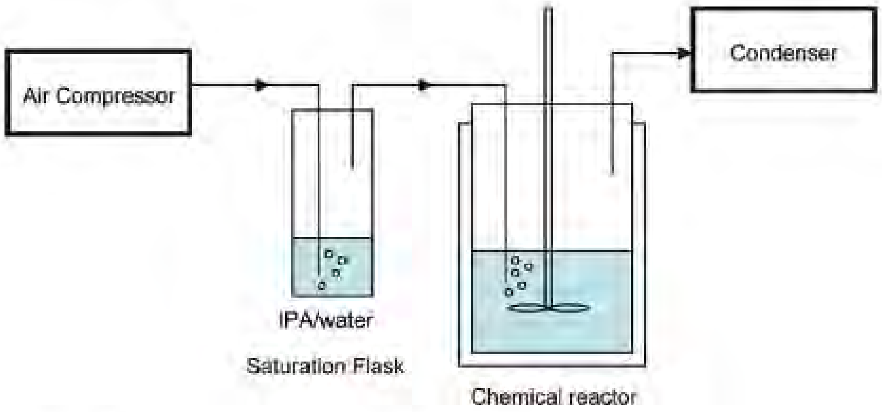

Figure 7. IPA/H_2O-saturated air sparge.

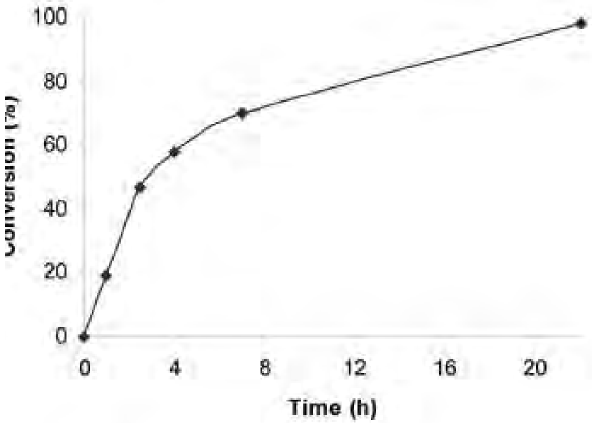

Figure 8. Reaction profile of the bioreduction of ketone 1 to alcohol (*S*)-3 at 100-g scale.

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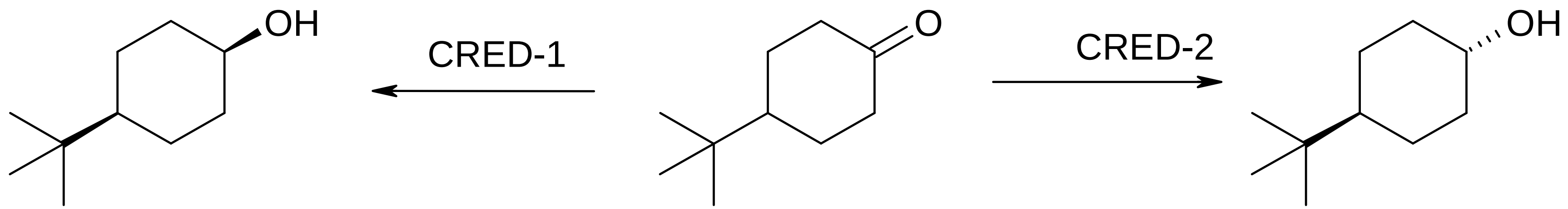
CRED – Woody Acetate



Bio route –

- <u>Cheap</u> cell pellet used for reduction
- One enzyme system

- 99% cis
- 99% trans

•>90% yields

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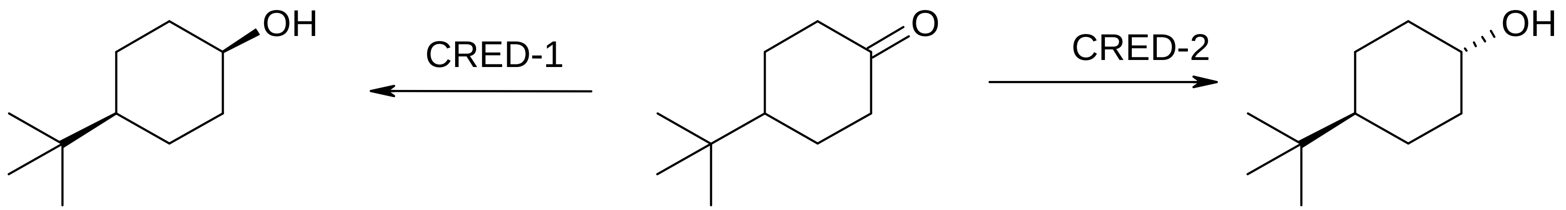
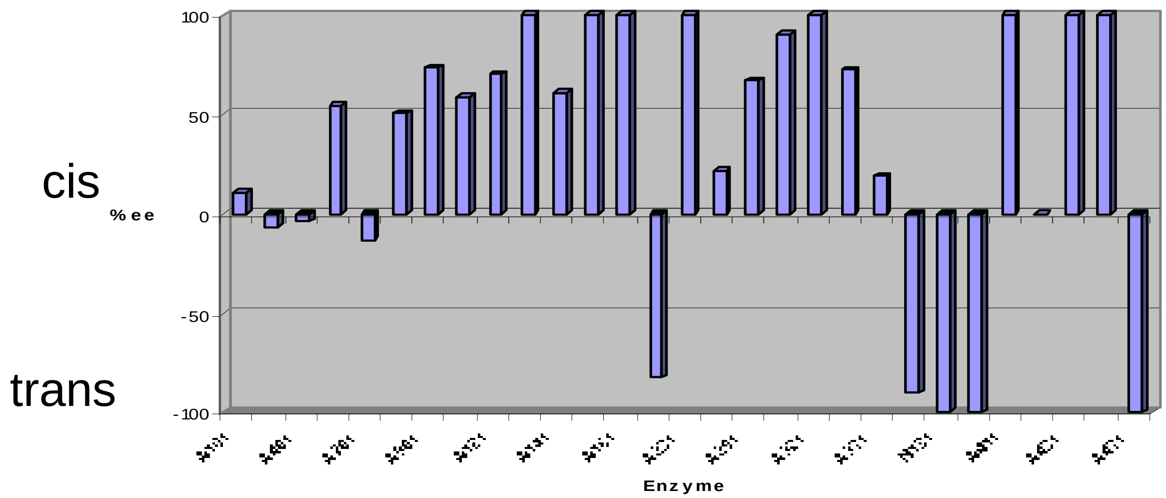
CRED – Woody Acetate





A161 used as enzyme for development

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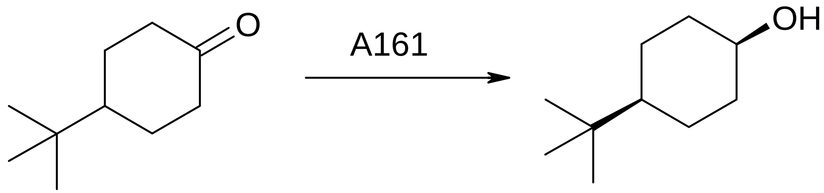
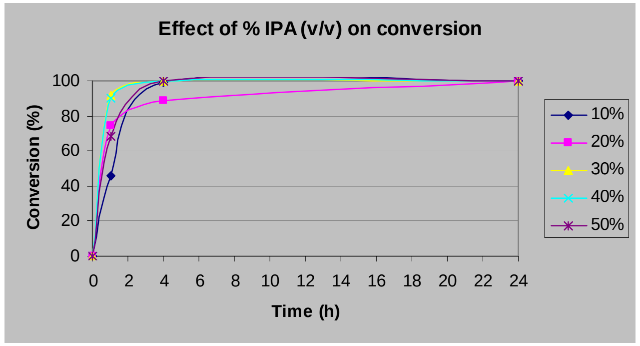
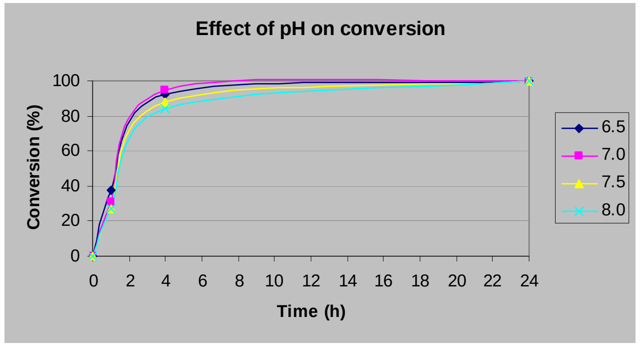
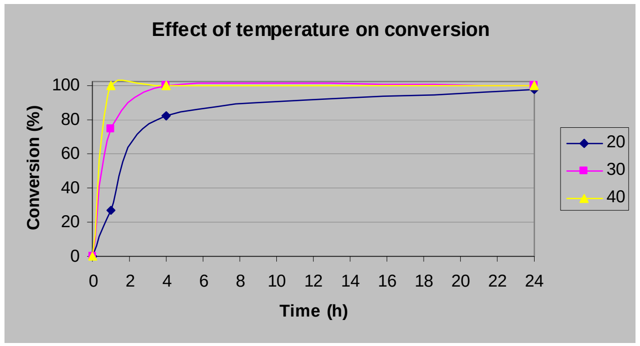
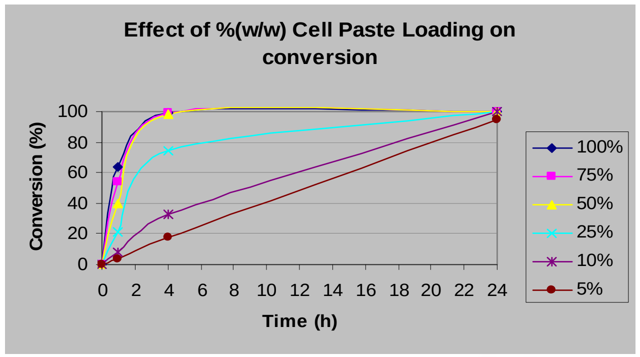
CRED – Woody Acetate



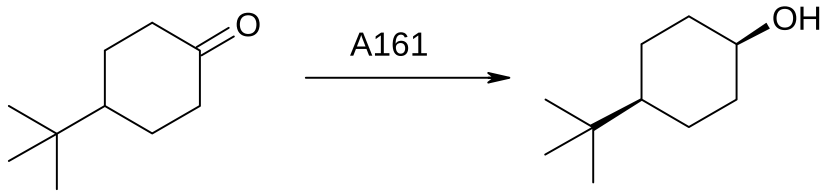


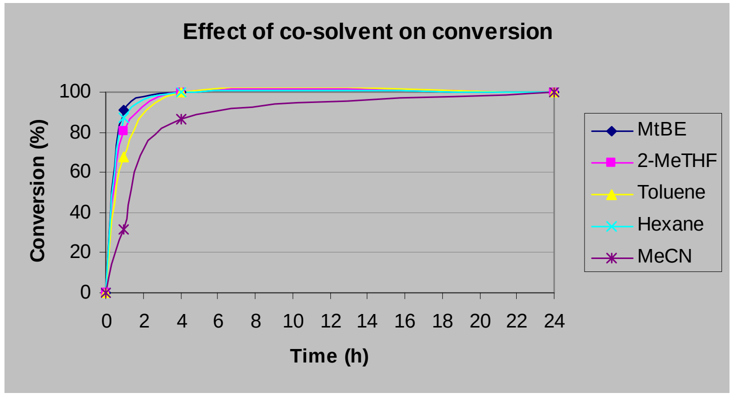


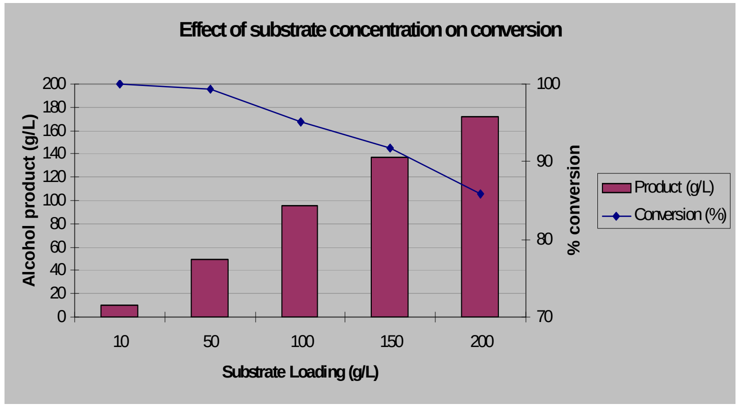




CRED – Woody Acetate







Cheap whole cell used for bioreduction

IPA used as "hydrogen" source

Demonstrated reduction at ~500g/L

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CASE STUDIES

Biooxidation

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Metabolites

Metabolite Identification and Synthesis

The selectAZyme™ platform is used for rapid metabolite synthesis, isolation, and structural identification.

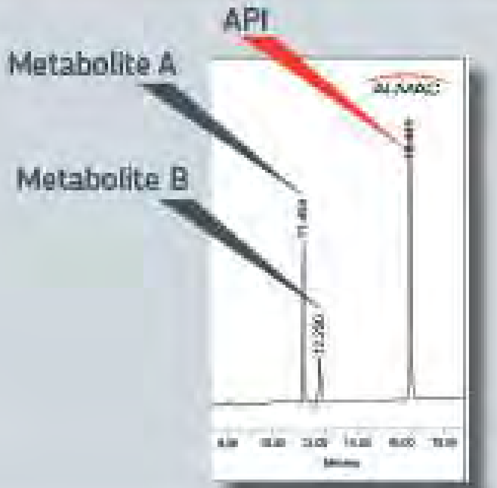
Metabolite Synthesis
- Cloned Human P_{450}
- Cloned Microbial P_{450}
- Extensive microbial collection

Metabolite Isolation/Structure ID
- Analytical and prep LC
- LC/MS
- High-field NMR

Case Study:
Rapid Structural Identification

Customer detected two metabolites at >10% of total drug load by HPLC. Almac selected the best biocatalyst for production of each metabolite from its enzyme and microbe collections.

- mgs prepared
- Preparative LC separation
- Structure ID by LC-MS and NMR



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cGMP Whole Cell Biooxidation
Case Study Sorbitol Dehydrogenase

Project background

- Biooxidation required as part Multistep cGMP API synthesis
- Recombinant Membrane bound dehydrogenases are not easy to obtain or handle
- Whole cell system provides all co-factors and recycling systems (via P_{450})

- Example: Gluconobacter oxydans mediated selective oxidation of an azasugar drug intermediate
- Reaction uses resting cells, integrity of cells is pre-condition for reaction

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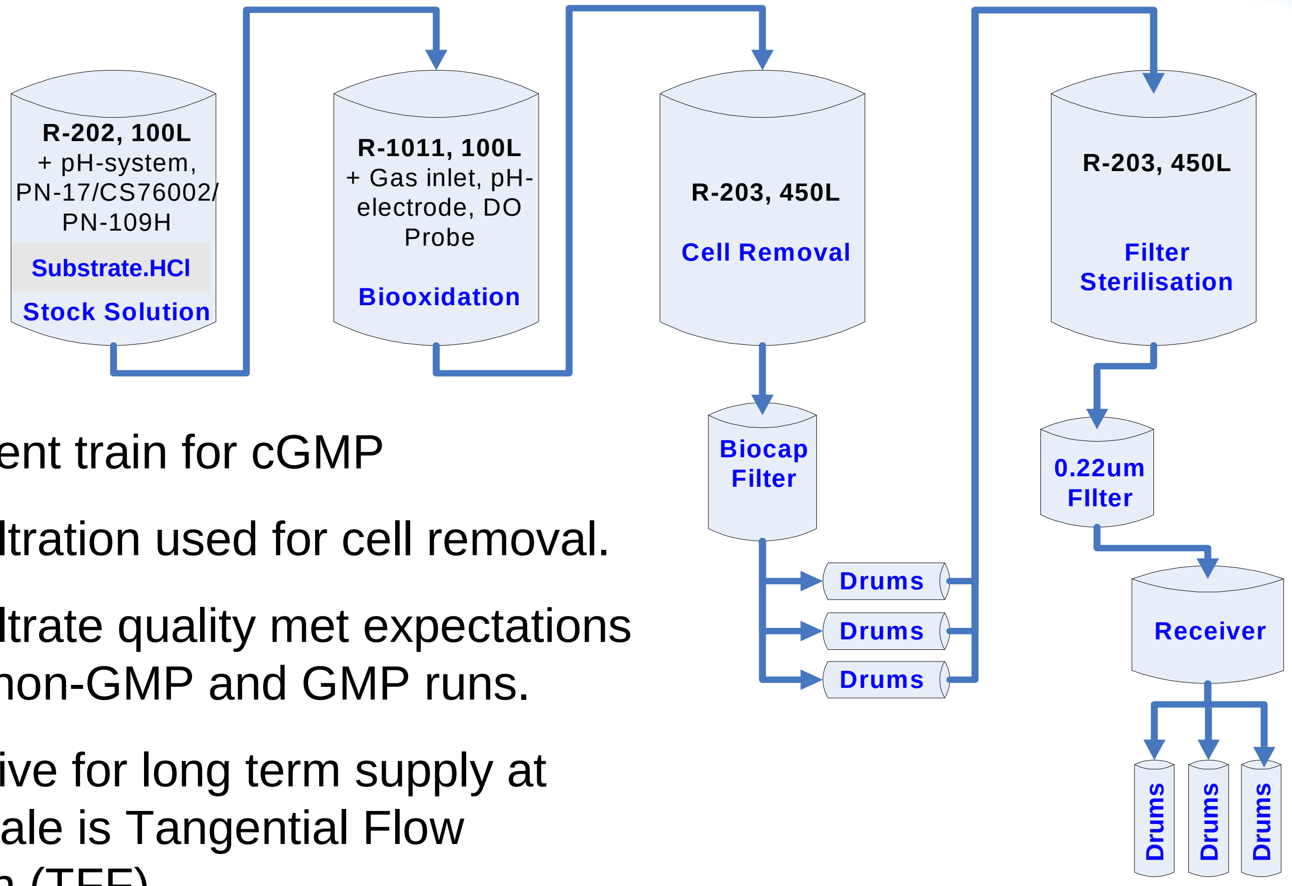
cGMP Whole Cell Biooxidation
Case Study Sorbitol Dehydrogenase



Equipment train for cGMP

Depth filtration used for cell removal.

Depth filtrate quality met expectations in both non-GMP and GMP runs.

Alternative for long term supply at large scale is Tangential Flow Filtration (TFF)

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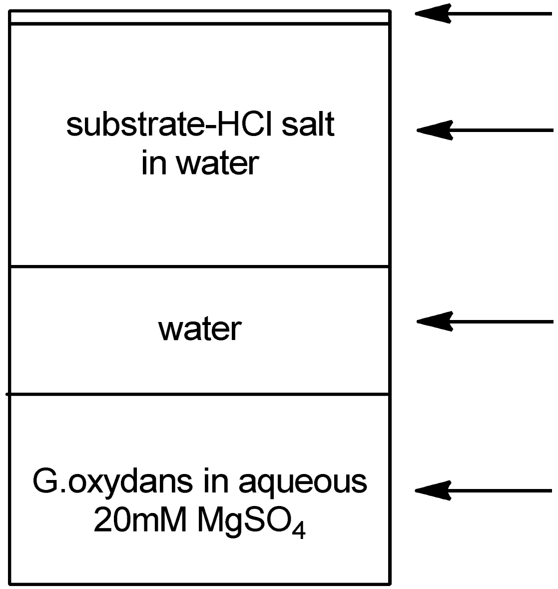
cGMP Whole Cell Biooxidation
Case Study Sorbitol Dehydrogenase

Total Batch Volume

Dedicated Batch Volume Fractions



up to 1% for pH-adjustment and anti-foam

46.5% reserved for substrate-HCl salt stock; adjustable for stock strength variation, **target 18.5% w/w substrate free base in total bx volume**

23.5% reserved for water; adjustable to compensate for reagent strength and charge variation

31% reserved for G.oxydans in 20mM $MgSO_4$; adjustable for cell content and activity

Step 2B biooxidation: composition of reaction mixture

Biomass charge is verified by lab use test as part of biomass release testing.

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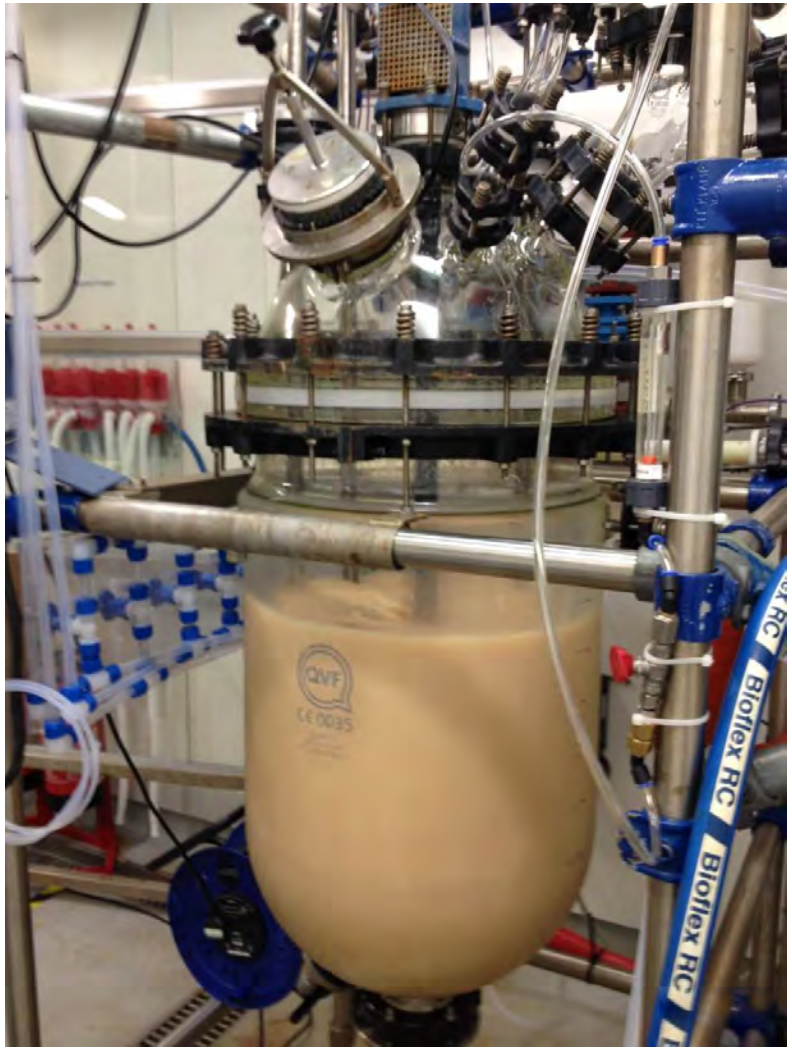
cGMP Whole Cell Biooxidation
Case Study Sorbitol Dehydrogenase



R-1011 in use for step 2B

At close to 1M concentration, oxygen demand is high.

Balance to be struck between adequate mixing/aeration and control of shear impact on the cells.

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cGMP Whole Cell Biooxidation Case Study Sorbitol Dehydrogenase

Ion exchange setup in

Almac's manufacturing plant

Used for product purification in catch and release mode.

Smaller units applicable to e.g. process stream de-salting

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CASE STUDIES

Transferase Enzymes

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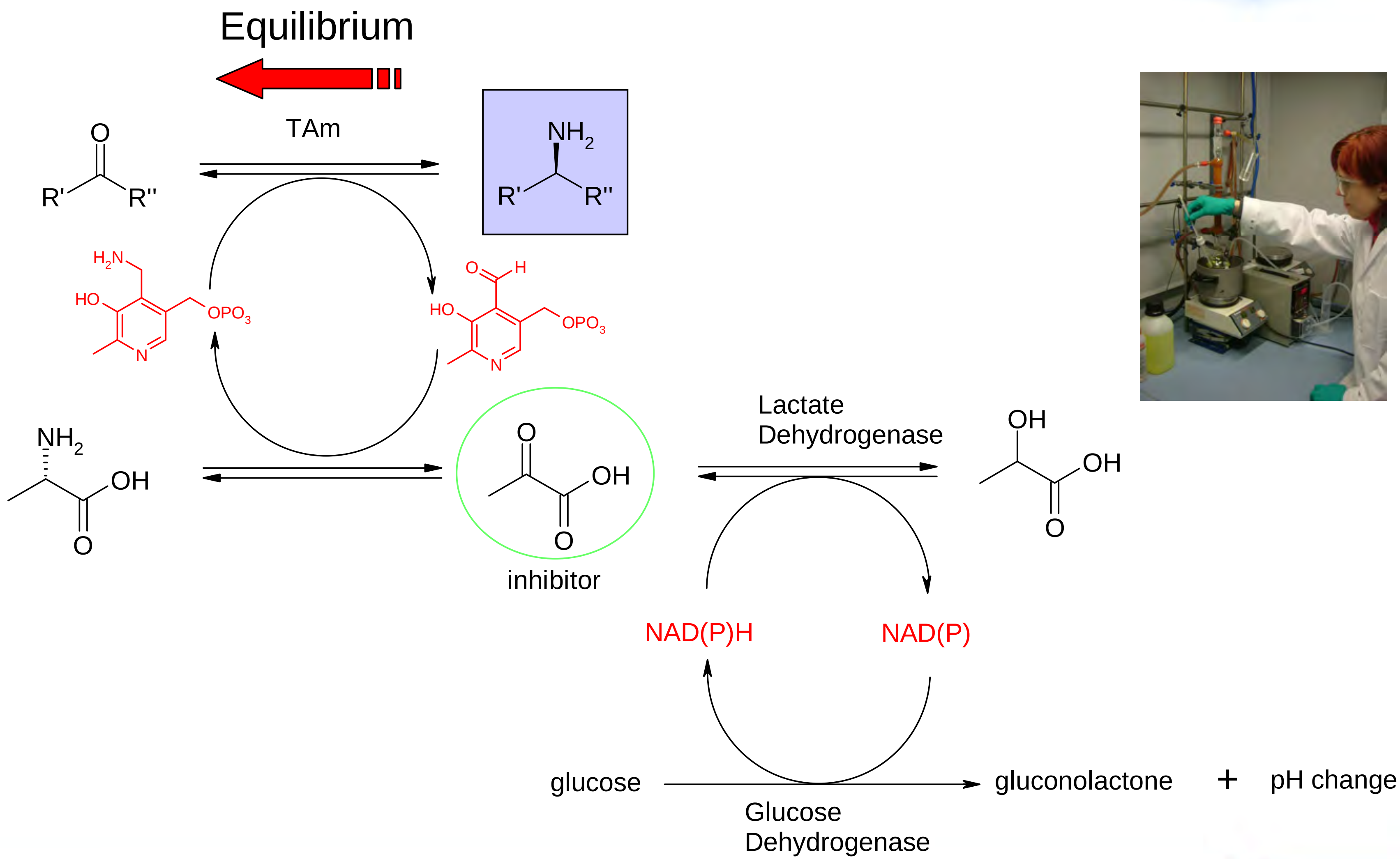
Transaminase



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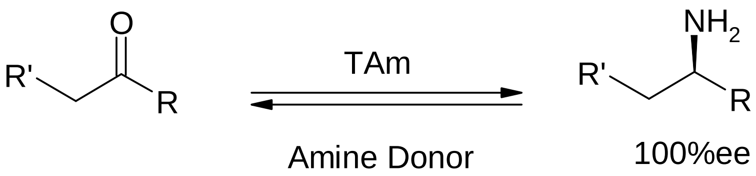
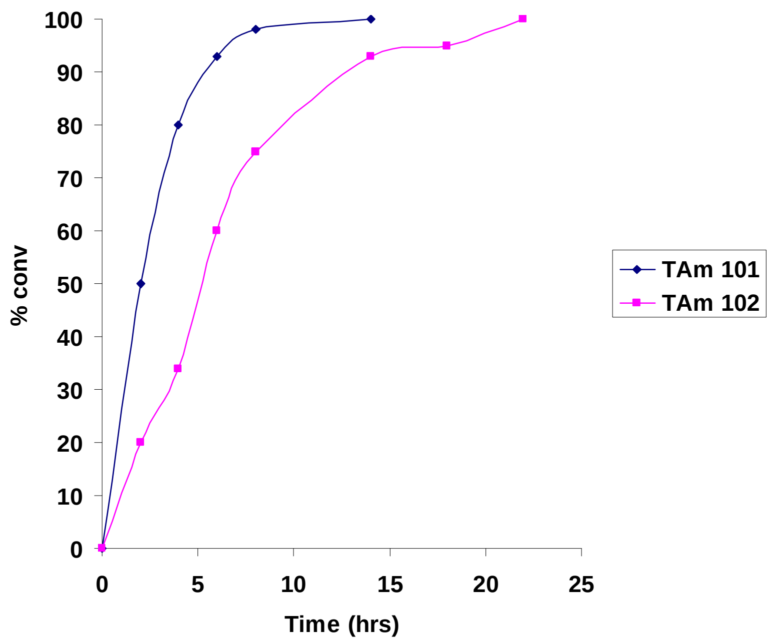
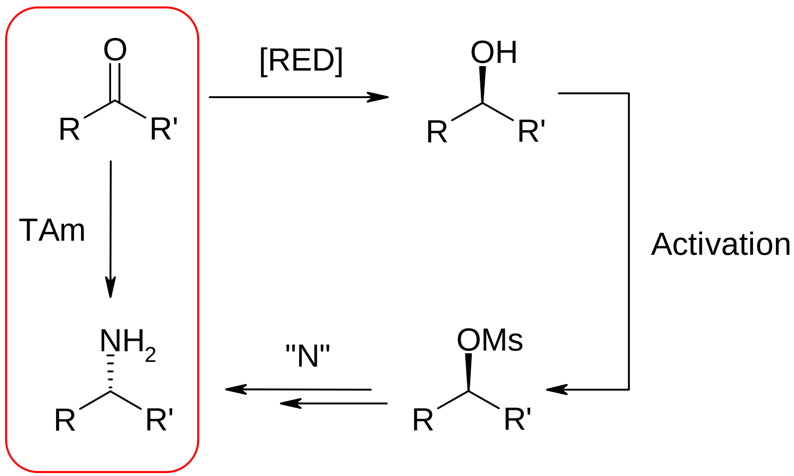
Transaminase





Shortening the Path – TAm technology



Reaction carried out in < 2vol buffer

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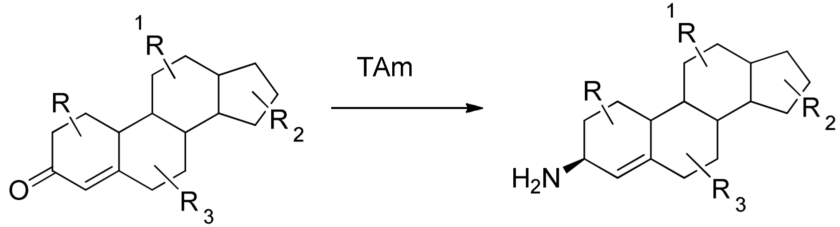
Transaminase



Modular Project Deliverables

- TAm screen and TAm identification
- TAm optimisation
- Delivery of chiral product

TAm resulted in removal of 8 steps of chemistry

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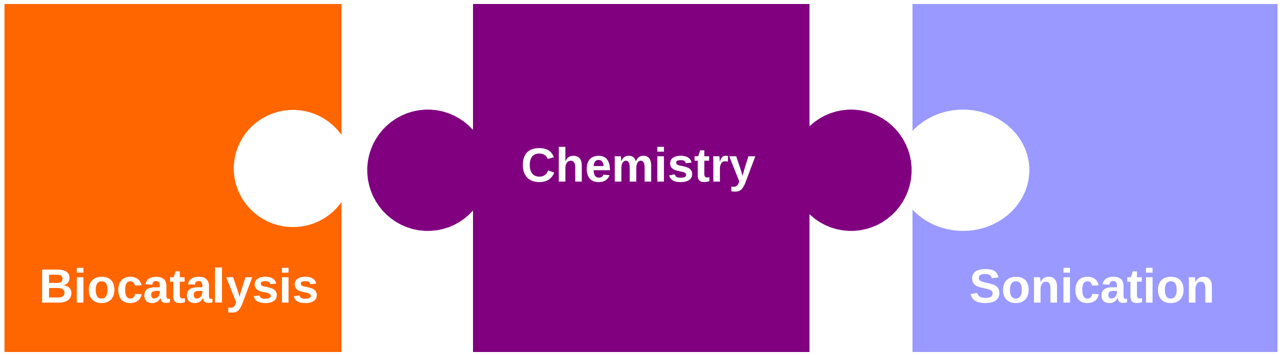
New Technology

Technologies



Our Chemistry group is linked to Biocatalysis and Sonication technologies for isolation of chemical products

Sonication

Celbius

selectAZyme™

- Chemical substrates
- Biomass sugars
- Sugars and starches

Feedstocks

Sonication

- Lower enzyme cost
- Faster reactions
- Higher yields

- Enzymes
- Bacteria
- Fungi

Biocatalysts

Higher productivity

Lower cost of goods

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Natural Product Extraction

- An efficient technique for extracting bioactive compounds from natural and synthetic matrices
- Advantages include
  - Reduced solvents
  - Reduced temperature
  - Reduced time for extraction

- Acoustic extraction is very scaleable
- Especially useful for the extraction of thermolabile and unstable compounds

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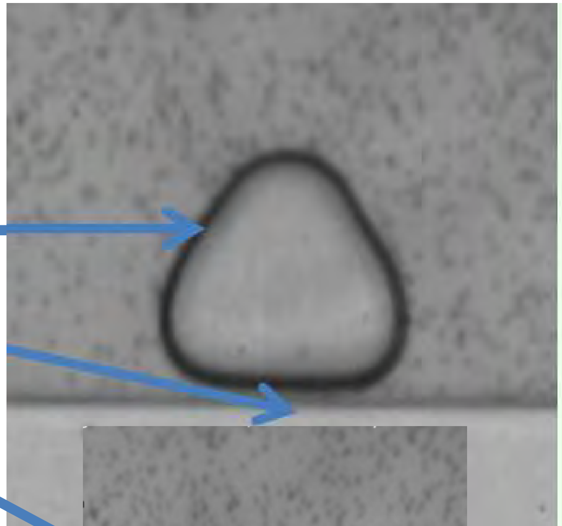
Acoustic Cavitations

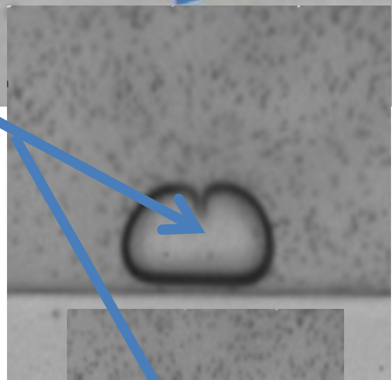
Celbius

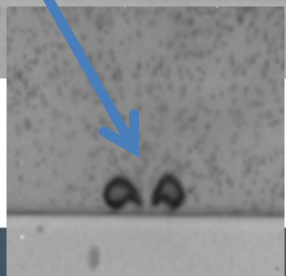
selectAZyme™

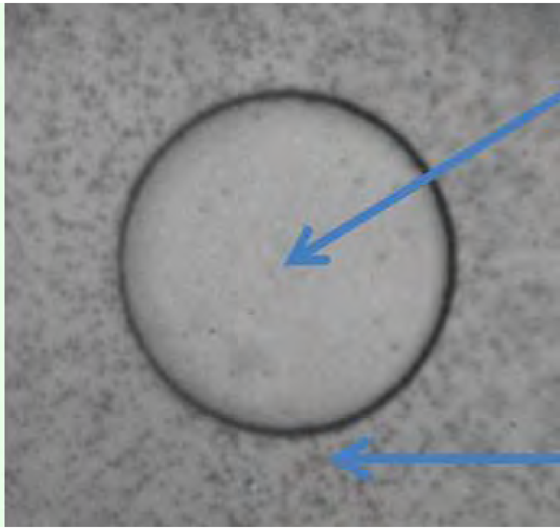
Acknowledgement Prof. T Mason, Uni. of Coventry

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

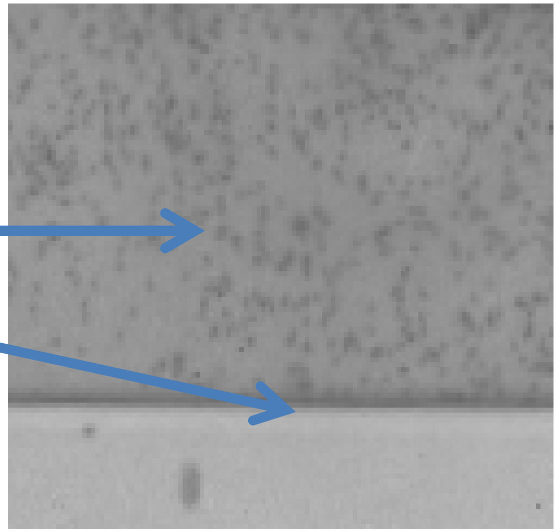
Acoustic Cavitations

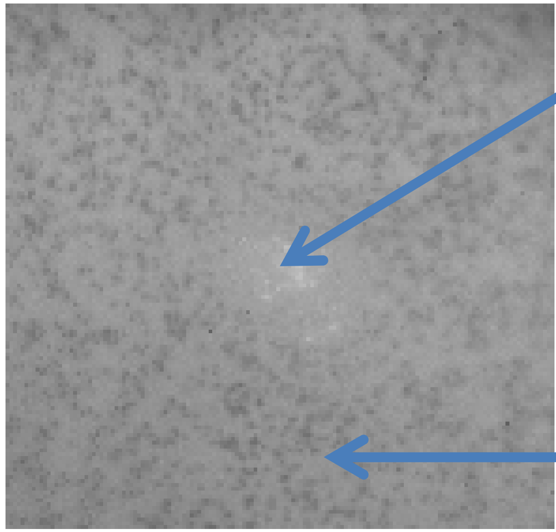
Celbius

selectAZyme™

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 5000ºC and 2000 atmospheres

IN THE BULK MEDIA
intense shear forces

- Lignocellulose weakening
- (Bio)polymer degradation
- Crystallization
- Cell permeation

- 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

Natural Product Extraction

Celbius

selectAZyme™

Sono-probe

Stirred vessel

Continuous flow

- Simple continuous lab set up
- Increased product extraction profiles
- Altered composition of extracted products from natural sources
- Potential for new compositions of products from fermenting biomass

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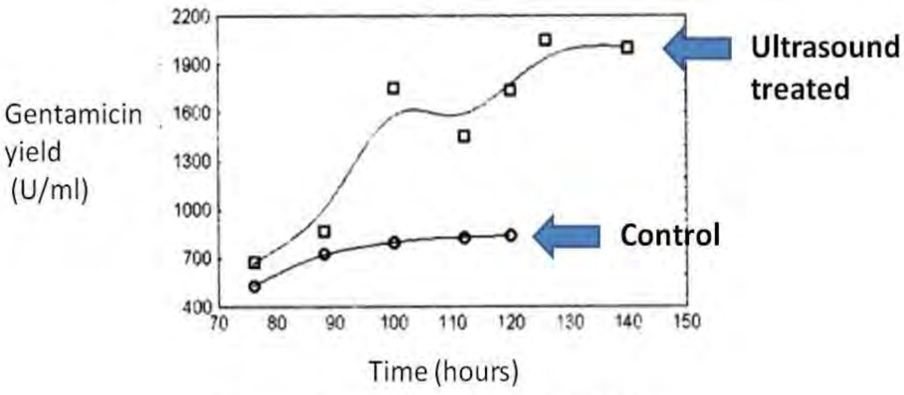
Sono Biocatalysis

Striking improvements in bioprocess performance can be achieved………..

Fermentation for Antibiotics

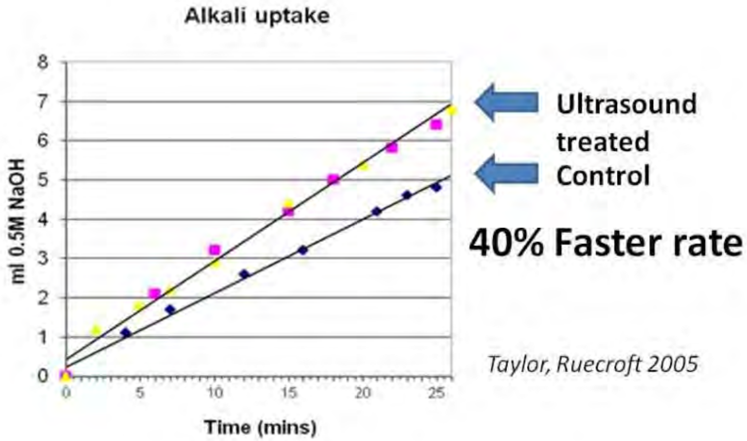
Increased antibiotic production (lab scale)



Process Biochemistry, 2000, 35, 569-572

Drug Intermediate Manufacture

Faster Enzyme Reactions: Model ester cleavage by a lipase enzyme



Taylor, Ruecroft 2005

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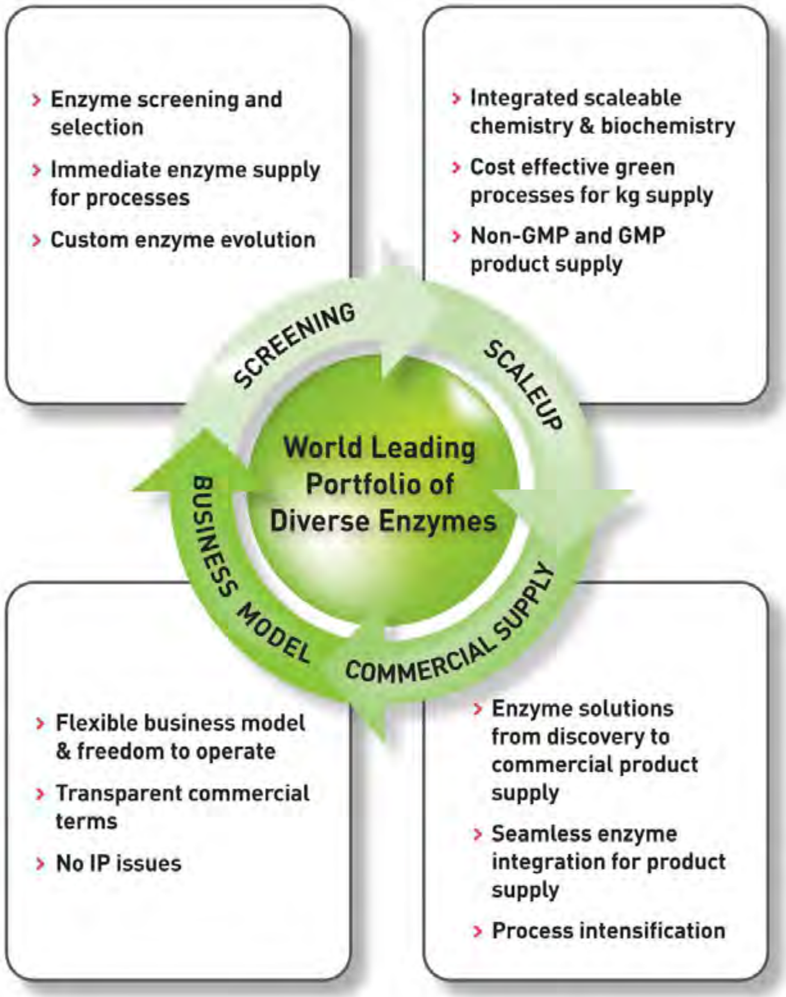
Summary



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Future Outlook

- Tailor-made/designed biocatalysts
- Solvent free environments
- Continuous mode operation
- Tandem biocatalysis
- Conversion of waste to chemicals
- Lower downstream waste

Integration from drug discovery to commercialisation

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Almac Bio-group

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