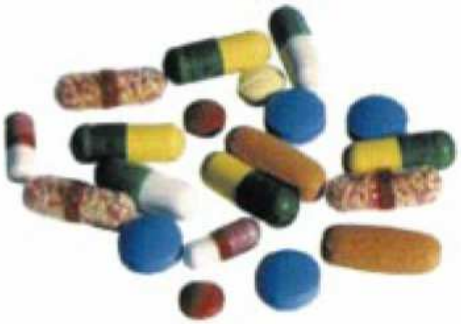
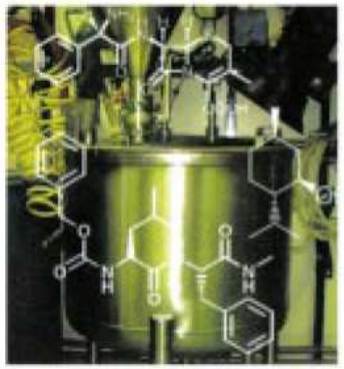
Naproxen

From skunk work to scale-up





LH Steenkamp
D Brady

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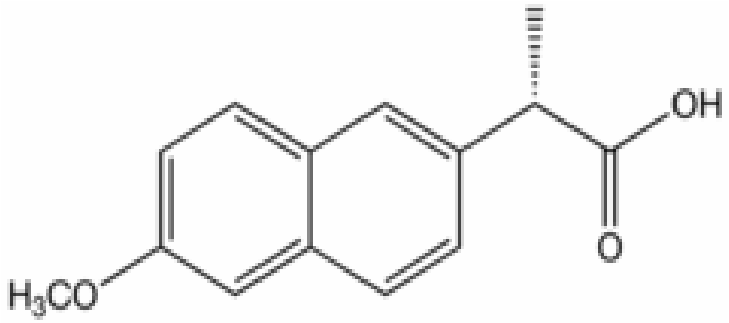
Introduction

- Aspirin – first generation anti-inflammatory drug discovered 150 years ago
- Indomethacin – second generation anti-inflammatory drug discovered in the 1960s
- Lower side effects than aspirin
- At the same time S-naproxen was introduced
- Possesses a 50 times higher activity than aspirin



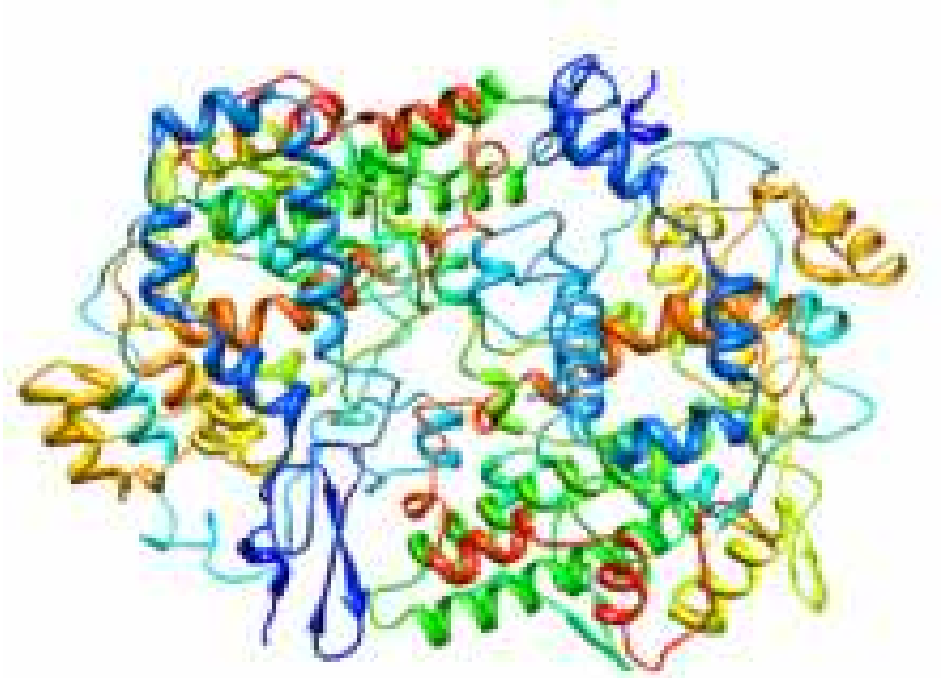
Naproxen

- (S)-(+)-6-methoxy-α-methyl-2-naphthaleneacetic acid
- Non-steriodal anti-inflammatory drug
- Used for reduction of moderate to severe pain, fever, inflammation and stiffness
- Used for treatment of osteoarthritis, rheumatoid arthritis, gout, tendinitis, bursitis and injuries such as fractures
- (S)-enantiomer is thirty times more active than the (R)-enantiomer
- (R)-enantiomer has unwanted side effects

Naproxen

- (S)-naproxen works by inhibiting the COX1 and COX2 enzymes
- COX-cyclooxygenase – an enzyme that is responsible for important biological mediators called prostanoids
- The prostanoids include prostaglandins, prostacyclin and thromboxane.
- One of the functions of prostaglandins is to sensitize the spinal neurons to pain so that the individual will stop his actions following injury and prevent further damage
- Inhibition therefore of COX will prevent formation of prostaglandins and therefore the sensitisation to pain – therefore the name non-steroidal anti-inflammatory drug.

Structure of the COX1 enzyme



Naproxen pharmaceuticals

Naproxen

- It is an odourless, white to off-white crystalline substance. It is lipid-soluble and practically insoluble in water. It has a melting point of 153ºC
- Synthesised via asymmetrical syntheses or
- Resolution from its racemate
  - Resolution via the diastereoisomeric salt formation
  - Enzyme kinetic resolution

- The original synthesis was disclosed by Syntex in 1967
- AECI has a patent on the synthesis of racemic naproxen which can then be resolved via a biocatalysis reaction

Biocatalysis

- Hydrolytic enzymes are the biocatalysts most commonly used in organic synthesis. Two of the most useful hydrolase enzymes are esterases and lipases, the classes of enzymes that catalyse the hydrolysis and formation of ester bonds.
- For a resolution process to be valuable, it must have a high selectivity
- For a commercial process it is important to determine the enantiomeric ratio (E) for the process
- E is the parameter that is characteristic of the enantioselectivity of a particular enzyme and the degree of conversion that occurs. It is independent of time

Calculating Enantiomeric ratio

$$E = \frac{\mathrm{Ln}[(1-c)(1-ee_s)]}{\mathrm{Ln}[1-c)(1+ee_s)]} = \frac{\mathrm{Ln}[1-c(1+ee_p)]}{\mathrm{Ln}[1-c(1-ee_p)]}$$

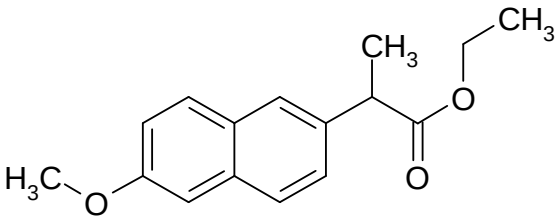
Enantiomeric excess (ee) is determined by the following equations:

$$ee_s = \frac{[A]-[B]}{[A]+[B]} \qquad ee_p = \frac{[P]-[Q]}{[P]+[Q]}$$

$$\text{and conversion (c)} = \frac{ee_s}{ee_s + ee_p}$$

where [A] and [B] are relative concentrations of the starting material enantiomers (s) and [P] and [Q] are concentrations of the product antipodes (p).

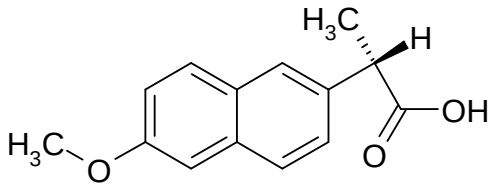
Reaction scheme for the proposed enantioselective hydrolysis



2-(6-Methoxy-2-naphthly)-propionic acid ethyl ester

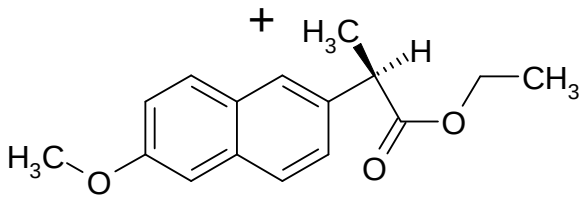
ChiroCLEC-CR

H_2O/PEG, pH 5



(*S*)-2-(6-Methoxy-2-naphthyl)-propionic acid

+



(*R*)- 2-(6-Methoxy-2-naphthyl)-propionic acid ethyl ester

+

Ethanol

Experimental work

- More than 80 commercially available esterases and lipases screened
- All sample analyses were performed by HPLC.
  - The % m/m was determined using a 25 cm C18 ODS 2 column
  - (R/S) ratios were determined on an (S-S)-Whelk/0/25 cm column
- Racemic naproxen was esterified to yield the racemic naproxen ester
- *(R,S)*-naproxen was suspended in methanol/ethanol or vinylacetate. Chlorine gas was bubbled into the reaction by evolution from NH_4Cl in H_2SO_4.
- Methylesters are the preferred substrate for esterases, while lipase prefer longer side-chains.
- Vinylester reactions are irreversible

Screening of the enzyme activity and selectivity

- The initial screen was done using the ethylester of racemic naproxen
- Reactions were done at 1ml scale in either PEG/acetate pH 5 buffer (lipases) or MOPS buffer at pH 7 for the esterases
- 50 mg of substrate was used
- Reactions were run for 4 hours at 37ºC
- Reactions were stopped with acetonitrile and filtered before analysis
- Analysis was first performed to determine if any conversion resulted
- Only positive reactions were then analysed for R/S ratio
- The lipase enzymes were also screened in water-saturated organic solvents

Results of the screening

- Eight enzymes were found to have some selectivity for production of *S*- naproxen (ee > 10% *S*) from the hydrolysis of NEE:
  - NOVO 388;
  - NOVO 398;
  - Boehringer hog liver esterase;
  - *Aspergillus niger* (Fluka 62294);
  - *Mucor miehei* (Fluka 62298);
  - *Aspergillus oryzae* (Fluka 62285);
  - ESL001-01
  - ChiroCLEC CR.
- The conversions are in terms of R,S naproxen and therefore a maximum of 50% conversion can be achieved.

Results of the Stats Design

Enzyme	% Conversion	ee(%)	E	% Improvement
NOVO 388	17.8	38	2.4	239
NOVO 398	8.4	70.6	6.2	189
Hog liver esterase	38	33	2.4	22
A niger lipase	4.4	43.4	2.6	4
Mucor miehei lipase	3	27.8	1.8	4
A oryzae lipase	5.2	68	5.5	6
ESL001-01	13.5	87	16.3	17
ChiroCLEC CR	40	99	>100	4

Results from other researchers

- Xin *et al* - *Candida rugosa* lipase in hydrolysis between an aqueous and an aqueous-organic biphasic system for enantiomeric resolution of (*S*)-(+) Naproxen. In the biphasic system a 42% conversion with 93% ee product was achieved providing an E of approximately 50
- Xin *et al* - also found that naproxen ester hydrolysis by a lipase in water-saturated organic solvent could be improved by linking it to a photo-hydrolytic reaction of methanol product to drive the reaction forward, but although the ee was good, only 25% conversion was achieved.
- Cui *et al* -achieved an ee of approximately 100% and 33.5% conversion (over 11 days) through lipase-catalysed esterification of racemic naproxen in organic solvent.

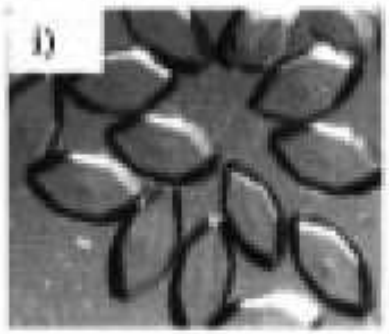
Optimisation of ESL001-01

- More effort was spent to optimise ESL001-01 as it is a thermostable enzyme which can result in faster reactions due to higher temperatures
- ESL-001-01, isolated from a metagenomic library obtained from a high temperature extreme environment by Diversa
- A series of factorial designs proved useful for determining the optimal conditions for both conversion and enantioselectivity, in addition to evaluating interactions between the factors under investigation.
- The highest ee value obtained throughout the experiments with ethylester was 0.89, and the enantiomeric ratio (E) was within the limits of 12- 20 over the range of 10 – 40% conversion
- With methylester, the E was increased to 75

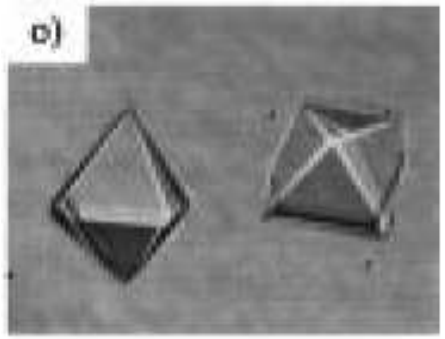
ChiroCLEC CR

- A technology for forming stable (cross-linked) enzyme crystals (CLECs) was developed by Altus Biologics Inc (USA)
- Microcrystals are cross-linked with a bifunctional agent such as glutaraldehyde
- These crystals are highly pure
- The CLEC of *Candida rugosa* lipase (ChiroCLEC-CR) is expected to have improved enantio- and regioselective properties compared to crude preparations

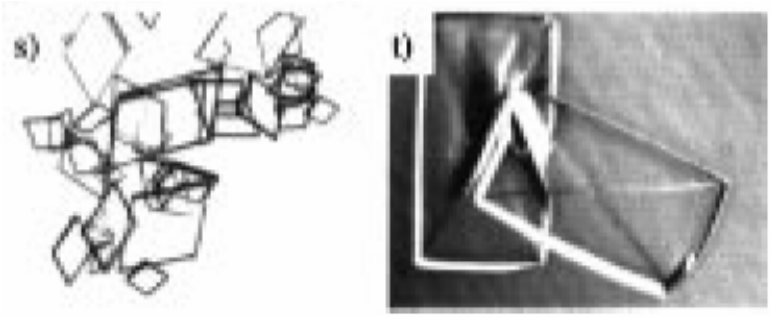
Examples of CLEC crystals



Savinase enzyme



Urease from Jack Beans



s- Lipase from Candida rugosa,
t – Penicillin acylase form E coli

AL Margolin, MA Vavia. Angew Chem Int Ed 2001, 40, 2204

Optimisation of ChiroCLEC CR

- Statistically designed experiments were performed to optimise:
  - Temperature
  - enzyme to substrate ratio
  - substrate concentration
  - Agitation
  - reaction time
  - pH
  - buffer concentration
  - co-solvent addition.

Optimisation of ChiroCLEC CR

- Optimisation efforts resulted in a more than 20-fold improvement of activity, while the excellent enantioselectivity of the enzymes was maintained
- In particular, the addition of PEG 1000 as a co-solvent improved conversion rates 10-fold.
- The ChiroCLEC-CR enzyme provided excellent enantioselectivity
  - enantiomeric excess (ee) of 99%
  - 45% conversion of substrate
  - yielding an enantiomeric ratio (E) of 500) for the hydrolysis of (*R,S*)-NEE to (*S*)-naproxen
- The specific activity of the ChiroCLEC-CR lipase under reaction conditions was improved more than 20-fold

Interesting observations

- In a sonication water bath, the reaction was 6 times faster than the stirred reaction.
- The enzyme was not damaged by sonication, but scale-up of a sonicated reaction could present problems such as design of specific equipment and effective mixing.
- Three metals had a distinct negative influence on the activity (Cu^{2+}, Na^{+} and Al^{3+})
- Some metal cations (Mg^{2+}; Ca^{2+} and Co^{2+}) and the non-metallic boron cation (B^{3+}) may activate or stabilise the enzyme crystals at the concentrations tested.

Interesting observations

- The half-life at 40°C (with or without stirring) was 72 hours, while at 55°C the half-life was 20 hours, demonstrating that higher temperatures are highly detrimental to enzyme stability
- All co-solvents except PEG added for solubilisation of the substrate was detrimental to the reaction
- Only PEG1000 permitted the highest activities with the excellent %ee of 99
- Due to the high cost of the enzyme ($100 000/kg) and its instability it was not suitable as a commercial process

Biocatalysis with Carboxylesterase NP

- Researchers at DSM (previously Gist-Brocades) isolated and identified a *Bacillus subtilis* (Thai I-8) strain
- This strain produced carboxylesterase NP,
- This enzyme can enantioselectively hydrolyse naproxen esters
- The gene coding for the esterase, one of several related genes in *B. subtilis* was identified, sequenced, and cloned into a host organism, resulting in the recombinant-DNA strain *B. subtilis* I-85/pNAPT-7.
- The enzyme expression by this recombinant-DNA strain was 800 times higher than that of the wild type strain Thai I-8.

Carboxylestarase NP

- At high substrate concentrations, a lack of enzyme stability was encountered and irreversible inactivation was observed when carboxylesterase NP was incubated with 30 g/ℓ *R, S*-naproxen ester.
- The enzyme inactivation by naproxen or ibuprofen was proposed to be due to the interaction of the carboxylic acid products with the amino groups of basic amino acids, particularly lysine.
- Therefore the carboxylesterase is chemically modified with formaldehyde to block the lysine side-chain amines.
- Carboxylesterase NP treated with 1% formaldehyde proved to be stable on incubation with naproxen (15 mg/mℓ) for 1.5 hours at 40ºC; conditions that lead to complete inactivation of the untreated enzyme.

Aim of the work with Carboxylesterase NP

- The specification of the final product was 99% ee
  - can be obtained from crystallisation of material with an ee of at least 97.5%
  - the target of this research was to synthesise the active *S*-naproxen with an enantiomeric excess of at least 97.5%
  - an E>200
  - reaction parameters consistent with a viable large scale process.

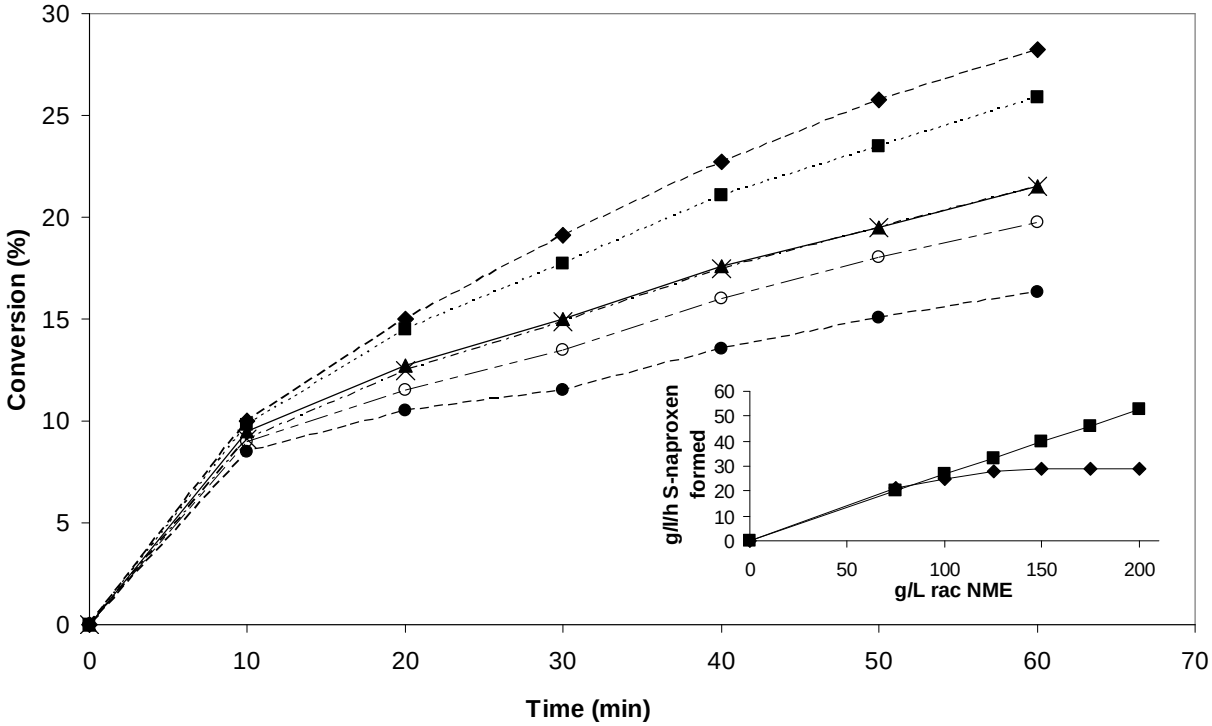
Comparison of substrate efficiency and enantioselectivity in the presence of different buffer and titration additive. The substrates were NME and NEE

Buffer	pH control	Substrate	(%) Conversion	% ee	E
MOPS	NH_4OH	NEE	49.1	94.2	106
Phosphate	NaOH	NEE	45.5	94.0	77
H_2O	NaOH	NEE	21.8	90.6	26
MOPS	NaOH	NEE	46.7	95.0	102
MOPS	NH_4OH	NME	49.3	99.0	810
TRIS	NaOH	NME	44.7	98.8	406
Phosphate	NaOH	NME	40.0	99.0	397

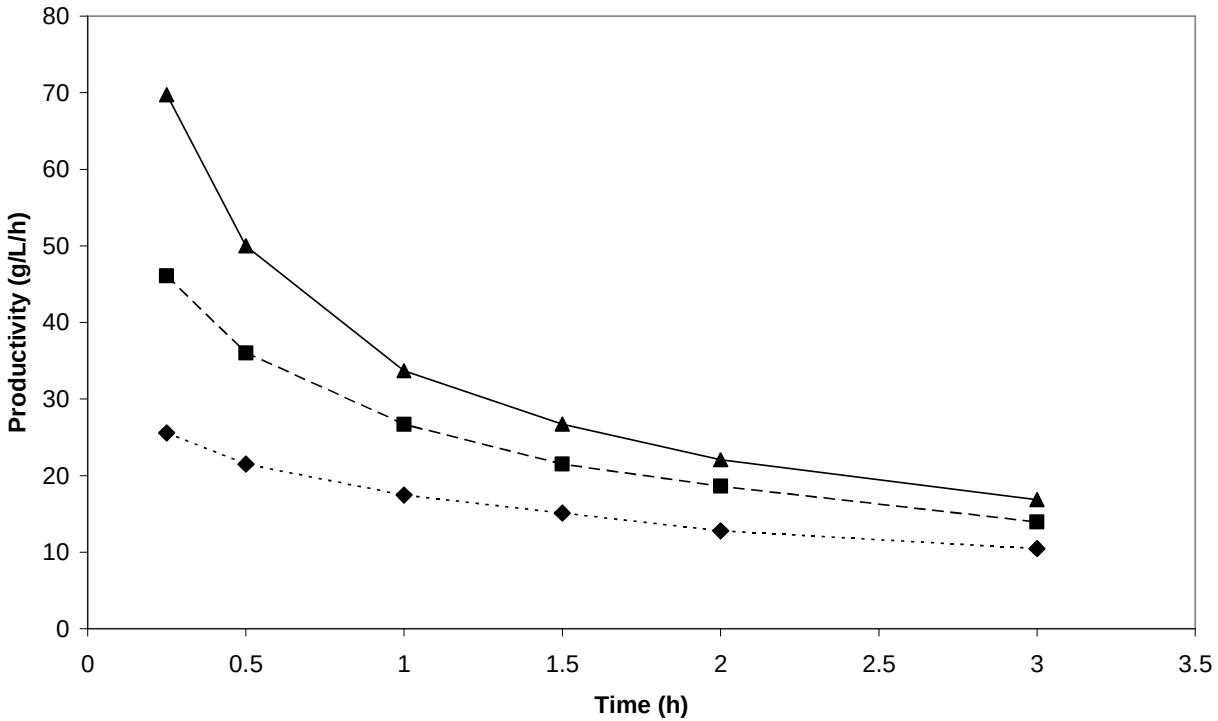
Enzyme stability

- Carboxylesterase NP had been stabilised to naproxen using formaldehyde at DSM laboratories
- Lower than expected conversions were obtained
- The enzyme was re-treated with formaldehyde and comparative resolution reactions were then performed using 265 g/ℓ NME and 18 units of enzyme/g ester
- After 5 hours, the conversions were 44.4% (22.2 g/ℓ/h) for re-treated enzyme compared to approximately 21% conversion by the stock enzyme
- This indicated that the protein reaction with formaldehyde was reversible. Hence, only re-treated Carboxylesterase NP was used in subsequent experiments.

Influence of substrate concentration on conversion of NME (75 g/ℓ ◆ ; 100 g/ℓ ■ ; 125 g/ℓ ▲ 150 g/ℓ x ; 175 g/ℓ ○; and 200 g/ℓ ●) by carboxylesterase_NP. The reactions were performed with constant ratio of enzyme to substrate, at 10 standard units of enzyme/g ester.



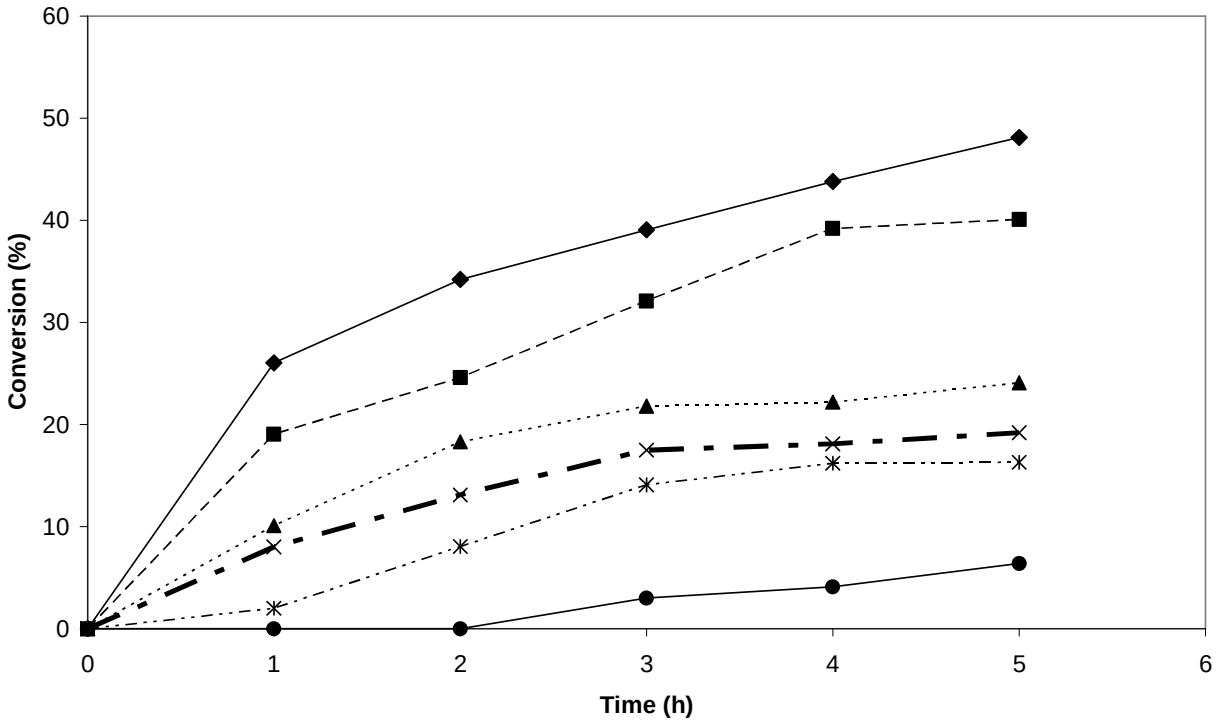
Initial rate results expressed in terms of productivity with a fixed substrate concentration and varying enzyme concentrations (♦) 5 U/g ester, (■) 10 U/g ester and (▲) 20 U/g ester.



Inhibition of the enzyme

- To isolate the influence of the unreacted enantiomer *R*-NME, which could potentially be a competitive inhibitor, experiments were run with *S*-NME alone.
- The results (not shown) indicated that *R*-NME did not influence the reaction rate of the resolution
- S-naproxen (product) was added to the start of the reaction
- From the results it was clear that the presence of *S*-naproxen was detrimental to the enzyme activity
- Methanol is formed as a by-product during the reaction. Inhibition by methanol was therefore
- From the results it was clear that methanol slightly lowered the reaction rate (a 5% decrease in total conversion

Inhibition of Carboxylesterase NP by product: ♦ 0%; ■ 10%; ▲ 20%; X 30% ; * 40% ; and ● 50% mol/mol *S*-naproxen added (0 to 47 g/ℓ).



Conclusions

- Up to 46.9% conversion was achieved in only 5 hours (13.3 g/ℓ/h) with an ee of 99.0% and an E of 576
- The efficiency of the enzyme was high, and the enzyme catalyst at 10 U/g ester only contributed approximately 3% to the cost of manufacture of *S*-naproxen.
- The water insoluble substrate forms a slurry in the reaction mixture, and particle size had an influence on reaction rate, with the smaller particle size providing the faster conversion rate
- Increasing the enzyme concentration also enhanced the reaction rate – by increases process cost.
- Only Tween 80 added for increased solubility, provided acceptable resolution rates and was not detectable in the product after re-crystallisation.

Validation

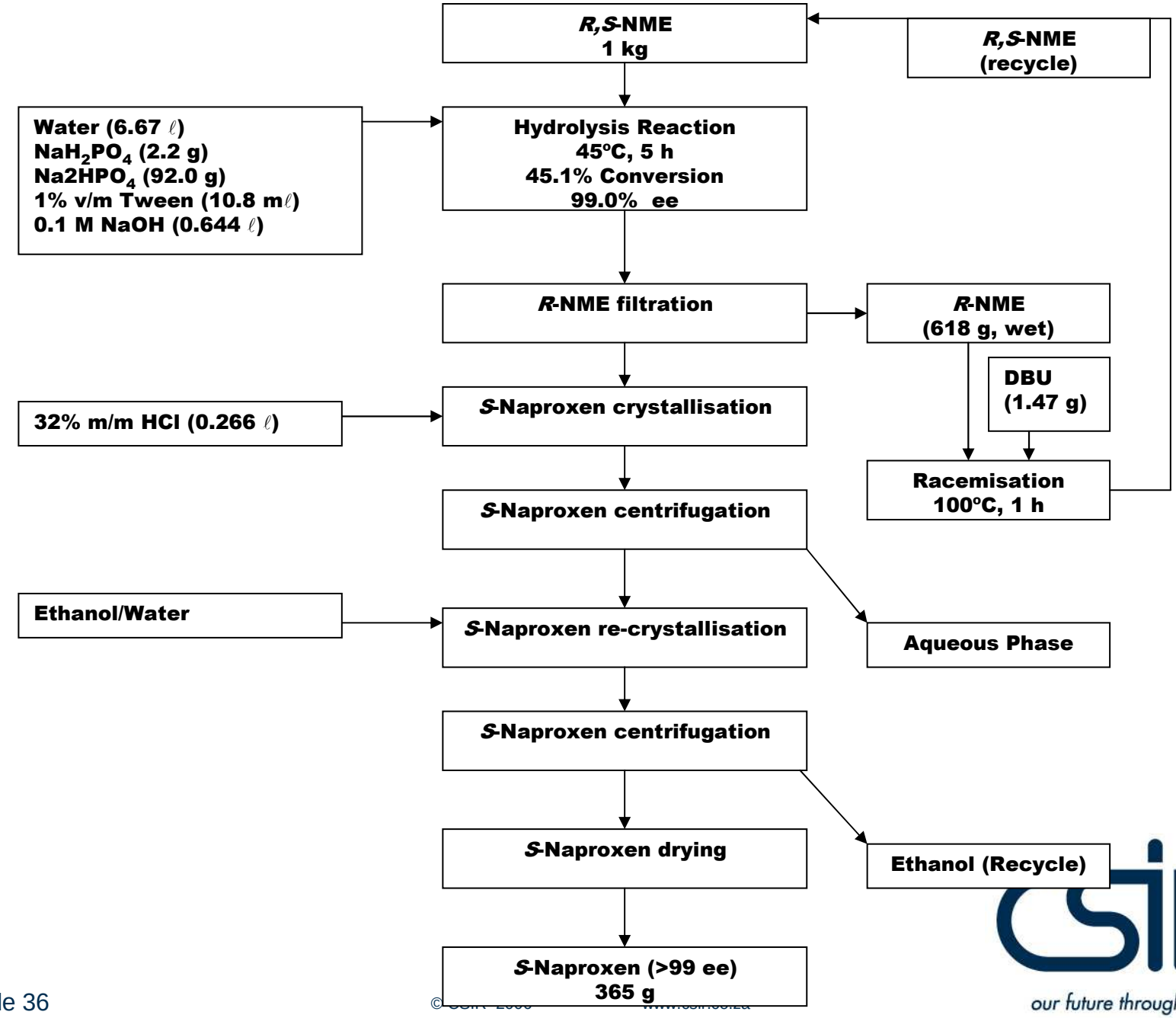
- This study has demonstrated that the biocatalyst was robust and performed well under process conditions.
- The process was validated by determination of the parameters which are quality critical.
- It was found that temperature was the most critical parameter.
- Increasing the temperature above 57°C not only decreased the conversion rate but also had a negative effect on the enantiomeric excess of the product
- At the lower temperatures (35-45ºC) increasing pH did not influence ee_p

Downstream Processing

- Recycling of the *R*-NME via racemisation with DBU improved the commercial viability of the process
- Successful integration of the reaction into a full process depended on the influence of the racemisation agent (DBU) that would be carried in the reaction through substrate recycling.
- DBU at catalytic concentrations was found to have no significant influence on the biocatalytic resolution reaction rate or quality of the product during substrate recycling experiments.
- The soluble product, *S*-naproxen could easily be separated from the insoluble substrate

Product quality

- The S-Naproxen yield on *rac*-NME for the isolated biocatalysis step was ~ 45%.
- With racemisation and recycle of unconverted NME the yield was increased to > 90%.
- The overall yield for the whole process starting with R,S-naproxen and esterification thereof to the racemic NME, conversion, racemisation and purification was ~77%
- Final product purity was >99%, with residual surfactant, protein, and formaldehyde levels in the ppm range.



Acknowledgements

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The project has resulted in 3 peer reviewed articles, 1 patent and a technology package which was licensed to an international pharmaceutical company

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