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Optimisation of the enantioselective biocatalytic hydrolysis of naproxen ethyl ester using ChiroCLEC-CR

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Abstract

In a biocatalytic reaction the immobilized lipase ChiroCLEC-CR enantioselectively hydrolysed a naproxen ethyl ester racemate, yielding (*S*)-naproxen with an enantiomeric excess of more than 98%, an enantiomeric ratio (*E*) of more than 100, and substrate conversion in excess of 40%. Statistically designed experiments were performed to optimise temperature, enzyme to substrate ratio, substrate concentration, agitation, reaction time, pH, buffer concentration and co-solvent addition. Optimisation efforts resulted in 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 kinetic parameters V_{max} and K_M were determined to be 0.359 μmol/min/mg and 17.6 mM, respectively.

The optimised reaction conditions were 10% (m/v) substrate, and enzyme to substrate ratio of 1:50, at 50 °C and pH 5 with addition of 41% PEG 1000. In spite of these kinetic improvements, the stability of the biocatalytic activity under these conditions was poor, limiting the number of possible recycles.
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Keywords: *S*-Naproxen; Naproxen ester; Hydrolysis; ChiroCLEC-CR; Enantioselectivity; Lipase; *Candida rugosa* lipase

1. Introduction

Although in nature lipases catalyse the hydrolysis of triacylglycerols to an alcohol (glycerol) and free fatty acids, they have attracted the interest of the chemical industry due to their wide substrate range and the fact that they do not require cofactors. Currently, approximately 25% of the enzymes used in industrial biotechnology applications are lipases, where they are utilised for hydrolysis, esterification, transesterification and inter-esterification reactions. This class of enzymes has found particular application in the hydrolysis of unnatural, water-insoluble esters, of which the substituted arylpropionic acid (such as naproxen) is an example [1].

The ability of enzymes to discriminate between the enantiomers of racemic substrates makes them valuable tools in the preparation of optically pure compounds. However, lower enantioselectivity has often been the experience when using commercial crude enzyme preparations, which are commonly chosen over highly purified enzymes because of availability, low cost, and higher operational stability [2]. This is generally caused by the presence of contaminating competing enzymes that may have opposing stereo- and regioselectivities, and in this way can reduce the optical purity of the final product. A typical example is the commercial enzyme preparation of *Candida rugosa* lipase (CRL) (EC 3.1.1.3) [3], which is one of the most versatile and widely used enzymes in the resolution of esters, acids and alcohols [4] in both aqueous and organic media. However, in its most widely used form, this preparation is a mixture of several hydrolases [4] resulting in non-reproducibility and variable enantioselectivity.

Such problems in biocatalyst variability can be addressed by purifying enzymes followed by immobilization onto solid supports [5]. Although expensive, purification and immobilization may be necessary for the synthesis of optically pure drugs, which obviously requires a reliable catalyst.

Unfortunately, immobilization provides its own problems, such as the dilution of the specific catalytic activity by the inclusion of the support. Hence, a significant volume of the reaction vessel may be occupied by non-catalytic mass of the support, which consequently diminishes the reactor volumetric productivity. To overcome this problem a technology for forming stable (cross-linked) enzyme crystals (CLECs) was developed by Altus Biologics Inc. (USA), where microcrystals are cross-linked with a bifunctional agent such as glutaraldehyde [4,6]. These crystals are highly pure and

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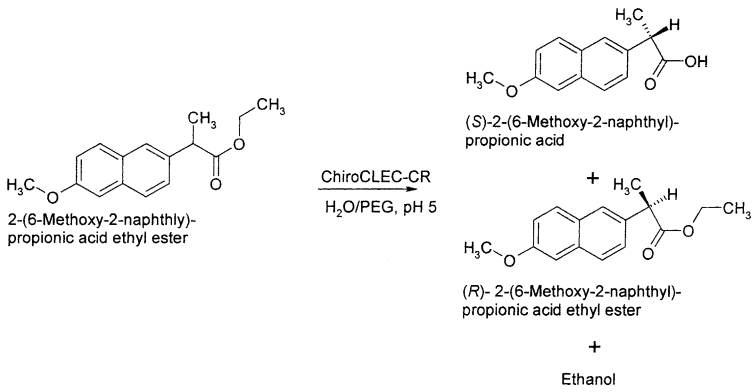

Fig. 1. Biocatalytic stereoselective hydrolysis of naproxen ethyl ester.

hence the CLEC of *C. rugosa* lipase (ChiroCLEC-CR) is expected to have improved enantio- and regioselective properties compared to crude preparations, thereby permitting kinetic resolution of chiral compounds through hydrolysis of carboxylate ester precursors. It was therefore chosen as the catalytic agent for this study.

Naproxen (2-(6-methoxy-2-naphthyl) propionic acid) is a commonly available non-steroidal anti-inflammatory drug. The *S*-isomer of naproxen is 28 times more active than the *R*-isomer (which also causes gastrointestinal disorders) [7]. Therefore, separation of the enantiomers has become an important part of any new (*S*)-naproxen synthetic process. The use of lipases for this technology has been studied extensively [8], but improvements in the efficiency of the biocatalytic reactions are still required to provide a commercial process.

The aim of the investigation was to demonstrate the improved chiral resolution properties of ChiroCLEC-CR on naproxen ethyl ester (NEE) (Fig. 1) and optimise the reaction kinetics.

2. Materials and methods

2.1. Chemicals

The substrates used in the experiments (*R,S*)-naproxen, (*S*)-NEE and (*R,S*)-NEE (where NEE is naproxen ethyl ester) were synthesised in these laboratories from (*S*)-naproxen (Sigma, USA). The racemisation of (*S*)-naproxen as well as the esterification of (*R,S*)-naproxen have been reported earlier [9]. ChiroCLEC-CR was obtained from Altus Biologics Inc. (USA). All other chemicals used were of analytical or reagent grade and purchased from commercial suppliers.

2.2. Analysis

The naproxen and NEE % (m/m) analyses were performed by HPLC using a 25 cm C18 ODS 2 column, eluted isocratically using a mixture of 70% acetonitrile and 30% buffer (aqueous 0.1% phosphoric acid). The *R*/*S* ratio was determined on a (*S,S*)-Whelkl/O/1 25 cm column eluted isocratically with a mobile phase of hexane:ethanol:acetic acid (95:5:0.5).

2.3. Biocatalytic hydrolysis of (R,S)-NEE

2.3.1. Standard reaction conditions

Hydrolysis experiments were performed in pre-weighed 5 ml reaction vials equipped with magnetic stirrer bars. Except where stated, the reaction volume was a total of 1 ml, consisting of 0.2 M ammonia acetate buffer (pH 5.0), 250 mg/ml NEE, and 2 mg/ml enzyme; the reaction was maintained at 55 °C for 5 h with agitation at 600 rpm. Reactions were terminated by addition of 1–3 volumes of acetonitrile to the suspension, which also dissolved any insoluble substrate or product. The reaction vials were then re-weighed to determine the reaction mass (i.e., total content of the vial) and subsequently the reaction mixture was filtered through pre-weighed Pasteur pipettes plugged with cotton wool (to retain the enzyme), into pre-weighed analysis vials. The concentration of product (% mass product/reaction mass) was determined by HPLC.

ChiroCLEC-CR was supplied as a slurry, and therefore for accurate determination of the exact amount of enzyme used in each reaction (and hence calculated specific activity) the dry enzyme mass was recovered and measured after the reaction. The cotton wool filters (mentioned above), were rinsed with 3–4 ml of methanol (to dissolve and elute residual PEG 1000), dried within the Pasteur pipettes at ∼80 °C overnight, and the enzyme dry mass determined by subtraction.

2.4. Biocatalytic hydrolysis of (R,S)-NEE

2.4.1. Michaelis–Menten enzyme kinetics

An important goal of reaction optimisation would be to overcome the reaction rate limitation imposed by the poor solubility of the substrate (NEE). Therefore, the maximum

Table 1
Solubility of (*R*,*S*)-naproxen ethyl ester and (*S*)-naproxen in PEG acetate buffer

Temperature (°C)	NEE solubility (g/kg)	(*S*)-Naproxen solubility (g/kg)
30	1.5	31.4
40	2.6	35.7
50	4.1	63.7
60	6.4	109.1
70	12.7	187.3

specific activity of the biocatalyst was determined in order to provide a target for reaction optimisation investigations.

Eight substrate concentrations (between 0.05 and 3 mg/ml) were chosen which fell within the solubility range of NEE in PEG 400/acetate buffer (Table 1). The reactions were performed with agitation in 20 ml of 50:50 PEG 400/acetate buffer pH 5. ChiroCLEC-CR (0.5 mg) was added to each reaction. Samples of 1 ml were taken at 0, 2, 4 and 6 h.

2.4.2. Influence of reaction conditions on enantioselective hydrolysis of racemic naproxen ethyl ester by ChiroCLEC-CR

To determine which reaction conditions had a significant influence on the specific activity, a statistically designed experiment was performed. The design was a 2^{5-1} (half factorial) with four midpoints designed with Design-Ease (Stat-Ease Inc., USA), resulting in 20 reactions. The reaction volume was fixed at 1 ml, while temperature (20 and 50 °C), stirring speed (200 and 600 rpm), acetone concentration (0 and 10% v/v) to improve the solubility of the substrate, enzyme to substrate ratio (1:200 and 1:50), and substrate concentration (10 and 25% m/v) were variables. The buffer used was a 50:50 PEG 1000/0.2 M acetate buffer pH 5. Four midpoint replicate experiments were included using the conditions: 37.5 °C, agitation speed of 400 rpm, 17.5% (m/v) substrate, 5% acetone, and an enzyme to substrate ratio of 1:125.

2.5. Effects of solubilisation and dispersion of NEE

Due to the low solubility of NEE in aqueous media it forms solid particles in suspension under the reaction conditions. This may result in a sub-optimal reaction rate owing to poor substrate availability through low solubilization rates. Addition of organic co-solvents, PEG, detergents and sonication were investigated with the aim of increasing the solubility of NEE, while retaining the enzyme activity.

Ten water-miscible solvents (ethanol, isoamylalcohol, dimethyl formamide (DMF), dimethyl sulfoxide (DMSO), acetone, tetrahydrofuran (THF), acetonitrile, PEG 400, dioxane and ethanediol at 2 and 10% v/v), and 7 water-immiscible solvents (chloroform, dichloromethane (DCM), toluene, diisopropyl ether (DIPE), cyclohexane, heptane and ethyl acetate at 10 and 50% v/v), were added as co-solvents and compared to a control reaction with 50% PEG 1000.

As the enzyme is usually washed with solvents (*t*-butanol and *t*-amyl alcohol) [6] at the end of a batch reaction, additional experiments were performed to determine their effect on enzyme activity in concentrations of 10 and 20%. Olive oil and soya oil were also tested at 20% (m/v) [10].

Three polyethylene glycol (PEG) species (namely PEG 400, (±)-2-methyl-2,4-pentanediol (hexylene glycol) and PEG 1000), in concentrations of 10–90% (v/v), were compared as solubilisation agents for NEE. Tween 80 at concentrations of 2 and 5% and a mixture of Tween 80 and PEG 1000 were also tested for their effect on activity. Substrate (NEE) concentration was 10% (m/v) and the reaction time was shortened to 2 h. Based on the results of these experiments, PEG 1000 was selected for further investigation and tested at concentrations of 50–100% (v/v) in 2 h reactions.

Sonication was attempted in order to disperse granular substrate and to form an emulsion, thereby increasing substrate availability to the enzyme. The reaction (50 mg NEE and 2 mg enzyme in PEG/acetate buffer) in a final volume of 1 ml, was sonicated for 4 h in a sonication water bath. In follow up experiments, a microtip was used for sonication over a range of 0.5–2.75 power output using a Vibracell Sonicator (Sonics and Materials Inc., Danbury, CT, USA) and sonication times tested (on-time set between 10 s and 1 min, and the off-time between 1 and 30 min).

To establish whether the enzyme crystals were being damaged by the ultrasound, a comparative experiment was done, in which the substrate was sonicated prior to addition of the enzyme crystals.

2.6. Determination of optimum reaction conditions

To determine optimum temperature, a PEG 1000/ammonium acetate buffer was used with 25% (m/v) substrate and 5 mg enzyme. The temperature range was explored between 40 and 70 °C (testing at increments of 5 °C). The optimum initial pH was determined using the same conditions with the pH being varied from 4.5 to 6 with increments of 0.5 at 55 °C.

For the purpose of reaction kinetic studies, the linear response range was determined (enzyme activity with time) in order to calculate the initial reaction velocity (υ). NEE (10% m/v) in PEG 1000/ammonium acetate buffer at pH 5 was incubated with 2 mg enzyme (1:50 enzyme to substrate ratio) in a total volume of 1 ml at 55 °C and the reaction sampled hourly for 7 h.

The optimum racemic substrate concentration (*R*,*S*-naproxen) was determined by performing the reactions at 55 °C, 50% (m/m) PEG 1000 for 2 h and varying the substrate concentration from 1 to 20% (m/v) in a reaction volume of 2 ml. As 2 mg enzyme was added, the substrate ratio therefore ranged from 1:10 to 1:200.

2.7. Investigation of enzyme inhibition

As this study was aimed at developing a reaction for a commercially viable process, the effects of process steps up-stream of the biocatalytic reaction had to be considered. Trace amounts of metal and other ions may be carried through to the reaction mixture from the substrate synthesis, and could have possible inhibitory effects on the reaction. To investigate this possibility, metal salts were each tested at three different concentrations (see Section 3). Reaction conditions were 55 °C, 10% (m/v) NEE, 2 mg enzyme, and a reaction time of 2 h.

The products ethanol and (*S*)-naproxen and the unreacted substrate (*R*)-NEE were also investigated for possible inhibition effects under reaction conditions. Ethanol was added to the reaction mixture prior to substrate addition, and was equal to the maximum molar concentration of (*S*)-naproxen that can be produced from 5% (m/v) (*R*,*S*)-NEE (11.3 μl per 2 ml reaction). Amounts of (*S*)-naproxen between 1 and 50 mg were added to a 2 ml reaction to investigate inhibition by this product. In a third set of reactions, combined inhibition by the two products was investigated. Pure (*R*)-NEE was unavailable for the inhibition studies, and hence variable amounts of enantiomerically pure (*S*)-NEE was added to (*R*,*S*)-NEE racemate (80 mg) to determine the effect of the *R* to *S* substrate ratio. Hence, the starting reaction mixtures contained 40 mg *R*-NEE and amounts of *S*-NEE between 40 and 150 mg.

2.8. Reaction scale-up

Accurate productivity and specific activity measurements required a larger scale reaction. Enzyme (40 mg) was added to NEE (2 g) in 20 ml PEG/acetate buffer and the reaction stirred at 55 °C for 24 h. Samples (1 ml) were taken every 2 h for the first 8 h and after 24 h the reaction was terminated with 20 ml acetonitrile.

2.9. Stability of ChiroCLEC-CR

Due to the expense of generating CLECs, a commercial process for resolving naproxen would demand enzyme recycle or use in a continuous process. Hence, the stability of this biocatalyst was of critical importance, and required evaluation. To each of two 25 ml round bottom flasks, 15 ml of a 0.2 M PEG 1000/acetate buffer pH 5 was added, followed by a 10 mM solution of $CaCl_2$ (as a stabilising compound [6]) and 30 mg of the enzyme. No substrate was included. The entire contents were stirred at 600 rpm for several days. One flask was incubated at 40 °C and the other at 55 °C to gauge thermal influence on stability.

The residual activity was determined by taking duplicate 1 ml samples from each flask, adding them to 10% (m/v) (*R*,*S*)-NEE in a reaction vial (enzyme to substrate ratio 1:50) and performing the standard reaction. Biocatalyst stability was also determined in a comparative experiment without stirring the round bottom flasks, to isolate the effect of agitation.

3. Results and discussion

A considerable problem with the NEE hydrolysis reaction is the very low solubility of the substrate. The solubility of (*R*,*S*)-NEE and (*S*)-naproxen was determined in PEG at different temperatures (Table 1), where it was observed that addition of PEG increased the solubility of (*R*,*S*)-NEE and naproxen compared to unmodified buffer or water (data not shown).

To determine the maximum possible reaction velocity, enzyme kinetics were performed with substrate concentrations within solubility limits at 55 °C. The Michaelis–Menten graph as well as the direct linear plot, were constructed from the data (Fig. 2). From this the V_{max} was estimated to be 4.95 g/g/h (0.359 μmol/min/mg CLEC) and the K_M 4.05 g/l (17.6 mM naproxen ethyl ester). In the present study, the specific activity of a commercial sample of free *C. cylindracea* lipase (Sigma) was determined to be in the order of 2.3 mg/mg of enzyme preparation/h under the same reaction conditions.

3.1. Influence of reaction conditions on enantioselective hydrolysis of racemic NEE

The statistically designed experiments were performed to determine the conditions that may have a significant influence on the reaction. The presence of acetone as a co-solvent did not improve the reaction rate. High temperature (50 °C) and high enzyme to substrate ratio (1:50) gave the best results. Under these conditions, at 25% (m/v) substrate (specific activity 210 mg/g h, *E* value 205, ee 99%) was more favourable than a substrate loading of 10% (m/v) (176 mg/g h, *E* of 67, ee of 97%). As the biocatalysts are

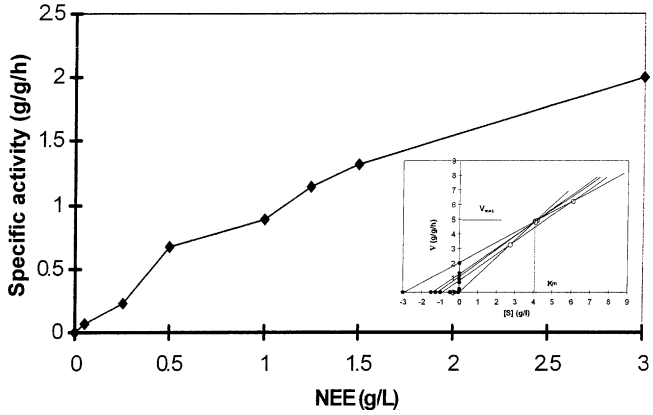

Fig. 2. Plot of the Michaelis–Menten equation of substrate conversion by ChiroCLEC-CR. Note the curve is not the typical rectangular hyperbola due to substrate solubility limitation. The insert is a direct linear plot of the hydrolysis of NEE by ChiroCLEC-CR.

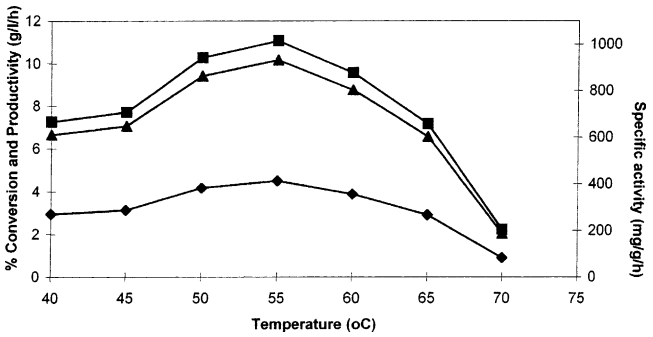

Fig. 3. ChiroCLEC-CR hydrolysis of NEE, % conversion (▲), productivity (◆) and specific activity (■) as a function of temperature. The conversion refers to the % substrate converted to product. PEG 1000/ammonium acetate buffer was used with 25% (m/v) substrate and 5 mg enzyme. The temperature range tested was between 40 and 70 °C.

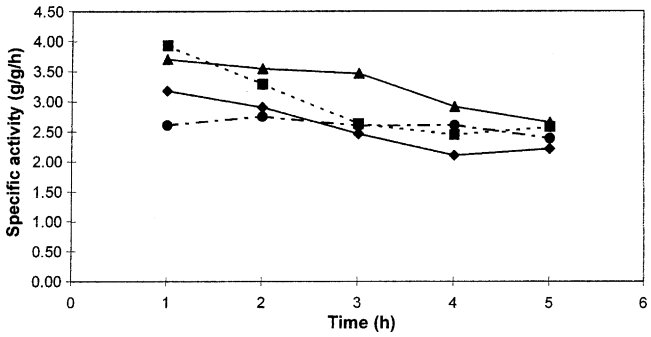

Fig. 5. NEE hydrolysis as a function of initial reaction pH, at pH 4.5 (◆); pH 5.0 (■); pH 5.5 (▲); pH 6.0 (●). The acetate buffer concentration was 0.2 M. The reaction conditions were temperature at 55 °C, NEE (10% m/v) incubated with 2 mg enzyme in a total volume of 1 ml and the reaction sampled after 2 h.

enzyme crystals, it was possible that the bulk transfer of substrates and products may be a limiting factor, and hence we explored the effect of substrate concentration and stirring speed (agitation). However, these two factors did not seem to have a significant influence on the activity over the range tested.

Subsequently, further optimisations of the reaction conditions were undertaken. The optimum temperature was confirmed to be 55 °C, with only 30% relative activity at 70 °C (Fig. 3). The ee of the product was always in excess of 99%.

Fig. 4 shows the influence of substrate concentration on the hydrolysis of NEE by CLEC-CR; 5% (m/v) (*R*,*S*)-NEE in 0.2 M ammonium acetate buffer was found to be the optimum. Fig. 5 shows the progress of the reaction at different pH's, indicating that pH 5.5 is optimal. The optimisation of all the conditions collectively led to an increase of the specific activity from 0.187 to 3.9 g/g h.

3.2. Solubilisation and dispersion of NEE

Investigation of co-solvent addition indicated that the 50% PEG 1000 (control) was better than any of the other co-solvents investigated for simultaneously enhancing specific activity and enantioselectivity. A decrease in either or both enantioselectivity and the specific activity with respect to the control reaction (Table 2) was observed in all cases. The effect of *t*-butanol, *t*-amyl alcohol, olive and soya oil on ChiroCLEC-CR activity resulted in a 60–85% decrease in activity and almost 25% decrease in enantioselectivity, while Tween 80 gave approximately 50% less activity than

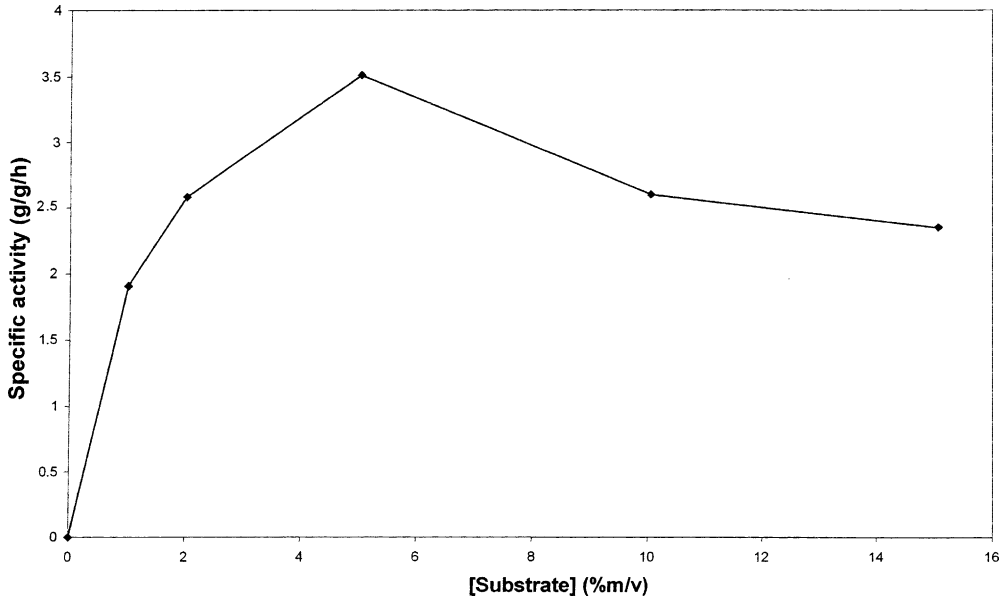

Fig. 4. Optimum substrate (NEE) concentration for hydrolysis by ChiroCLEC-CR. Reactions were performed at 55 °C, 50% (m/m) PEG 1000 for 2 h and varying the substrate concentration from 1 to 15% (m/v) in a reaction volume of 2 ml. As 2 mg enzyme was added, the enzyme to substrate ratio ranged from 1:10 to 1:200.

Table 2
Results of the effect of addition of co-solvents on hydrolysis of NEE

% Co-solvent	% ee (*S*)-Naproxen	*E* (*S*)-Naproxen	Specific activity (mg/g/h)	Relative activity (% of control)
2% Ethanol	98.6	149	73	36
2% Isoamylalcohol	98.4	127	87	43
10% Ethylacetate	0.0	1	0	0
2% DMF	99.0	211	117	58
2% DMSO	98.0	104	192	95
2% Acetone	94.6	38	73	36
2% THF	96.4	59	183	91
2% Acetonitrile	93.8	32	73	36
2% PEG 400	96.8	64	130	64
2% Dioxane	96.8	67	158	78
2% Ethanediol	96.8	67	126	62
50% PEG 1000[a]	99.0	219	202	100
10% Chloroform	0.0	1	0	0
10% DCM	0.0	1	0	0
10% Toluene	0.0	1	0	0
10% DIPE	98.4	136	59	29
10% Cyclohexane	97.0	71	55	27
10% Heptane	95.8	47	55	27
10% Ethanol	89.6	18	77	10
10% Isoamylalcohol	93.0	28	103	13
50% Ethylacetate	0.0	1	0	0
10% DMF	97.2	72	361	47
10% DMSO	98.0	106	577	75
10% Acetone	90.8	21	93	12
10% THF	96.0	57	276	36
10% Acetonitrile	91.2	23	98	13
10% PEG 400	97.4	82	494	65
10% Dioxane	97.8	90	164	21
10% Ethanediol	97.7	88	444	58
50% PEG 1000[a]	99.2	275	765	100
50% Chloroform	0.0	1	0	0
50% DCM	0.0	1	0	0
50% Toluene	0.0	1	0	0
50% DIPE	96.0	51	77	10
50% Cyclohexane	96.4	56	93	12
50% Heptane	91.6	24	94	12

[a] A PEG 1000 control was used as a reference for each set of experiments.

the PEG control. PEG 1000 permitted the highest activities compared to PEG 400 and (±)-2-methyl-2,4-pentanediol (Fig. 6). The optimum concentration of PEG 1000 was determined to be between 70 and 90% (v/v) PEG 1000. However, as this provided only an incremental improvement on the activity obtained at 50% (v/v) PEG 1000, and because of the cost and high viscosity of the reaction mixture at levels above 50%, it is doubtful whether these levels will be viable at large scale.

Interestingly, using an alternative approach, the successful application of PEG in aqueous systems to increase the hydrolysis rate (the primary positive factor in this study) has been applied to esterification in organic solvent by linking it to the enzyme [11] rather than use in the bulk medium.

In a sonication water bath, the reaction was six times faster than the stirred reaction. Sonicating the substrate before addition of the enzyme doubled the conversion compared to the

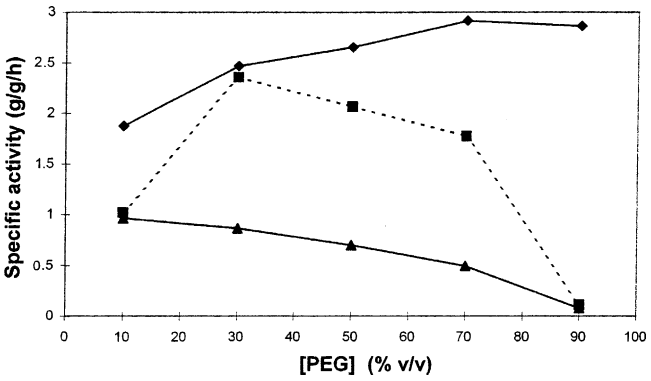

Fig. 6. Comparison of the influence of different PEG species on specific activity. PEG 1000 (◆); PEG 400 (■); (±)-2 methyl 2,4-pentanediol (▲). The PEG concentrations are given as % v/v. The reaction conditions were temperature at 55 °C and NEE concentration at 10% (m/v) with 2 mg enzyme in ammonium acetate buffer in a total volume of 1 ml. The reaction time was 2 h.

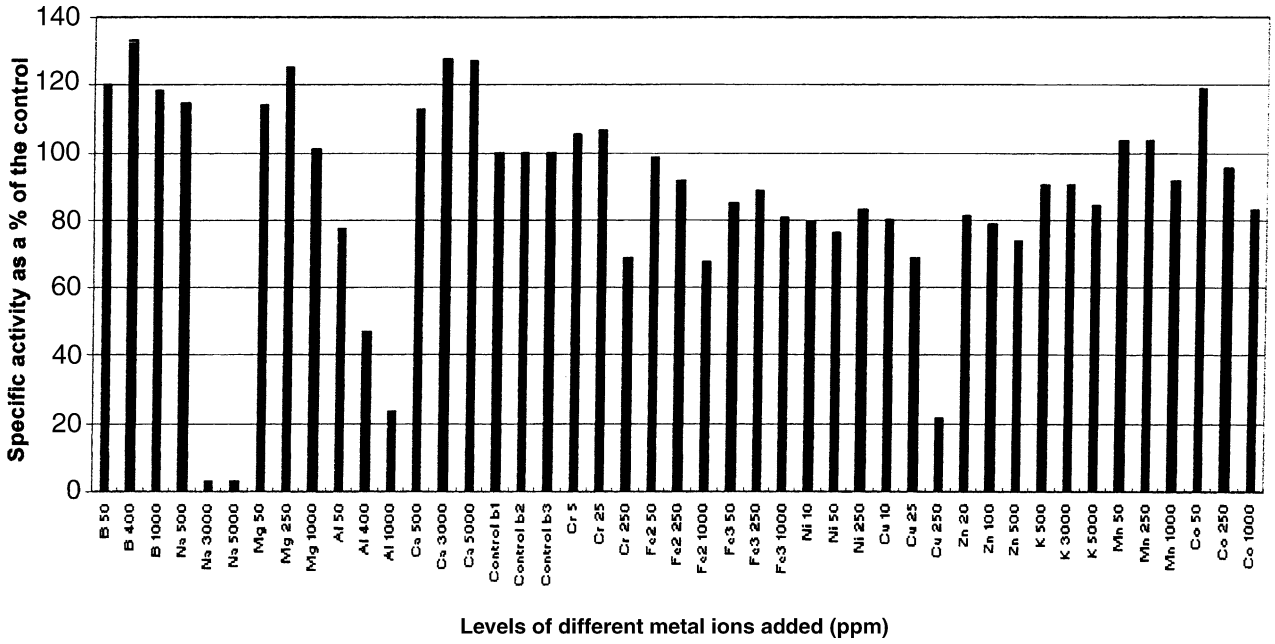

Fig. 7. Influence of metals and boron on the hydrolysis rate of ChiroCLEC-CR. Reaction conditions were temperature at 55 °C, NEE at 10% (m/v), enzyme concentration 1:50 (2 mg) and reaction time of 2 h.

control reactions, and may be explained as the increased accessibility of the substrate. 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.

3.3. Enzyme inhibition

Metals, particularly those used for catalysis during substrate synthesis, may be present in the bulk substrate synthesised under production conditions. Fig. 7 shows the influence of the different metals as a percentage of the control reaction. Three metals had a distinct negative influence on the activity (Cu^{2+} (250 ppm), Na^{+} (>500 ppm) and Al^{3+} (>50 ppm)), while 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. There was some similarity with results obtained by Ghazali and Lai [10] who determined that the free enzyme used in a reverse micellar system with different vegetable oils as substrate was inhibited by metal ions such as Hg^{2+}, Cu^{2+}, Fe^{3+}, Al^{3+}, Zn^{3+} and Ca^{2+}.

The influence of the substrate ((*S*)-NEE) and the two products of hydrolysis, ethanol and (*S*)-naproxen, were investigated. None of the factors tested were significantly reduced the reaction rate under optimised reaction conditions (data not shown).

3.4. Reaction productivity

The value and applicability of a biocatalytic reaction in a process for the production of (*S*)-naproxen depends in part on its volumetric productivity. The reaction progress was followed on a 20 ml scale in a batch stirred tank reactor under the conditions optimised above. The volumetric productivity (g/l/h) and the specific activity (g/g/h) decreased as the reaction progressed (Fig. 8). Under the conditions used, the volumetric productivity started at approximately 1.7 g/l/h and decreased almost linearly to 0.9 g/l/h over 24 h, while the specific activity decreased from 3.4 to 1.7 g/g/h.

3.5. Stability of the ChiroCLEC-CR

Agitation whilst incubating the enzyme in buffer does not seem to accelerate the inactivation of the enzyme to

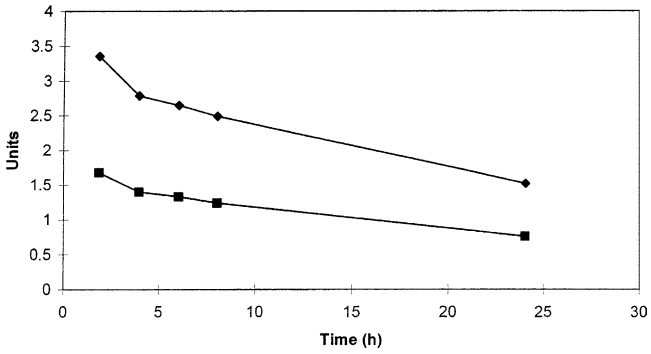

Fig. 8. Productivity (g/l/h) (◆) and specific activity (g/g/h) (■) with time. Enzyme (40 mg) was added to NEE (2 g) and 20 ml PEG/acetate buffer and the reaction stirred at 55 °C for 24 h. Samples (1 ml) were taken every 2 h for the first 8 h and after 24 h the reaction was terminated with 20 ml acetonitrile.

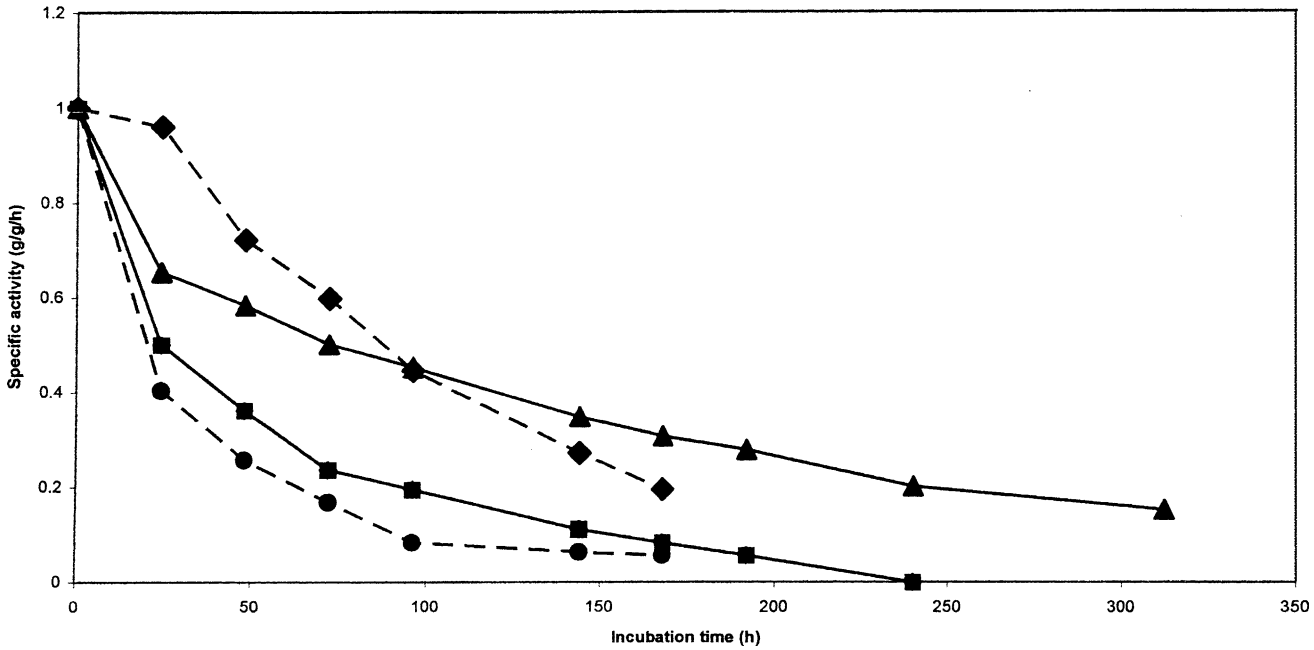

Fig. 9. Thermostability profile of ChiroCLEC-CR enzyme at temperatures of 40 °C (◆) and 55 °C (●) with stirring (600 rpm); and without stirring at 40 °C (▲) and 55 °C (■). 30 mg of the enzyme was incubated in 15 ml of a 0.2 M PEG 1000/acetate buffer pH 5 in the presence of 10 mM solution of $CaCl_2$ (no enzyme substrate included). The residual activity was determined by taking duplicate 1 ml samples from each flask, adding it to 100 mg (*R*,*S*)-NEE in a reaction vial (enzyme to substrate ratio 1:50) and performing the standard reaction.

an appreciable extent, and is therefore probably not a significant destabilising factor. The half-life at 40 °C (with or without stirring) was 72 h, while at 55 °C the half-life was 20 h, demonstrating that higher temperatures are highly detrimental to enzyme stability under these reaction conditions (Fig. 9). Although the present study demonstrates a low CLEC stability for this particular reaction, there is still potential for the application of this biocatalyst in other biocatalytic reactions where high specific activities and enzyme purity is desirable. ChiroCLEC-CR has recently been shown to mediate the conversion of racemic ibuprofen into (*S*)-ibuproxam [12]. Xin et al. [13] demonstrated that the use of alkali in a two chambered membrane reactor permits a dynamic kinetic resolution of naproxen ester racemate with yields greater than 60% and enantioselectivity of 96% using *C. rugosa* lipase.

4. Conclusion

Optimisation of reaction conditions for the enantioselective resolution of racemic naproxen ethyl ester catalysed by ChiroCLEC-CR was performed. The ChiroCLEC-CR enzyme provided excellent enantioselectivity (with an enantiomeric excess (ee) of 99 at 45% conversion of substrate, yielding an enantiomeric ratio (*E*) of 500) for the hydrolysis of (*R*,*S*)-NEE to (*S*)-naproxen, which is comparable with other purified lipase preparations [1]. Similarly Lee et al. [14] used *C. rugosa* lipase for enantiospecific resolution of racemic naproxen methyl ester through hydrolysis, yielding optically pure (*S*)-naproxen with an ee of >98% and an (*E*) of 100. Their optimal reaction conditions for hydrolysis rate and enantioselectivity were 37 °C and pH 6.0 at 156 h.

In the present study the target enantiomeric ratio of 100 was exceeded, and the specific activity of the ChiroCLEC-CR lipase under reaction conditions was improved more than 20-fold (to 3.9 from 0.187 g/g h). As the calculated V_{max} for the reaction was 4.95 g/g h, this represents a 78% efficiency of the reaction, which provided acceptable volumetric productivity. However, instability of the enzyme under the optimal reaction conditions, combined with the high manufacturing cost of the CLEC biocatalyst, adversely affected the economics of the reaction. The rapid loss of biocatalytic activity in the present study, compared to that reported for other immobilised systems [8] may be due to the higher temperature selected. However, at lower temperatures the conversion rate halved, with a dramatic decrease in volumetric productivity.

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References

[1] Hernaiz MJ, Sanchez-Montero JM, Sinisterra JV. Comparison of the activity of commercial and semipurified lipase of *Candida cylindracea* in the hydrolysis of ester of (*R*,*S*)-2-aryl propionic acid. Tetrahedron 1994;50:10749–60.

[2] Wu SH, Guo ZW, Sih CJ. Enhancing the enantioselectivity of *Candida* lipase catalysed hydrolysis via noncovalent enzyme modification. J Am Chem Soc 1990;112:1990–5.

[3] Kazlauskas RJ, Weissfloch ANE, Rappaport AT, Cuccia LA. A rule to predict which enantiomer of a secondary alcohol reacts faster in reactions catalyzed by cholesterol esterase, lipase from *Pseudomonas cepacia*, and lipase from *Candida rugosa*. J Org Chem 1991;56:2656–65.

[4] Lalonde JJ, Govardhan C, Khalaf N, Martinez AG, Visuri K, Margolin AL. Cross-linked crystals of *Candida rugosa* lipase: highly efficient catalysts for the resolution of chiral esters. J Am Chem Soc 1995;117:6845–52.

[5] Chibata J, editor. Immobilised enzymes. Tokyo, New York: Kodansho-Halsted Press; 1978.

[6] ChiroCLEC-CR information booklet. Version 2.0 September 20, 1995. Published by Altus Biologics Inc., Cambridge, USA.

[7] Roszkowski AP, Rooks WH, Tomolonis AJ, Miller LM. Anti-inflammatory and analgesic properties of *d*-2-(methoxy-2′-naphtyl)-propionic acid (naproxen). J Pharmacol Exp Ther 1971;179:114.

[8] Battistel E, Bianchi D, Cesti P, Pina C. Enzymatic resolution of (*S*)-(+)-naproxen in a continuous reactor. Biotechnol Bioeng 1991;38:659–64.

[9] Steenkamp LH, Brady D. Screening of commercial enzymes for the enantioselective hydrolysis of (*R*,*S*)-naproxen ester. Enzyme Microbiol Technol 2003;32(3/4):472–7.

[10] Ghazali HM, Lai CS. Hydrolytic activity of *Candida rugosa* lipase entrapped in aerosol-OT/isooctane reversed micelles: effect of temperature, pH, ionic strength, substrate concentrations and cations. Asia-Pacific J Mol Biol Biotechnol 1996;4:67–73.

[11] Zacchigna M, di Luca G, Lassiani L, Varnavas A, Boccu E. Properties of methoxy(polyethylene glycol)-lipase from *Candida rugosa* in organic solvents. Farmaco 1998;53:758–63.

[12] Hacking MAPJ, van Rantwijk F, Sheldon RA. Lipase catalysed acylation of hydroxylamine and hydrazine derivatives. J Mol Catal B Enzymatic 2001;11(4/6):315–21.

[13] Xin JY, Li SB, Chui JR, Xia CG. Dynamic enzymatic resolution of naproxen methyl ester in a membrane bioreactor. J Chem Technol Biotechnol 2001;6:579–85.

[14] Lee EG, Won HS, Chung BH. Enantioselective hydrolysis of racemic naproxen methyl ester by 2-step acetone-treated *Candida rugosa* lipase. Proc Biochem 2001;37(3):293–8.