1 **Optimisation of stabilised Carboxylesterase NP for enantioselective hydrolysis of**

2 **naproxen methyl ester**

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7

Abstract

Although the enantioselective kinetic resolution of ester racemates of the non-steroidal anti-inflammatory drug naproxen is a common demonstration for biocatalysis, the enantiomeric excess of the reactions is usually insufficient to warrant commercialisation. However, optimised reactions using heterologously expressed carboxylesterase NP provided highly enantioselective hydrolysis of racemic naproxen methyl ester. Up to 46.9% conversion was achieved in 5 hours in the presence of 10 Units enzyme/ g ester with an ee of 99% and E of approximately 500. The final optimised conditions were found to be 150 g/l of substrate in 0.01 M sodium phosphate buffer pH 8.75 at 45°C in the presence of 1% Tween 80 and controlling the pH with 2.5 M NaOH at 8.75. Additional stabilisation of the enzyme with > 2000 ppm formaldehyde resulted in a volumetric productivity of 21.2 g/l/h substrate at an enzyme loading of 18 Units enzyme/ g ester. DBU, used for the racemisation of the unwanted enantiomer, was recycled with the substrate but did not influence the conversion rate. Reaction kinetics revealed that the naproxen formed causes product inhibition, but not enzyme toxicity, and resulted in the decrease in reaction rate with time. The *R*-NME (unwanted enantiomer) did not have a significant influence on the reaction rate.

Keywords

Naproxen methyl ester, *S*-naproxen, carboxylesterase NP, conversion, enantiomeric excess.

Introduction

Biological activity and absolute chemical configuration are closely related and therefore often only one enantiomer of a racemic drug or agrochemical shows the desired level of therapeutic or biological activity while the other enantiomer might have highly undesirable side effects, or no activity at all. The demand for single enantiomer pharmaceuticals is increasing rapidly, with 80% of new drugs entering the market by expected to be chirally pure. However, separation of racemic mixtures is not always simple. As a result of their enantioselectivity and ability to work under mild conditions, enzymes have become one of the more favoured methods of resolution of racemic mixtures [1,2].

Hydrolytic enzymes are the biocatalysts most commonly used in organic synthesis [3 – 5], in particular esterases (including the sub-class lipases), which catalyse the hydrolysis and formation of ester bonds. Their ability to discriminate between the enantiomers of racemic substrates make them valuable tools in the preparation of optically pure compounds [6].

2-Aryl propionic acids, which include non-steroidal anti-inflammatory drugs (NSAID), are examples of chemical compounds with the activity restricted to one of the enantiomer. Today the NSAID, naproxen [(+)5-2-(6-Methoxy-2-naphthyl)propionic acid] is administered in the homochiral *S*-configuration, which is up to 150 times more active than *R*-naproxen [7] and in addition, the *R*-isomer gives rise to unwanted gastro-intestinal disorders [8].

56

57 Several research groups have already described enzymatic routes for the production of

58 *S*-naproxen [9 – 13]. Some of the recent papers include the work by Giorno et al [14]

59 in which they tested their two phase enzyme membrane reactor by immobilizing

60 lipase in presence of emulsion and then using the enantioselective hydrolysis of

61 naproxen ester as a model. The enzyme immobilised this way gave an improved ee

62 from 74 to 97% for the methyl ester and the enantioselectivity was raised form 96 to

63 100% for the butyl ester. The conversion was however very low. Chen and Tsai [15]

64 have used a *Carica papaya* lipase and obtained excellent ee values, but the substrate (

65 *R*,*S*-naproxen 2,2,2-trifluoroethyl ester) concentrations used were low (1 to 4.5mM).

66 Koul et al [16] used a whole cell microorganism, *Trichosporon* for stereoselective

67 resolution of *S*-naproxen and achieved an ee of >99%, and an E of approximately 500

68 at a volumetric productivity of 0.8 g/ℓ/h.

69

70 Researchers at DSM (previously Gist-Brocades) isolated and identified a *Bacillus*

71 *subtilis* (Thai I-8) strain which produced carboxylesterase NP [17]. The gene coding

72 for the esterase was identified and cloned into a *B. subtilis* I-85 organism, resulting in

73 the recombinant-DNA strain *B. subtilis* I-85/pNAPT-7. The enzyme expression of

74 this recombinant-DNA strain was more than 800 times higher than the wild type strain

75 Thai I-8. At high substrate concentrations, a lack of stability was encountered and

76 irreversible inactivation was observed when carboxylesterase was incubated with 30

77 g/ℓ R, S-naproxen ester. the enzyme was inactivated by naproxen formed, possibly

78 by interacting with the amino groups of basic amino acids at the surface of the

79 enzyme, thereby allowing the hydrophobic bulk of the naproxen to interfere with the

80 tertiary protein structure. Therefore the carboxylesterase was chemically modified

with formaldehyde and was confirmed to be more resistant to the high acid-denaturing conditions. The enzyme treated with formaldehyde concentration of 1% or higher, proved to be stable on incubation with naproxen (15 mg/ml) for 1½ hours at 40ºC; conditions that lead to complete inactivation of the untreated enzyme. All the modifying agents (glutaric anhydride, succinic anhydride, glyoxal, glutaraldehyde or formaldehyde) showed an improved performance, the best results being obtained with formaldehyde, glutaraldehyde or glyoxal [1].

The present work is concerned with the optimisation of the carboxylesterase NP enzyme for the resolution of racemic naproxen (Scheme 1) to yield the active *S*-naproxen with an enantiomeric excess of 97.5% and an E>200, under reaction parameters consistent with a viable large scale process.

Materials and Methods

2.1 Analytical conditions

HPLC: The quantitative naproxen, and naproxen methyl ester (NME) and naproxen ethyl ester (NEE) analyses were by HPLC using a 25 cm C18 ODS 2 column. The system was run isocratically using a mixture of 70% acetonitrile and 30% aqueous acidified with 0.1% phosphoric acid. The *R*/*S* ratios of all the isomers were determined on an (S,S)-Whelk/0/25 cm, column run isocratically with a mixture of hexane:ethanol:acetic acid (95:5:0.5).

Substrate: *R*,*S*- and NME were synthesised as described previously [18]. Carboxylesterase NP (165 U/mℓ) was kindly provided by DSM (the Netherlands)

106

107 **Enzyme activity:** Standard reactions were performed wherein solid racemic substrate

108 was added to 18 mℓ of a 0.1M Tris buffer (pH 8.75) and 2 mℓ of a 10% v/m Tween 80

109 solution. The pH was adjusted to and maintained at 8.75 using 1 M NaOH.

110 Subsequently, 16.5 µl enzyme was added and the temperature controlled at 40°C.

111 Samples of 0.5 mℓ were taken at time intervals, and the reaction products were

112 weighed, filtered through non-absorbent cotton wool and analysed. The units of

113 enzyme activity were defined as the amount of enzyme that hydrolysed 10^{-6} mol (1

114 µmole) *S*-NEE per minute.

115

116 **2.2 Reaction conditions**

117

118 **Initial rates:** To determine the initial rates of the reactions due to enzyme

119 concentration, the reactions were performed using 3 g (0.615 mole) of NME in 20 mℓ

120 made up of buffer and a final concentration 1% v/m Tween 80 with regard to the

121 ester. The reactions were run at 45°C and the pH was maintained at 8.75. The

122 reactions were initiated with 5.5, 10 and 20 units enzyme/g ester. Samples (0.5 mℓ)

123 were taken at intervals up to 3 hours, weighed, acetone added (2 mℓ), and analysed

124 by HPLC.

125

126 **Reaction Rates:** Reactions were performed under standard reaction conditions,

127 varying only the substrate concentration at 25 g/ℓ intervals between 75 g/ℓ - 200 g/ℓ.

128 The enzyme volume was adjusted for each reaction proportionately to the substrate to

129 correspond to 10 units of enzyme per gram ester.

130

131 Solid substrate particle size: NME was sieved to give different fractions, one with
132 particle size greater than 53 μm and another with particle size smaller than 53 μm.
133 The standard reaction conditions were used to test the different particle size samples
134 to determine the effect on the reaction rate.

135

136 Volumetric productivity: The enzyme concentration necessary to give optimal
137 volumetric productivity was tested, with the rate of reaction using 100 g/ℓ or 150 g/ℓ
138 substrate with 10, 20 or 30 units of enzyme/g ester.

139

140 **2.3 Enzyme stability**

141

142 Reactant denaturation: Naproxen The enzyme was pre-treated with formaldehyde by
143 DSM to stabilise the enzyme. The enzyme was pre-incubated with naproxen for 2, 5
144 and 20 hours at 45°C before addition of the substrate to test the stability of the
145 enzyme in the presence of product. An experiment was done in which the enzyme
146 was re-treated with formaldehyde to determine if it has an influence on the enzyme
147 stability. Following the results a statistically designed experiment was done to find
148 the optimum conditions for re-treating the enzyme with formaldehyde in terms of
149 formaldehyde concentration, temperature and time.

150

151 **2.4 Enzyme inhibition**

152

153 For the inhibition study 0, 10, 20, 30, 40 and 50% m/m (0 - 0.94 g) *S*-naproxen was
154 added to the start of the reactions (0.94 g being the maximum *S*-naproxen which can

155 be formed from 2 g of racemic naproxen). The enzyme concentration was 10 units
156 enzyme/g ester. Samples (0.5 mℓ) were taken every hour and weighed before addition
157 of acetone (2 mℓ).
158
159 Methanol is a by-product of the resolution reaction. Experiments were performed as
160 described previously using 2 g NME, with 0 - 50% v/m methanol added to test the
161 influence thereof on the resolution rate.
162
163 The control reaction was run with NME (3 g), while 1.5 or 3.0 g *S*-NME was used to
164 test the influence of *R*-NME on the reaction. The reactions were as described for the
165 standard reaction at 45°C and at pH 8.75. Samples (0.5 mℓ) were taken every hour for
166 5 hours, weighed, acetone (2 mℓ) added, and analysed by HPLC.
167
168 In the envisaged process diazabicyclo[5.4.0]undec-7-ene (DBU) is used to racemise
169 the unreacted *R*-NME to *R*,*S*-NME for recycling back into the reaction [20]. The
170 DBU carries over into the resolution reaction and therefore the influence thereof was
171 tested by addition of 0.25, 0.5 and 10% DBU to the standard reaction mixture. In
172 other experiments, DBU racemised NME containing DBU was used, and in four
173 further experiments recycled NME isolated from methanol or methanol/water
174 solutions was used in subsequent resolution reactions.
175

176 **2.5 Reaction optimisation**

177
178 Physical Parameters: The interaction of temperature and pH was tested in a
179 statistically designed experiment as the 4 points of a square to test the failure of ee

180 (set as 97.5%) at various combinations of temperature an pH. The temperatures were
181 chosen as 35°C and 50°C (and in a second set as 35°C and 57°C) and the pH values as
182 8 and 10. The standard reaction protocol was used except where temperatures and pH
183 were adjusted.
184
185 Lower pH values might influence the separation of NME and naproxen as naproxen
186 starts to precipitate at 7.5, while above pH 10.5 chemical hydrolysis may occur. The
187 standard reaction conditions were used, changing only the pH of the reaction either to
188 7.5, 10.5 or 11.00. Lower temperatures (35°C) have been found to affect only
189 conversion and not quality. The higher temperatures may denature the enzyme
190 (causing a decrease in conversion or ee) or cause chemical hydrolysis of NME
191 (causing a decrease in ee). The temperatures tested were 45°C, 57°C and 65°C using
192 the standard reaction conditions. The influence of agitation on conversion was
193 determined by stirring at 250, 450 and 650 rpm.
194
195 Detergents: The effect of different Tween 80 concentrations on the rate of resolution
196 was examined using 0.1, 5 and 10% v/m Tween 80 in different reactions. The effect
197 of methanol was determined on conversion by replacing Tween 80 with 1, 2 and 5%
198 v/m methanol. Polyethylene glycol (PEG) 400 is more water soluble than Tween 80
199 and might be more efficiently removed from the product during DSP. This was tested
200 in standard reactions.
201
202 Co-solvents: IPA (isopropyl alcohol) or tBA (*tert*-butyl alcohol) was tested at 1 and
203 10% concentrations as replacements for Tween 80 as they could make the
204 downstream processing easier.

205

206 **2.6 Process integration**

207

208 **Enzyme:** High enzyme load can result in carry-over of protein and formaldehyde in
209 the final product and may also potentially have an influence on ee by virtue of the
210 enzyme action on R-NME. The standard reaction conditions were used, changing
211 only the enzyme concentration to 100 and 200 units/g ester, from the normal level of
212 10 units/g ester.

213

214 **Buffer:**

215 The standard reaction conditions were used, 150 g/ℓ, 10 units enzyme/g ester, 1%
216 Tween at 45°C or 57°C, pH 8.75 or 10, and buffer concentrations were varied
217 (0.01 M, 0.5 M and 1.0 M). Comparative studies were undertaken with MOPS or
218 Tris, as well as titration with base (either NaOH or NH_4OH). Reactions were also
219 performed where the pH was controlled at 9.00, 9.50 or 10.0 to determine the
220 sensitivity of enzyme enantioselectivity with pH.

221

222 **Results and Discussion**

223

224 Resolution of racemic naproxen esters can be achieved by enantioselective hydrolysis
225 of the *S*-naproxen ester, yielding *S*-naproxen and the R-naproxen ester. However,
226 for S-naproxen to be pharmaceutically acceptable, a high enantiomeric excess (ee)
227 must be achieved during hydrolysis, yielding enantiomeric ratio (E) of greater than
228 100 [18]. An initial screen [18] determined that none of a broad selection of
229 commercial enzymes, could provide an economically viable process for resolution of

230 naproxen, either due to insufficient enantioselectivity or due to biocatalyst cost [19].
231 However, carboxylesterase NP, developed by Gist Brocades (now DSM)
232 demonstrated superior enantioselectivity (>97%ee) [17], and hence was selected for
233 further reaction optimisation.

234

235 **3.1 Reaction conditions**

236

237 **Enzyme activity**

238 Substrate selection: The substrate preference, ethyl or methyl esters of naproxen, was
239 evaluated in the presence of various buffers (Table 1). It was evident from the results
240 that the methyl ester allowed for better enantioselectvity than the analogous longer
241 carbon chain ethyl substrate. NME was therefore selected for further experiments.

242

243 **Initial enzyme to substrate ratio.** Experiments using 180 g/ℓ of the NME showed
244 that 10 units of enzyme gave better conversion than 5.5 units (Table 2).

245

246 **Reaction Kinetics**

247 Reactions were done using the standard reaction conditions, varying only the substrate
248 concentration between 75 g/ℓ and ending at 200 g/ℓ at 25 g/ℓ. The enzyme units were
249 adjusted proportionately for each reaction to correspond to 10 units of enzyme per
250 gram ester. The conversion results (Fig. 1) indicated that the % conversion
251 decreased with an increase in substrate concentration, possibly due to the limitations
252 of dissolution of the solid substrate.

253

254 **Reaction Productivity and conversion:** The resolution reaction was done on a
255 number of naproxen ester substrates from different sources. The first hour of the
256 reaction was always relatively fast and then dropped (Fig 2). The productivity of the
257 reaction in the first hour was typically 30 - 35 g/ℓ/h in the first hour and then
258 decreased to approximately 20 g/ℓ/h in the second hour and ended at approximately
259 9 g/ℓ/h after 5 hours.

260

261 **Initial reaction rates**

262

263 The productivity is important from a scale-up perspective as the amount of product/ℓ
264 broth/h determines the economics of the resolution step. A fixed substrate
265 concentration of 150 g/ℓ racemic NME was used but the amount of enzyme was
266 varied. The initial rates of the reactions were measured and results expressed in terms
267 of productivity (Fig 3). The conversion of the substrate initially was dependent on the
268 amount of enzyme added and this relationship is almost linear. The productivity
269 results were in correlation with the conversion results, with the productivity in terms
270 of grams naproxen formed per litre broth per hour being the highest for 20 units of
271 enzyme. From all the results a relationship between the rate of reaction and amount
272 of enzyme was evident.

273

274 **3.2 Enzyme stability**

275

276 **Enzyme Thermal Denaturation**

277 In all the resolution runs, the activity/productivity is very high in the first hour and
278 then drops quite dramatically. It was thought that this could be due to loss of enzyme

279 activity at high temperatures. Experiments were done in which the enzyme was pre-
280 incubated with naproxen for 2, 5 and 20 hours before addition of the substrate, to test
281 this theory (Figure 4). From the results we found that ageing of the enzyme did lead
282 to a decrease in the conversion rate, but the same trend as before was still noticeable,
283 that is, in the first hour the activity was the highest and then it dropped quite
284 significantly. This trend may be due to product-inhibition.

285

286 **Reactant Related Denaturation**

287

288 Carboxylesterase NP had been stabilised to naproxen using formaldehyde at DSM
289 laboratories. However, lower than expected conversions were obtained, and hence the
290 enzyme was re-treated 2300 ppm formaldehyde for 4 hours at 40°C [1] and
291 comparative resolution reactions were then performed using 265 g/l and 18 units of
292 enzyme/g ester. After 5 hours, the conversions were 44.4% for re-treated enzyme
293 compared to approximately 21% conversion of the untreated enzyme. This indicates
294 that the reaction with formaldehyde is reversible.

295

296 **3.3 Enzyme inhibition**

297

298 **Enzyme inhibition by R-NME:** To determine the influence of the unreacted
299 enantiomer *R*-NME, which could potentially be a competitive inhibitor, experiments
300 were run with *S*-NME alone. Reactions were performed using *S*-NME (1.5 g or 3 g)
301 which would correspond to the normal available *S*-NME and total NME
302 concentrations respectively. The results (not shown) indicated that *R*-NME did not

303 influence the reaction rate of the resolution, as the S-NME reactions preceded at the
304 same rate as the racemic substrate.
305
306 **Enzyme product inhibition by S-naproxen**: Conversion of racemic NME to an
307 optimum of 50% *R*-NME and 50% *S*-naproxen was calculated on a mass basis. Based
308 on this, 0 - 50% (0 - 47 g/ℓ) *S*-naproxen was added to the reaction being performed at
309 100 g/ℓ and the results are given in (Fig 5). From the results it was clear that *S*-
310 naproxen formed in this reaction was detrimental to the enzyme activity. This could
311 either be due to product inhibition, either reversible or irreversible (enzyme toxicity).
312
313 **Enzyme product inhibition by methanol**
314
315 S-naproxen and methanol are the products of the resolution reaction. Methanol is
316 formed as a by-product during the reaction when they methyl group is eliminated and
317 reacts with water to form methanol. The influence of methanol was therefore tested
318 on the reaction rate.
319
320 From the results it was clear that methanol slightly lowered the reaction rate but this
321 effect was insignificant compared to the effect of the *S*-naproxen. A 5% decrease in
322 total conversion (45 to 40%) was experienced from 0 to 50% methanol.
323
324
325 **3.4 Reaction Optimisation**
326

327 **Effect of high pH on the enantioselectivity of the enzyme:** For the reaction to be a
328 scalable, it should not be overly sensitive to pH, as localised pH variations are
329 common at large scale during titration. Reactions (100 g/ℓ rac-NME) were controlled
330 at pH 9, 9.5 and 10 and conversions were 43.4, 41.5 and 39% respectively, while the
331 ee was .99.2% in each case.
332
333 **Agitation:** The influence of agitation on the conversion of the substrate to *S*-naproxen
334 was determined by using three different stirring speeds, i.e. 250, 450 and 650 rpm.
335 The reactions were stopped after 5 hours and revealed that there were no differences
336 in conversion using different rates of agitation (data not shown).
337
338 **Influence of detergents and co-solvents on conversion:** As the substrate is poorly
339 soluble, this may limit the reaction kinetics. The surfactant Tween 80 increases the
340 solubility of the substrate and might therefore influence the rate of reaction. Inclusion
341 of 0, 1, 5 and 10% (v/m) Tween 80 in the reaction had a strong positive influence on
342 the reaction rate, indicating that substrate solubility is indeed a limiting factor (Table
343 3). The main benefit is achieved at relatively low levels of surfactant as there was a
344 negligible difference for the 1 to 10% Tween 80 concentrations.
345
346 Reactions were done with 1 and 10% PEG 400 instead of Tween. The reaction with
347 1% PEG 400 gave a conversion of only 9.9% in five hours and the 10% PEG reaction
348 a conversion of 13.4%, moreover the ee_p fell to 97.8 and 98.4 respectively, indicating
349 that this is not a viable option. To determine the influence of IPA and tBA on the
350 reaction rate, 1 and 10% of these solvents were added to the reactions instead of
351 Tween 80. The IPA and tBA used in the reactions were detrimental to the reaction

352 rate with the highest conversion being 9.1% in 5 hours with 1% IPA. Conversions
353 between only 0.8% and 4.6% were recorded with the higher IPA concentrations and
354 either concentration of tBA.
355
356 **Influence of particle size on the resolution rate:** To test the effect of particle size on
357 the reaction rate, the substrate was sieved to give fractions with a particle size either
358 larger or smaller than 53 µm. The particles larger than 53 µm were visibly coarser
359 and resulted in a 14% slower reaction rate with a conversion of 31.1% compared to
360 36.2% for the smaller particles. Again this indicates that substrate solubility and
361 dissolution rates are the limiting factor in the reaction.
362
363 **Determination of the pH and temperature limits:** Lower pH values (eg 7.5) may
364 influence down stream processing of the reactants, as naproxen precipitates as the free
365 acid at and below this pH value. This would complicate its separation from the
366 insoluble NME, reducing product purity. Conversely high pH values may cause
367 chemical hydrolysis of the ester bond, leading to a decrease in ee_p.. Reactions
368 maintained at pH values between 7.5 to 11.0 (at 45°C or 57°C) were evaluated (Table
369 4). At 45°C the ee was not noticeably sensitive to pH and was within the limits set,
370 but naproxen yield was decreased at pH 7.5, either due to reduced enzyme activity or
371 product precipitation. At 57°C the pH was critical and caused the ee to fail at pH
372 10.00, while conversion was less in each case.
373
374 Temperatures of 45, 57 and 65°C were tested at the standard pH value of 8.75. The
375 conversion and ee were both affected negatively by increase in temperature (Table 4).

376 A two factorial Design-Ease model was used to test the interaction of pH and
377 temperature and to determine if such an interaction will result in a failure of ee
378 (minimum specification limits set at 97.5%). In the first set of experiments the pH
379 was chosen to be 8 and 10 and the temperature 35°C and 50°C with reactions in
380 duplicate. The response factors were taken as conversion (%) and ee (%). The R-
381 squared value was 1.00 and the F value (significance) 563.24 revealing that the
382 interaction between temperature and pH is significant and working at high pH gives
383 the best conversion at either of the temperatures investigated (Fig 6A).

384

385 The conversion does not directly influence the quality of the final product, but will
386 make the DSP more difficult and increase production costs. However the real effect
387 on quality is reflected in the ee. The R-squared value for the interaction of pH and
388 temperature in terms of ee was 0.89 and the F-value 11.33. The probability was not
389 significant compared to the conversion and the failure limit of 97.5% ee was not
390 reached. The ee's of the reactions were between 99.0 and 98.4 (Fig 6B).

391

392 **Enzyme Concentration:** The standard reaction used 10 units of enzyme per gram
393 ester. Addition of 10 and 20 times the normal amount was investigated (Table 5).
394 The conversion was faster with a 10 fold higher enzyme concentration, yielding a
395 conversion of 47.2% in only 3 hours, and an the ee of 98%. A 20 fold enzyme
396 provided only a 0.4% improvement in conversion, while dropping the ee to 97.2% as
397 the enzyme begins to convert increasing amounts of the R-enantiomer.as the S-
398 enantiomer is depleted.

399

400

401 **Buffer concentration limits:** The standard buffer concentration is 0.1 M sodium
402 phosphate at pH 8.75. In the initial set of experiments 10 times lower and 5 and 10
403 times higher buffer concentration was used (Table 6). Using a lower buffer
404 concentration was expected to make pH control very difficult, and therefore adversely
405 affect conversion or ee_p. However, reducing the concentration of phosphate had a
406 positive effect on the conversion result and no significiant effect on the ee, while
407 conversely increasing the buffer concentration had a negative effect on conversion
408 and also started to influence the ee at 1.0 M strength.
409
410 **3.5 Process integration**
411
412 Scale up parameters:
413 The optimised reaction conditions for the resolution step were set as 150 g/ℓ of the
414 NME in 0.1 M sodium phosphate buffer at pH 8.75 and 45°C in the presence of 10
415 units enzyme in 20 ml.
416
417 A larger scale (600 mℓ) reaction was done to determine the ease of scale-up. A
418 conversion of 44% was achieved after 5 hours at 40°C starting with 150 g/ℓ NME and
419 10 units of enzyme. The ee of the product was 99%.
420
421 **Substrate recycling:**
422 Recycling of the residual *R*-NME, is achieved using DBU. However it is anticipated
423 that DBU would be carried through to the biocatalytic reaction, although the exact
424 amount would vary with the final process steps and parameters. Hence we

425 investigated the influence of DBU on the biocayalytic resolution or *rac*-NME.
426 Whether it was the racemised NME or NME spiked with DBU, inclusion of 0.25%
427 DBU resulted in the same ee_p and conversion as the control (~ 38%), both after 5
428 hours. However the racemised NME containing 10% DBU caused a decrease in
429 conversion to only 30.8% and a drop in ee to 96%. NME racemisation reactions
430 indicated that a DBU concentration of 0.5% is sufficient for complete racemisation,
431 and hence DBU does not have to be removed before the resolution of the racemised
432 material provided that it is not allowed to accumulate in the re-cycle stream. As a
433 demonstration of this, unreacted *R*-NME at the end of the resolution reaction run was
434 isolated and racemised using DBU before for recycling back into a subsequent
435 resolution reaction. The results (Table 7) indicated that there were no adverse
436 effects on the resolution reaction or quality of the product.
437
438 **CONCLUSION**
439
440 We set out to provide an optimised naproxen resolution reaction with an E of >200
441 and an ee_p of > 97.5%. This specification was set as it was found to be possible to
442 recrystalise the product to within the required ee of 99% if the crude product has an
443 initial ee of 97.5%.
444
445 The resolution of *rac*-naproxen ester with carboxylesterase NP to yield *S*-naproxen
446 acid and *R*-naproxen ester was optimised. The optimised conditions were found to be
447 10 units enzyme per gram ester reacted with150 g/ℓ ester in 0.1 M sodium phosphate
448 buffer at pH 8.75 at 45°C in the presence of 1% Tween 80, and pH maintenance with
449 either 2.5 M NaOH or NH_4OH. Up to 46.9% conversion was achieved in only 5

450 hours with an ee of 99% and E of 576, which is over double that we previously
451 attained with CLECs of *Candida rugosa* lipase [19].
452
453 An extra stabilisation of the enzyme with 2000 - 2300 ppm of formaldehyde revealed
454 that the conversion of 265 g/l ester with 18 units of enzyme per gram ester was
455 possible with a conversion in excess of 40% in 5 hours with a good enantioselectivity.
456
457 The water insoluble substrate forms a slurry in the reaction mixture, and particle size
458 had an influence on reaction rate, with the smaller particle size providing the faster
459 conversion rate (36% compared to 31% in 5 hours) as would be expected. Dispersal
460 of the substrate was demonstrated using the surfactant Tween 80 and several
461 alternatives (PEG, methanol, IPA, tBTA), but only Tween 80 provided acceptable
462 resolution rates.
463
464 The process was validated by determination of the parameters which are quality
465 critical. It was found that temperature is the most critical parameter. Increasing the
466 temperature above 57°C not only decreases the conversion rate but also has an effect
467 on the enantiomeric excess (ee) which determines the quality of the final product. At
468 65°C at the normal pH the ee fails the set specification of 97.5%. As an isolated
469 factor, pH does not seem to be critical and even at pH 11 the conversion and ee do not
470 seem to be significantly influenced. At lower pH (8.0 and 7.5) the conversion rate is
471 much lower, but again the quality is not significantly changed.
472
473 The hydrolytic enzyme, Carboxylesterase NP has been found to be more efficient for
474 the enantioselective production of *S*-naproxen than any other reported enzyme. This

475 study has demonstrated that the biocatalyst was robust and performed well under
476 process conditions. Recycling of the *R*-NME via racemisation with DBU improves
477 the commercial viability of the process. Successful integration of the reaction into a
478 full process depended on the influence of the racemisation agent (DBU) that would be
479 carried in the reaction through substrate recycling. DBU was found to have no
480 significant influence on reaction rate or quality of the product during substrate
481 recycling experiments.

482

483 The conclusion therefore is that the enantioselective resolution of racemic naproxen
484 through hydrolysis of its ester can be achieved using Carboxylesterase NP under
485 conditions that could be implemented at an industrial scale. Formaldehyde stabilised
486 Carboxylesterase NP heterologously overpexpressed in *B. subtilis* provides the best
487 opportunity so far for commercial implementation of this biocatalytic process.

488

489

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495

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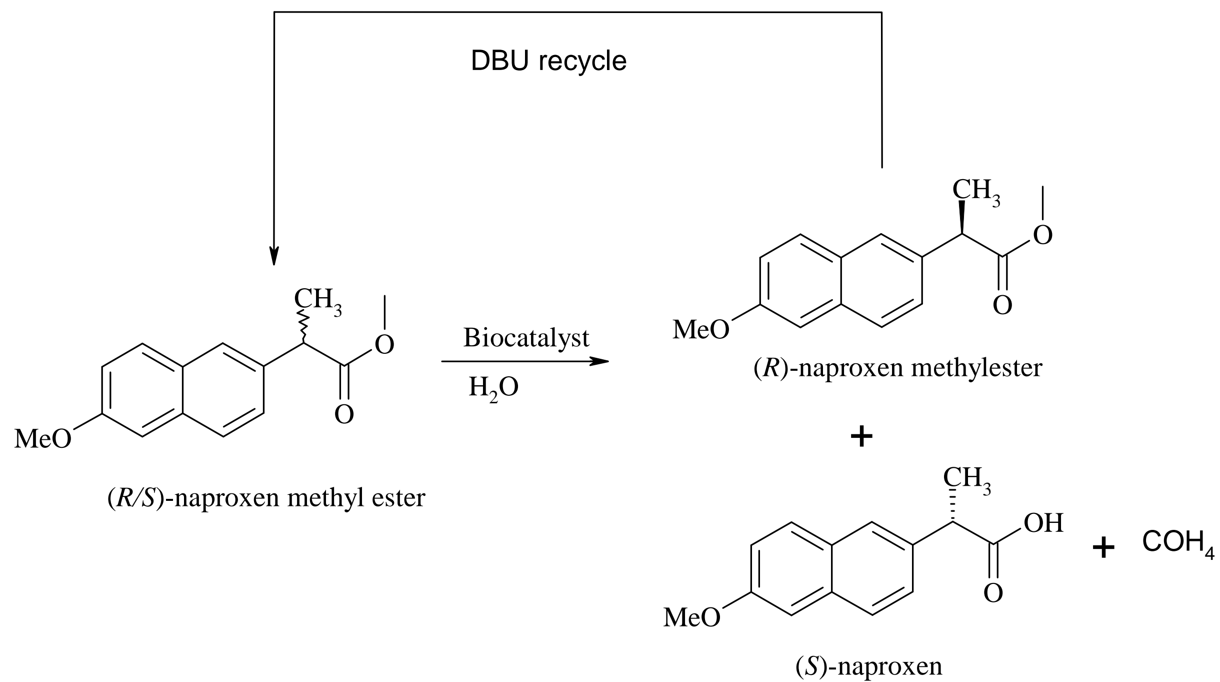

556
557
558 Scheme 1: Enantioselective hydrolysis of (*S*)-naproxen methyl ester.

559 **Table 1:** Comparison of substrate efficiency and enantioselectivity in the presence of

560 different buffer and titration additive

561

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	99.0	397

562

563 **Table 2:** Results of the reactions performed with the 5.5 and 10 U enzyme
564

Reaction	Reaction time	Enzyme conc. (U)	Conversion	% ee	E
A	5	10	39.6	99.0	391
	7		43.8	99.0	468
	23		46.9	99.0	576
B	5	5.5	25.0	99.2	344
	7		30.0	99.2	379

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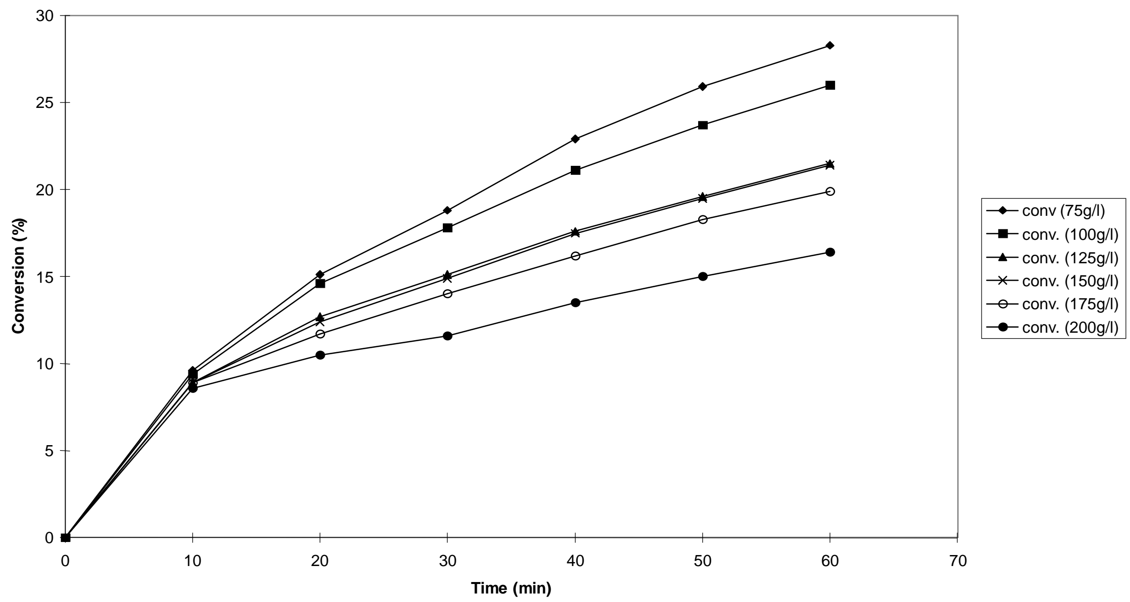
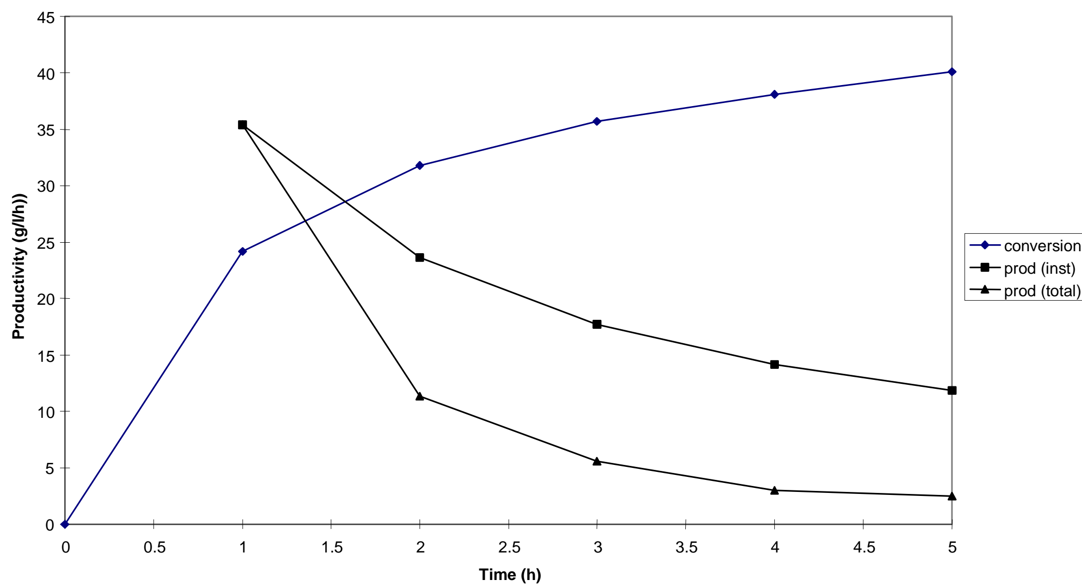


566
567
568 **Figure 1**: Conversion of NME by carboxylesterase_NP.. Influence of substrate
569 concentration normalised for enzyme activity (10 units of enzyme/g
570 ester)

571



572
573
574 **Figure 2**: The instantaneous and total productivities of a normal resolution reaction.
575 The average productivity was calculated from the total amount of naproxen formed
576 over a certain period divided by the amount of time, while the real productivity refers
577 to the exact amount of naproxen formed in a specific hour.

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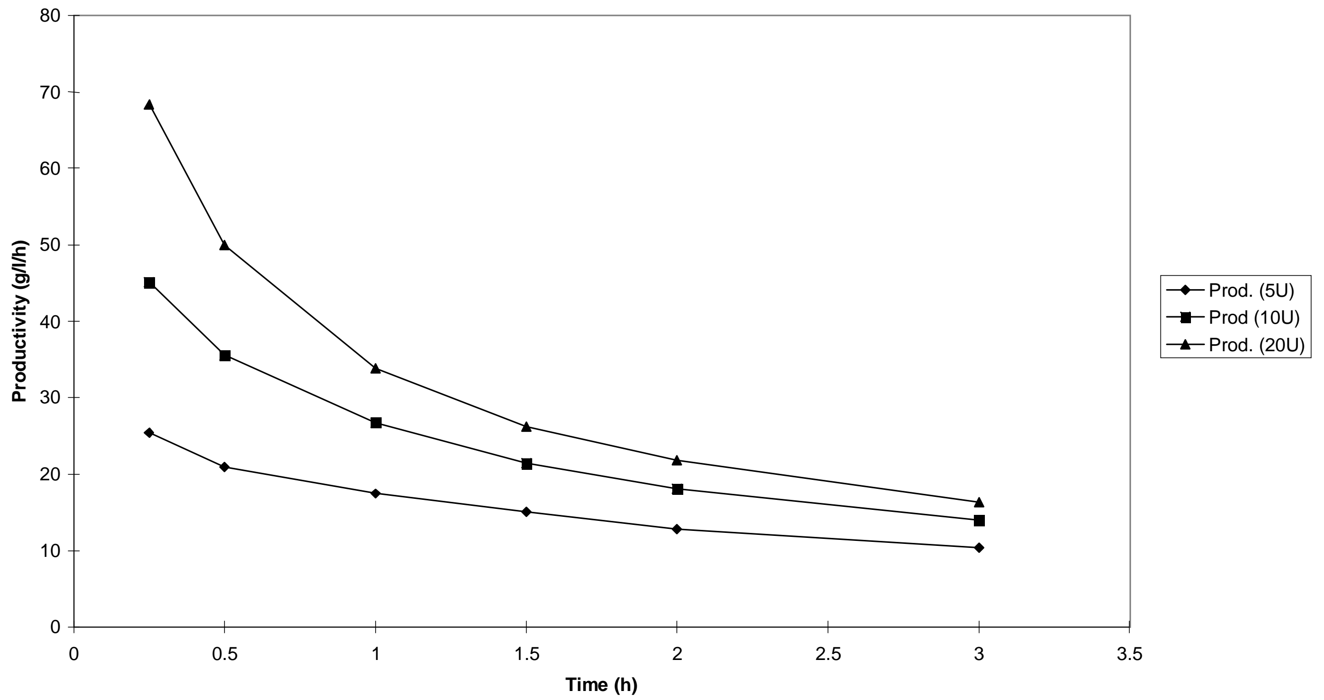
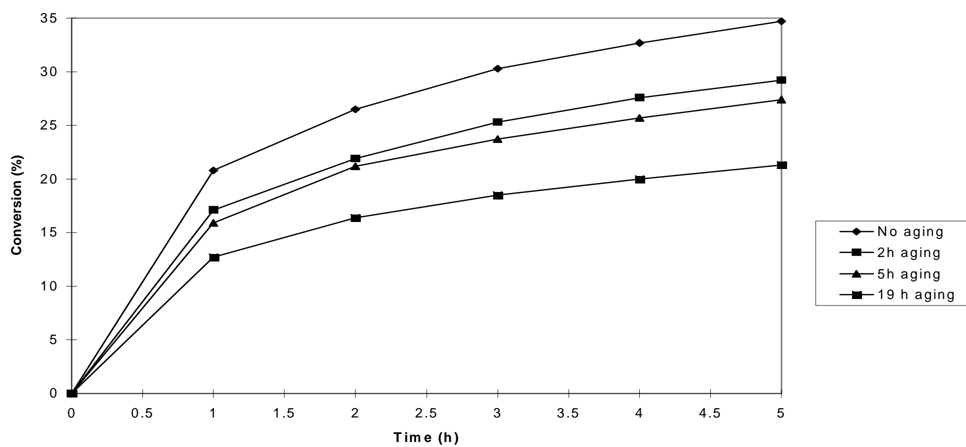


581
582 **Figure 3**. Initial rate results expressed in terms of productivity with a fixed substrate

583 concentration and varying enzyme concentrations (♦) 5 U, (■) 10 U and (▲) 20 U.

584



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586 **Figure 4:** Comparison of the conversion results following exposure of the enzyme to

587 naproxen (♦) Control, (■) 2 hours exposure, (▲) 5 hours exposure and (■) 20 hours

588 exposure.

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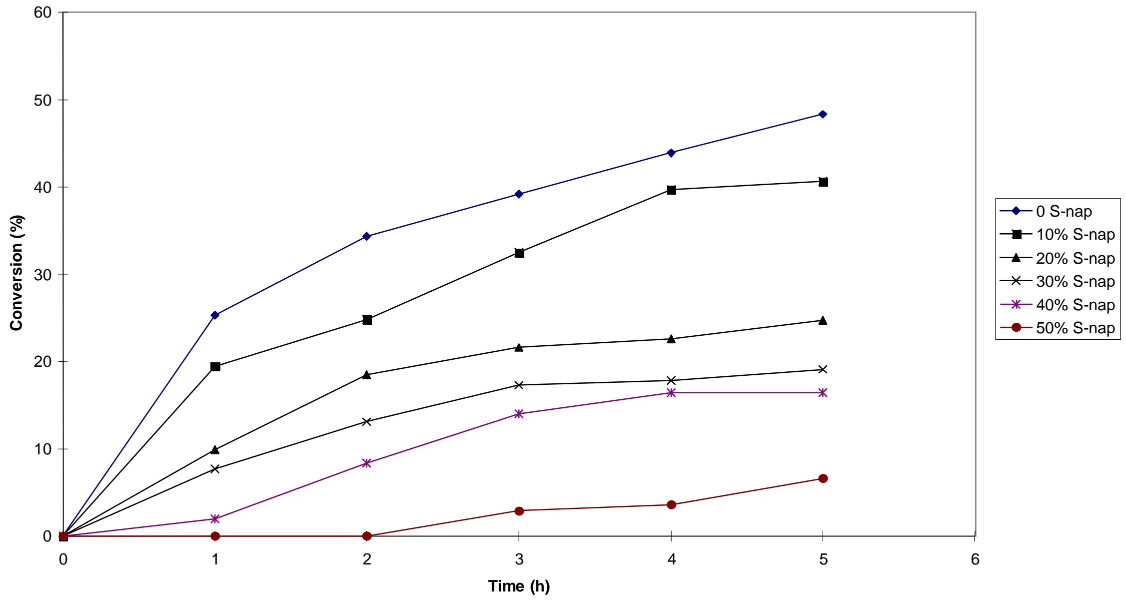

596 Figure 5: Inhibition of Ccarboxyesterase NP by product (S-naproxen between 0
597 and 47 g/ℓ).

598

599 **Table 3:** Results of different Tween concentrations on the conversion of the substrate
600

% (v/m) Tween 80 concentration	% Conversion (5 h)
0	12.9
1	35.5
5	37.5
10	38.5

601

602 **Table 4:** Influnce of pH and temperature on enantiomeric ratio, duplicate experiments
603

Temp (°C)	pH	Conv (%)	ee (%)	E
45	7.5	20.8	98.6	182.7
45	8.75	33.9	99.0	330.6
45	10.5	35.7	98.4	215.1
45	11.0	36	98.8	290.5
57	7.5	15.5	98.4	148.0
57	8.75	23.4	98.6	190.5
57	10.0	23.3	96.8	82.0
65	8.75	10.0	97.0	73.0

604

605 **Table 5:** Results of enzyme concentration on the reaction enantioselectivity.
606

Units enzyme	Conv (%)	Time (h)	ee (%)	E
10	33.9	5	99.0	331
100	47.2	3	98.0	287
200	47.6	3	97.2	208

607

608 **Table 6:** Results of the effect of buffer concentration on conversion and ee
609

Buffer conc. (M)	Conv (%)	ee (%)	E
0.01	38.7	98.8	315
0.1	33.9	99.0	331
0.5	22.3	98.8	219
1.0	9.7	98.2	122

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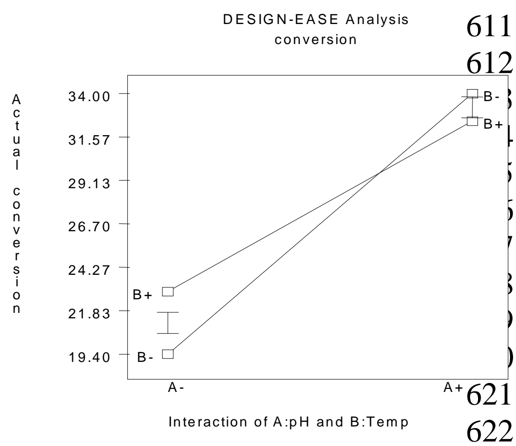

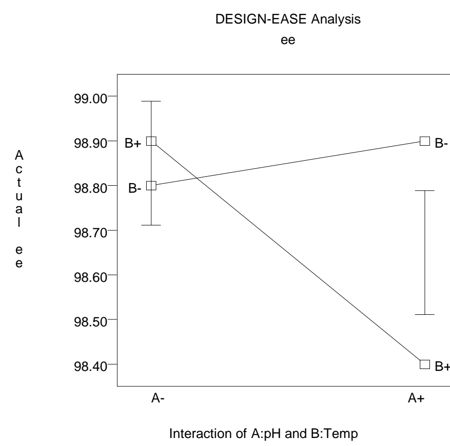

623

624 **Figure**. 6A **Figure**. 6B

625

626

627 **Figure 6**: Interpretation graph of actual conversion (6A) and ee (6B) respectively for

628 determination of interaction between (A) pH (8 and 10) and temperature (B) (35°C

629 and 50°C)

630

631
632 **Table 7:** Results of the laboratory scale (600 mℓ) duplicate recycling runs.
633

Recycle No.	Enzyme Conc (U)	Conv	ee_p	E
1	14	40.1	99.2	499
2	14	40.0	99.2	497
3	14	39.8	99.2	494
4	14	37.5	99.4	611
5	14	37.5	99.0	365

634