

Decisionmaking in the development of a biocatalytic route for resolution of *S*-naproxen – from screening to scale-up

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The non-steroidal anti-inflammatory drug naproxen is most effective as the single *S*-naproxen enantiomer. However, typical synthetic routes to naproxen yield the racemate of both the *R* and the *S* stereoisomers. Biocatalysts can be used to resolve racemic mixtures of naproxen esters using esterases or lipases. During research and development of this process we reached several decision points based on biocatalyst selection, reaction engineering, and process definition. These included reaction type (hydrolytic versus esterification options), substrate selection, biocatalyst selection, and reaction conditions. The study began with identification of a suitable lipase or esterase for biocatalytic enantiomeric resolution of *R,S*-naproxen to yield the single enantiomer *S*-naproxen with an enantiomeric ratio (E) in excess of 200. Approximately 650 unidentified fungi, yeasts and bacteria from culture collections were screened and more than 80 commercially available esterases and lipases. From this 9 enzymes were chosen to optimise using statistically designed experiments to find the most important factors which influenced the conversion and enantioselectivity. During the process development, decisions were made regarding hydrolytic versus esterification options, enzyme type, substrate size, co-solvent, and physical parameters. Final considerations were the optimised conversion and enantiomeric excess, reaction productivity, and enzyme cost to give a process which would be feasible on large scale.

The result was a commercially viable reaction yielding an E of approximately 500 and enantiomeric excess of 99%. The decisions behind the selection of the route are broadly applicable to other biocatalytic processes.

Keywords enantioselective catalysis; bioprocess engineering; enzyme; *S*-naproxen; enantiomeric ratio; enantiomeric excess; biocatalysis

1. Introduction

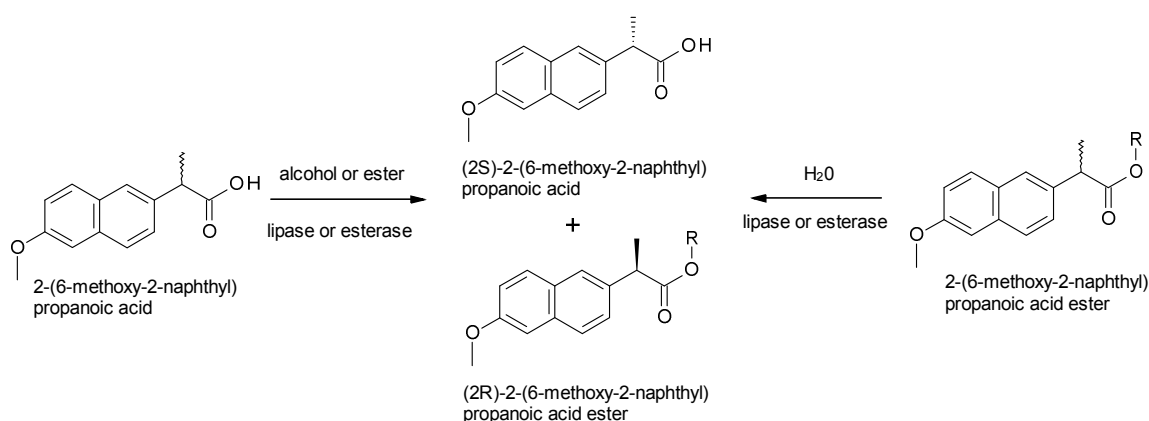
The demand for chiral pharmaceuticals is increasing rapidly, with 70% of new drugs entering the market in 2010 expected to be chirally pure [1]. The unwanted enantiomer is often associated with detrimental side-effects or lacks activity. However, separation of racemic mixtures is not always simple, and as a result of their stereospecificity, enzymes have become one of the more favoured methods for resolution of racemic mixtures [2].

Naproxen, (2-(6-methoxy-2-naphthyl) propanoic acid, is a non-steroidal anti-inflammatory pharmaceutical with a stereogenic centre. The *S*-isomer of naproxen is approximately 30 times more active than the unwanted *R*-isomer, which incidentally causes gastrointestinal disorders [3]. Therefore, separation of the enantiomers of naproxen is an important part of any synthetic process that yields the racemate as an intermediate. The use of lipases and esterases for stereoselective synthesis has been studied extensively [4] and therefore kinetic enantioselective resolution of naproxen esters by an esterase or lipase can provide the basis of a stereospecific synthetic route [5,6].

The overall aim of the research in our laboratories was to develop an enzymatic route for the production of *S*-naproxen which will be feasible on large scale. The purpose of this paper is to share the learning experiences obtained during the search for the ultimate process.

2. Reaction selection

The first decision point was to use either a hydrolysis, esterification or transesterification reaction according to Scheme 1. Esterification and transesterification are best performed in organic solvents, which provide the advantage of higher hydrophobic substrate solubility. Esterification is limiting in that it results in the formation of water, which will then allow for the reverse reaction to occur, creating equilibrium. The hydrolysis can be done in an aqueous solution, leading to an environmentally more friendly process, but the substrate has a very limited solubility in water and hence may limit reaction kinetics. Transesterification was investigated as the first option.



Scheme 1. Reaction scheme for conversion of racemic naproxen

2.1 Transesterification

Transesterification was attempted using the vinyl ester of racemic naproxen. The leaving vinyl group comes off as acetaldehyde and leaves the reaction as a gas, thus making the reaction irreversible. In initial experiments, transesterification reactions involved the alcohol methanol dissolved in *n*-heptane and were catalysed by *Candida antarctica* lipase (Fluka), Lipomax CXT (Genencor) and Lipolase (Novozymes) lipase enzymes. A sample taken after 24 hours indicated that almost complete conversion (93.98%) and an *ee* of 98% after 24 hours was achieved with a productivity of 2.9 g/L/h using *C. antarctica*. No reaction occurred when Lipomax was used, while the conversion using Lipolase was not significant enough to warrant further investigation.

The effect of various solvents on the rate of reaction, *ee* and conversion which together contribute to the enantiomeric ratio (*E*) utilising *C. antarctica* lipase as catalyst were also determined. It was found in a limited study that there was a direct correlation between the polarity of the solvent and *E* for a limited selection of solvents. The highest *E* was achieved in either cyclohexane (*E* = 12, polarity 0.2) or heptane (*E* = 10, polarity 0.1), and the lowest *E* in chloroform (*E* = 7.6, polarity 4.1). However this trend could not be extrapolated to other solvents in a larger study. This implies that broad solvent studies are important.

The fastest rate of reaction was observed in heptane where the *S*-naproxen vinyl ester was obtained in an *ee* of the substrate of 72.4% after 6 hours at a productivity of 6.5 g/L/h and specific activity of 268 mg/g/h. Cyclohexane also gave good results (42.2% *ee* after 6 hours, a productivity of 4.3 g/l/h and specific activity of 180 mg/g/h). In both case an *ee* of 98% was obtained after 22 hours. In the transesterification of vinyl ester and methanol, an *ee* of 98% was typically achieved at *S*-naproxen conversions of around 65 - 70% (giving an *E* of up to 168). However, a large quantity of *C. antarctica* lipase enzyme to substrate was required, which resulted in low specific activity which was not feasible for scale-up. This was also true for the other enzymes mentioned above.

Nine other solvents, differing markedly in their physical properties, were screened to determine their influence on the *C. antarctica* lipase catalysed transesterification with methanol. No trend was observed between any physical properties of the solvent (be it polarity, dipole moment, dielectric constant or log *P* value). Toluene, chloroform and xylene were unsatisfactory owing to the low final % *ee* of the vinyl ester and productivity, while the low *E* value for *n*-hexadecane excluded the use of this solvent. Using both cyclohexane and heptane as co-solvents, the effect of the alcohol used in the transesterification reaction, starting with methyl ester, was investigated. Ethanol, butanol and isopropanol were used, but GC and HPLC results revealed very low conversions, probably due to the fact that alcohols can participate in the transesterification reaction and compete with the vinyl acetate.

2.2 Stereoselective esterification of racemic naproxen

An experiment was done to determine if selective esterification was possible with the ChiroCLEC-CR, an enzyme which we had shown was highly selective in the hydrolysis of esterified racemic naproxen [6]. The results showed that although esterification did take place and conversions between 15 and 20% were reached with the alcohols tested (methanol, ethanol, 1-Hexanol, 2-propanol and isoamylalcohol), no stereoselectivity was displayed.

Due to the transesterifications and selective esterification in organic solvent not giving the required *E*, the time needed for the conversion and the amount of enzyme required which impacted on the specific activity, the decision was made to attempt stereoselective hydrolysis of racemic naproxen ester.

2.3 Hydrolysis

2.3.1 Microorganism screening

More than 250 uncharacterised fungi, yeasts and bacteria from the CSIR (South Africa) culture collection and 400 fungi and yeasts from the Mercer culture collection (University of the Free State in South Africa) were screened for esterase and lipase activity. Twenty one microorganisms from the CSIR collection with very strong extracellular lipase or esterase activity and 7 organisms from UFS were selected and preliminary optimisation done. None of the microorganisms exclusively hydrolysed *R,S*-NEE (racemic naproxen ethyl ester) to yield *S*-naproxen. This is probably due in part to the presence of multiple esterases and lipases in microorganisms. Overall activities were very low and gene isolation and over-expression would have been needed in order to evaluate the potential of each of these activities. Hence wild-type organisms were not suitable for cost-effective process development at the time.

2.3.2 Commercial Enzymes

For the hydrolysis evaluation, a rapid and small scale (1 ml) reaction method was used to screen for suitable biocatalysts for the hydrolytic resolution of ethyl esters of *R,S*-naproxen. Only a small number of enzymes, from the group of more than 80 hydrolytic enzymes, were able to provide some selectivity during conversion, as reported earlier [7]. From the screening process nine enzymes which displayed some enantioselectivity were selected for further optimisation in statistically designed experiments as shown in Table 1. During these experiments parameters such as pH, temperature, addition of surfactant, as well as substrate and enzyme concentration were investigated. The conclusion from the results was that physical parameters have different effects on enzyme activity and enantioselectivity, but even though large improvements were made in some of the enzymes, only three ESL001-01 (Diversa), ChiroCLEC-CR (Altus Biologics) and Carboxylesterase NP (DSM) gave results which warranted further investigation.

Table 1. The nine enzyme selected for further optimisation via statistically designed experiments for the enantioselective hydrolysis of racemic naproxen ethyl ester (NEE)

<i>Enzyme</i>	<i>Activity on NEE</i>	<i>%C</i>	<i>ee_p (%)</i>	<i>E</i>
NOVO 388	21.4 mg/ml/h	17.8	38	2.4
NOVO 398	16.9 mg/ml/h	8.4	70.6	6.2
Hog liver esterase	2 mg/mg/h	38	33	2.4
<i>A niger</i>	0.32 mg/mg/h	4.4	43.4	2.6
<i>Mucor miehei</i>	0.36 mg/mg/h	3	27.8	1.8
<i>A oryzae</i>	0.56 mg/mg/h	5.2	68	5.5
ESL 001-01	1.5 mg/mg/h	13.5	87	16.3
ChiroCLEC-CR	0.33 mg/mg/h	40	99	396
Carboxylesterase NP	0.96 g/ml/h	29.8	98	149.2

3. Hydrolysis Reaction development

Due to the encouraging results obtained during the screening for stereoselective hydrolysis using commercial enzymes, it was decided to optimise the ESL001-01 enzyme due to its lower cost than ChiroCLEC-CR (an immobilised form of *Candida rugosa* lipase) as well as the ChiroCLEC-CR and Carboxylesterase NP due to their excellent selectivities. Several parameters were investigated to overcome the problems associated with working with an almost insoluble substrate. Besides the normal parameters such as substrate type, solvent type, addition of surfactants and pH, one possibility was to find a biocatalyst that would be stable at higher temperatures where increased naproxen ester solubility occurs.

Thermostable enzymes are to be discovered in thermophilic organisms [8, 9], but these are often difficult to cultivate for commercial overproduction of enzymes. Hence we included thermostable enzymes isolated from a metagenomic library [10] from an extreme environment, in particular ESL-001-01 from Diversa. Immobilisation can also provide enhanced thermostability [11], and hence we applied a CLEC (Cross-Linked Enzyme Crystal, Altus Biologics) [12,13]. A third alternative was the application of chemical enzyme modification as reported by Mutsaers et al [14] on the Carboxylesterase NP.

3.1 Optimisation of the thermostable ESL001-01 for enantioselective hydrolysis of naproxen ester

Design of Experiments (DOE) using Design-Ease (Stat-Ease Inc USA) was used to optimise the performance of ESL-001-01. The factors investigated were pH (pH 5, 7, 9) and substrate concentration (2, 4, 6% m/v), enzyme to substrate ratio (1:50, 1:100, 1:275, 1:500), PEG 400 co-solvent concentration (50, 60, 70% m/v), and temperature (50, 60 and 70°C). The results of the 36 reactions indicated that enzyme to substrate ratio had a major influence on activity, demonstrating that the substrate was sufficiently soluble to be accessible during the course of the reaction. Under these conditions increased temperature exerted a slightly positive effect, the influence of pH over the range tested was neutral, while PEG addition as a co-solvent was detrimental at these concentrations.

From the set of experiments evaluating the 1:50 to 1:500 enzyme to substrate ratio, the concentration of the enzyme at 1:50 (enzyme to substrate) was most effective and this parameter was carried forward into the next experiments. A smaller study (a half level 2^3 factorial DOE, with 12 reactions) subsequently explored the interaction of PEG at lower concentrations (0 and 20 % m/v) in combination with variations in temperature (50 and 70°C) and pH (5, 7, 9). Under these conditions both temperature (70°C) and PEG 400 addition (20% m/v) had a positive effect. Again the influence of pH was small, and the pH optimum was located near neutral.

Methanol addition (25%) has been shown to increase the enantioselectivity of the thermostable enzyme from *S. solfataricus* P1 towards naproxen methyl esters from an enantiomeric ratio of 24 to 30 [15]. Therefore we further investigated the effect of co-solvent addition to the reaction mixture, with PEG 400 as a control. Higher activities (10 – 15%) resulted from the addition of DMSO, acetonitrile and PEG 1000. These two solvents were investigated at different concentrations (5 – 20% m/v). Only 10% m/v DMSO resulted in slightly higher conversion (7% higher than the control). However we also found a definite trend of lower conversion and *ee* with increasing concentration of DMSO. Similarly Suzuki, *et al* [16] found that acetonitrile and DMSO decreased the activity of the esterase from a strain of *Sulfolobus tokodaii*.

While it is clear that the water-miscible solvents, PEG, DMSO and acetonitrile had a positive effect on the enzyme, the enzyme activity was severely impaired in the presence of water-immiscible solvents, (dichloromethane, toluene and heptane at 5% m/v), with only 17% activity remaining in the presence of DCM and toluene and 40% in the presence of heptane.

The surfactant Triton X-100 had a positive effect on ESL-001-01 specific activity at both 0.5 and 5% (m/v), increasing it by 36% compared to the PEG 400 (5%) control reaction. Similar results were found by Sunna *et al* 2002 [17] for the thermoalkalophilic esterase from a *Bacillus* species, which was stimulated by 28% by Triton X-100 (0.1%). Jung *et al* 2003 [18], found that an esterase from *Bacillus megaterium* was stimulated to ~260% activity at 2% Triton X-100. The activity was increased by decreasing the K_m for the substrate (*p*-nitrophenyl caproate). However in the present study Triton X-100 also had the undesirable effect of decreasing the enantioselectivity of ESL-001-01 to an *ee* of 81%.

The response of ESL-001-01 to another non-ionic surfactant, Tween-80, was negative (48% activity of the control reaction). Although Jung *et al* [18], found that the 2% Tween 80 stimulated the esterase from *B. megaterium* by 300%, the same detergent completely inactivated the *Bacillus* species thermoalkalophilic esterase [17].

The strong influence of temperature identified in the optimisation experiment was investigated over a wider range. The kinetic rate of conversion of NEE by esterase ESL-001-01 under these conditions (neutral pH, 5% m/v PEG) had an optimum reaction temperature of 85°C. Under these reaction conditions the enantiomeric ratio (E) could be determined to be between 12 – 20 over the range of 0 – 40% conversion.

Increasing the reaction temperature had a negative effect on enantioselectivity, decreasing the *ee* from 86 to 81% at a similar degree of conversion (Table 2). Hence although the conversion increased, no significant increase in E could be achieved. The highest E was at 70°C and hence it would not be possible to use the enzyme at its optimum temperature.

Table 2. Influence of temperature on enantioselectivity of ESL-001-01.

Temperature °C	%Conversion	% enantiomeric excess (<i>ee_p</i>)	E
60	10	88	17.21
65	13	87	16.38
70	34	86	20.5
75	32	84	16.9
80	33	82	15.0
90	35	81	14.5

The role of temperature in the enantioselectivity of lipases and esterases has been shown to vary with different enzymes. Sehgal and Kelly [19] found that a thermostable esterase SsoEST1 (from *S. solfataricus*) exhibits decreased enantiomeric ratio (from 24 to 3) for naproxen methyl esters with increasing temperature over the range 50 – 70°C respectively. Conversely the same researchers found that the lipase from *C. rugosa* and the lipase Palatase® had

improved enantioselectivity with increasing temperature. However, in most cases the highest *ee* has been achieved at more moderate temperatures.

A mesophilic *Bacillus* strain was used as a whole cell biocatalysts capable of an *ee* of 86.47% at 40 – 50% conversion of *R*-naproxen from NEE [20], but this was at the relatively low reaction temperature of 30°C. Even better enantioselectivity was achieved using a lipase from a *Trichosporon* species to generate *S*-naproxen from naproxen methyl ester (NME) at an *ee* >99% and an *E* of approximately 500 [5], but again at only 32°C. The thermostable enzyme from hyperthermophile *S. solfataricus* P1 has been applied to naproxen methyl esters, providing an *ee* of 92.9% at low conversion (8.3%) at 50°C [9].

The influence of substrate type was investigated using the methyl ester of racemic naproxen (NME). In a reaction of 1:10 enzyme with PEG 400 the conversion was 39.95%, the *ee* was 91.3% providing an *E* of 40.9. The conversion with the same ratio of enzyme to substrate in the presence of Triton X100 was 46.3% in 5 hours with an *ee* of 93.2% resulting in an *E* of 70.

The conclusion from the work was that none of the general physical parameters such as pH, and temperature had a significant effect on the reaction *E*. The substitution however of the substrate type from the ethyl to the methyl ester gave a significant increase in *E*. The addition of Triton X100 instead of PEG 400 also contributed to an increase in the *E* from 40.9 to 70. Unfortunately an *E* of 70 with an *ee* of 93.2% was insufficient for a feasible large scale process and will not result in an *ee* of 98% for *S*-naproxen.

3.2 Optimisation of ChiroCLEC CR or enantioselective hydrolysis of racemic naproxen ester

The CLEC enzymes are enzyme crystals which are stabilised by cross-linking them with bifunctional reagents such as glutaraldehyde. These CLECs have been found to be catalytically very active even at higher temperatures and can be used in organic solvents. ChiroCLEC-CR is a CLEC of the *C. rugosa* lipase and was more effective than the free enzyme.

Unlike ESL001-01, this enzyme had a higher activity for NEE than NME. To determine the reaction kinetics, reactions were done with the substrate concentration within the solubility range. It was determined that V_{max} and K_m were 4.95 g/g/h and 4.05 g/L respectively for the reaction [6].

Statistically designed experiments (Design-Ease) were performed to optimise temperature, enzyme to substrate ratio, substrate concentration, agitation, reaction time, pH, buffer concentration and co-solvent addition. The addition of a full range of organic solvents, surfactants and PEG, revealed that PEG 1000 improved conversion rates by approximately 10 fold [6]. Further efforts to increase the activity of the enzyme involved the use of additives such as *tert*-butanol, *tert*-amyl alcohol, olive and soya oil. Addition of all four additives led to a dramatic decrease in activity and enantioselectivity - between 60 and 85% decrease in activity and almost 25% decrease in enantioselectivity.

Although the reaction rate could not be improved using acetone as co-solvent, improved results were obtained with increased temperature (from 30 to 50°C) and high enzyme to substrate ratio (1:50). Optimisation efforts [6] resulted in a more than 20-fold improvement of activity, while the excellent stereoselectivity of the enzymes was maintained, yielding a constant *S*-naproxen of >98% *ee*. The optimised conditions were determined to be 10% m/v substrate, and enzyme to substrate ratio of 1 : 50 at 50°C and pH 5 with addition of 41% PEG 1000. The optimisation of all the conditions collectively led to an increase of the conversion and the enantiomeric ratio. The specific activity was increased from 0.187 g/g/h to 3.9 g/g/h during the optimisation of the conditions and the biggest influence was due to the addition of PEG which resulted in a 10 fold increase.

Other efforts to increase the economical viability of the process included investigating the thermostability and recycling capabilities of the enzyme. The thermostability of the CLEC-CR was determined to be a half-life at 40°C of 3 days and at 55°C, 20 hours. The enzyme was recycled six times at 40°C over a total period of 48 hours. During this time the apparent specific activity dropped by 57%. The yield of product on substrate was 64%, but this could be improved by longer reaction times and better mixing. The yield of the product on enzyme was 17.2 g/g or about 21% of the theoretical maximum determined from kinetics and thermal denaturation experiments. Hence in spite of activity improvements and an excellent *E*, the cost to yield ratio of this reaction was not economical due to the inadequate half-life of the biocatalyst under these reaction conditions.

3.3 Optimisation of Carboxylesterase NP for selective hydrolysis of naproxen ester

The Bio-organic Chemistry Department at DSM (previously Gist-Brocades) did extensive screening on naproxen esters which led to the isolation and identification of a *Bacillus subtilis* (Thai I-8) strain [21]. HPLC fractionation of the crude enzyme from *Bacillus Thai I-8* demonstrated the presence of two enzymes: a highly enantioselective esterase (Carboxylesterase NP) and a contaminating, less specific lipase [21]. The gene coding for the esterase was identified and cloned into *B. subtilis* I-85 [22] resulting in the r-DNA strain *B. subtilis* I-85/pNAPT-7. The productivity of this r-DNA strain was more than 800 times higher than the wild type strain Thai I-8. The pure carboxylesterase had a molecular weight of 32 000 and a specific activity of 10 U/mg protein [21].

The researchers were faced with a problem well-known when using enzymes under industrial conditions, and that is their lack of stability. They encountered this problem with the carboxylesterase at high substrate concentrations.

Irreversible inactivation was noticed when Carboxylesterase NP was incubated with 30 g/L *R,S*-naproxen ester (pH 9, 40°C and 2% Tween 80). The carboxylesterase as such, without substrate and product being present, was stable for several hours. It turned out that the enzyme was inactivated by naproxen formed during the hydrolysis of naproxen ester. As a working hypothesis, the researchers assumed that the naproxen would interact with the amino groups of basic amino acids at the surface of the enzyme, thereby allowing the hydrophobic bulk of the naproxen to interfere with the folding of the protein structure. By chemically modifying the amino acids involved, it was anticipated that new enzyme preparations could be produced which are more resistant to the high acid-"stress" conditions. This hypothesis was confirmed by the modification of Carboxylesterase NP with increasing formaldehyde concentrations. It was shown that the untreated enzyme was completely inactivated on incubation with naproxen for 90 minutes at 40°C. Although formaldehyde treatment of the esterase gave rise to a partial loss of activity, the enzyme treated with formaldehyde concentrations of 1% or higher, proved to be completely stable on incubation with naproxen (15 mg/ml) for 90 minutes at 40°C. All the lysine modifying agents (glutaric anhydride, succinic anhydride, glyoxal, glutaraldehyde and formaldehyde) showed an improved performance, the best results were obtained with formaldehyde, glutaraldehyde and glyoxal [21, 22].

The enzyme was provided by DSM for further investigation. The activity of the enzyme was evaluated with respect to the methyl and ethyl esters of naproxen. From the results the conclusion could be made that the methyl ester allowed for better enantioselectivity than the analogous longer carbon chain ethyl substrate and for this reason NME was selected for further experiments. Reactions in MOPS buffer and with titration using NH₄OH instead of NaOH gave an E of more than double that in Tris and phosphate buffer due to almost complete conversion, however phosphate buffer was chosen due to the economics on larger scale [23].

The productivity is important from a scale-up perspective as the amount of product per volume and time determines the economics of the resolution step. A fixed substrate concentration of 150 g/L racemic NME was used but the amount of enzyme was varied. The initial conversion of the substrate was dependent on the amount of enzyme added until the enzyme was saturated with substrate, with the productivity in terms of grams naproxen formed (g/L/h) being the highest for 20 units of enzyme. From all the results a relationship between the rate of reaction and enzyme load was evident.

In all the resolution runs, the productivity was very high in the first hour and then decreased quite dramatically. It was thought that this could be due to loss of enzyme activity at high temperatures. Experiments were done in which the enzyme was pre-incubated with naproxen for 2, 5 and 20 hours before addition of the substrate, to test this theory. From the results we found that ageing of the enzyme did lead to a decrease in the conversion rate, but the same trend as before was still noticeable, that is, in the first hour the activity was the highest and then it dropped quite significantly.

Although Carboxylesterase NP had been stabilised using formaldehyde at DSM laboratories, lower than expected conversions were obtained, and hence the enzyme was re-treated with 2300 ppm formaldehyde for 4 hours at 40°C [23] and comparative resolution reactions were then performed using 265 g/L and 18 units of enzyme/g ester. After 5 hours, the conversions were 44.4% for re-treated enzyme compared to approximately 21% conversion of the untreated enzyme. This indicated that the reaction with formaldehyde was reversible.

The previous results indicated that the reaction might be hampered by inhibition potentially due to the unreacted R-NME, the product *S*-naproxen, or the byproduct, methanol. Following the inhibition studies it was clear that the major inhibition was caused by the product *S*-naproxen and very little inhibition resulted from the by-product, methanol [23].

As part of the optimisation, the interaction and influence of different parameters such as pH, temperature, agitation, co-solvent and surfactants were investigated on the enantioselectivity as well as the conversion. These parameters may have an influence on the scalability of the reaction. It was found that pH as well as temperatures as single factors did not have a significant influence on the quality of the product, but the interaction of these two parameters was important. If the temperature increased to 57°C while the pH was at 10, it resulted in decreased *ee*. Agitation had no influence on the reaction, while the addition of Tween 80 had a major impact on the conversion as it resulted in higher substrate concentration being available. This confirmed the influence of solubility and dissolution rate on the rate of conversion. Another important factor was the buffer concentration, which at lower molarity resulted in faster conversion without influencing the product quality. Increasing the buffer molarity to 1 M negatively influenced the conversion as well as the enantioselectivity. The reaction was scaled to using 90 g of racemic naproxen in 600 mL and then to 1 kg of racemic naproxen in 6.67 L of buffer. These runs were used to determine the scalability as well as influence of parameters to which the reaction may be sensitive on industrial scale, such as pH control and temperature control. *S*-naproxen was produced and optimised with an *ee* of >99%, a conversion of 46.9%, and an E of >500. This reaction was later developed into a full scale process [23].

4. Conclusion

The commercial naproxen ester resolution reaction required an E of >200 and a product *ee* of > 98%. The screening of commercially available enzymes resulted in a number of leads which were used to optimise conditions. From the leads only ESL001-01, ChiroCLEC-CR and Carboxylesterase NP gave promising results which were investigated further. The biggest influence on the ESL-001-01 was the addition of PEG 400 or Triton X-100 as well as the substrate type. Because the enzyme appears to be an esterase, the use of a methyl instead of an ethyl ester of naproxen, had a large

impact and the methyl ester resulted in an *E* increase of approximately 4 times to 70. This suggest that substrate engineering is perhaps one of the most important factors in biocatalytic reaction development.

The optimisation of ChiroCLEC-CR resulted in an excellent conversion and enantioselectivity with an *E* >300. The immobilised enzyme price and stability in this particular reaction environment, however made it unfeasible for scale up to a commercial process.

The optimisation of the Carboxylesterase NP enzyme resulted in a 78% overall yield from esterification, biocatalysis and downstream processing and an *ee* of >99% with an *E* of approximately 500. The conversion was rapid – approximately 5 hours with 10 Units of enzyme per gram of ester even though the solubility of the substrate was a limiting factor. The solubility and dissolution problem were overcome by the addition of the surfactant Tween 80. The resulting process can be implemented on commercial scale for the production of *S*-naproxen with a purity sufficient for pharmaceutical use (Table 3). In spite of the use of a poorly soluble substrate (which was a slurry) commercially relevant yields and productivities were obtained. This suggests that researchers should not be afraid of using slurries in biocatalytic reactions. In conclusion the overall decision process during the research and development used is depicted in Figure 1.

Table 3 Comparison of the most important parameters of ESL 001-0,1 ChiroCLEC-CR and Carboxylesterase NP

Parameter	ESL001-01	ChiroCLEC-CR	Carboxylesterase NP
Substrate concentration (g/L)	100	100	150
pH	6	5	8.75
Temperature (°C)	85	55	45
Surfactant	20% PEG 400	41% PEG1000	1% Tween 80
Conversion (%)	50	45	43
Time for conversion (h)	24	24	5
<i>ee</i> (%)	93.2	99	99.2
Volumetric productivity (g/L/h)	2.08	1.71	12.9
Specific acitivity (kg/kg)	33.5	20.5	190.9

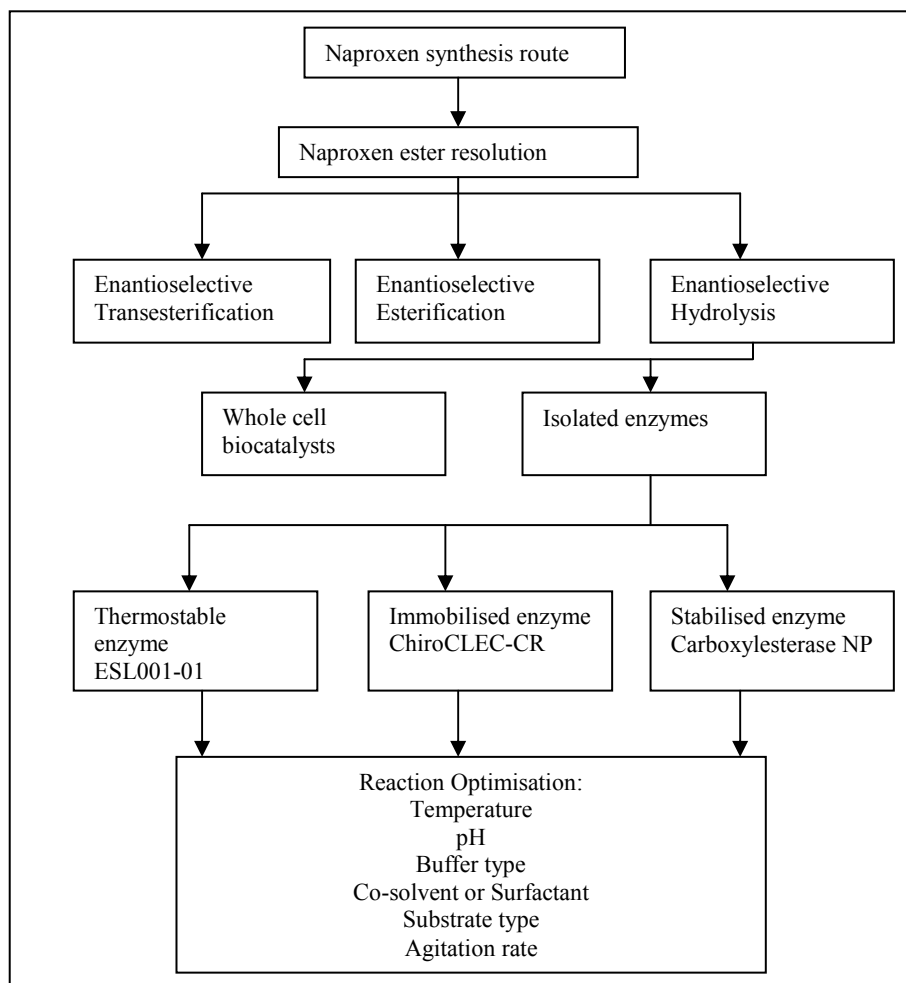


Fig 1: Decision tree for the resolution of racemic naproxen.

Acknowledgements AECI for funding, DSM for supply of the Carboxylesterase NP, the research team at CSIR as well as analytical staff for analysis. This work was presented in part at the CATSA conference, 9-11th November 2008, Parys, South Africa.

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