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Enzymatic resolution of naproxen

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Abstract—*Trichosporon* sp. (TSL), a newly found strain isolated from a locally fermented cottage cheese has been found to be highly stereoselective in the resolution of (*S*)-(+)-naproxen (ee >99%, $E\sim500$) from the corresponding racemic methyl ester. The process of resolution using whole cells has been scaled up to multi-kg level. Optimization of experimental conditions including downstream processing at 80–100 g/L substrate concentration with >90% recovery has been achieved. Changes in the physical parameters such as the particle size of the substrate play an important role in the resolution kinetics. A new strain of *Trichosporon* sp. having high cell density in cultivation (>60 g dry cell mass L^{-1} in 14–16 h) is found to be sufficiently stable for two years in dry powder form at 5–8°C. The viability of the resolution process has been further improved by the development of a simple racemization process for the enriched (*R*)-(−)-ester.

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1. Introduction

Aspirin, a first generation anti-inflammatory drug, discovered 150 years ago, is still in use because of its multiple biological activities. The introduction of indomethacin in the 1960s as a second-generation anti-inflammatory agent was followed by 2-arylacetic and propanoic acids by Chen et al.[1] and Adams et al.[2] (known as 2-APA's or profens) which had comparatively lower side effects such as gastrointestinal irritation, hepato-toxicity, papillary necrosis besides higher therapeutic index. Parallel to the study on 2-arylpropanoic acids,[3] another potent NSAID namely (*S*)-(+)-6-methoxy-α-methyl-2-naphthaleneacetic acid (naproxen) possessing eleven and fifty five times higher activity compared to phenylbutazone and aspirin respectively was reported by Harrison et al.[4] In terms of biological activity, the (*S*)-enantiomer is thirty times more active than the (*R*)-enantiomer.[5] Naproxen, therefore is one of the early chiral molecules developed and used as a single enantiomer.[6] Besides several asymmetric syntheses,[7–11] the two most commonly used resolution methodologies being adopted for the preparation of (*S*)-(+)-naproxen from its racemate are (a) resolution via diasteroisomeric salt formation[12,13] and (b) enzymatic kinetic resolution. The use of enzymes for their kinetic resolution began in the 1970s when Gist Brocades, in a patent, disclosed the preparation of (*S*)-(+)-naproxen through enantioselective hydrolysis of alkyl esters, using a biocatalyst from *Bacillus thai* and other microorganisms.[14] The resolution process was performed at low substrate concentration and required longer reaction time (5–6 days). Qu-Meng et al.[15] also reported bio-catalytic resolution of racemic esters at mmol scale using crude isolated lipase from *Candida cylindraceae* (CCL) and *Mucor rhizopus*. CCL was shown to hydrolyse (*S*)-(+)-ester to (*S*)-(+)-naproxen (acid) while other enzymes hydrolysed the (*R*)-(−)-ester to the corresponding acid. Bianchi et al.[16] in their patent reported a yield of 19% for the (*S*)-(+)-naproxen after 50 days of continuous reaction by using immobilized CCL. Water soluble esters were prepared by Matson et al.[17] for kinetic resolution studies, by means of a two stage extractive membrane, to obtain (*S*)-(+)-naproxen. In the last few years, an increasing number of enzymatic kinetic resolution reactions have been reported for the preparation of (*S*)-(+)-naproxen involving *trans*-esterification as well as hydrolytic methods[18–21] using nitrilases,[22,23] isomerases[24] and lipases and esterases.[25–41] Polyclonal antibodies have also been utilized as stereoselective catalysts for the hydrolysis of esters.[42–45] Resolution has also been reported with animal liver enzymes.[46]

Such a large number of patents and publications appearing in literature in the last decade and a half in the area of enzymatic resolution of (*S*)-(+)-naproxen underscores the importance of enzymatic processes,

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which are catalytic, simple, and environmentally friendly.

2. Results and discussion

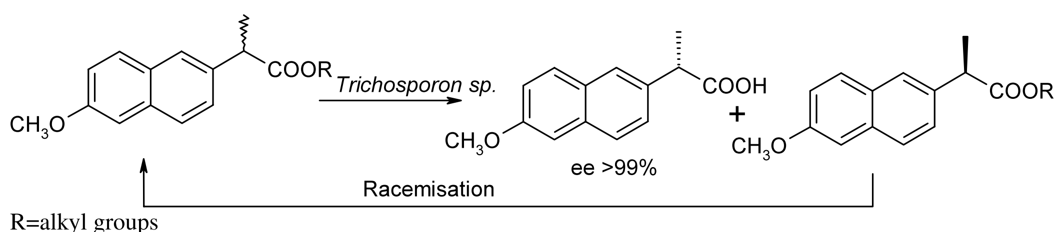
As a part of our study into the kinetic resolution of drugs, their intermediates and chiral auxiliaries[47–49] using indigenous microbial enzymes and also commercial ones, we herein report a highly stereoselective process for the preparation of (*S*)-(+)-naproxen using whole cells of *Trichosporon* sp.(TSL), a newly found strain isolated from a locally fermented cottage cheese. The culture has been deposited in Deutsche Sammlung von Mickroorganismen und Zellekulturen GmbH Braunschweig, Germany (DSMZ11829) and its hydrolytic activity has also been patented.[50,51] The effectiveness of the new yeast strain lies in its very high selectivity for the (*S*)-enantiomer in hydrolyzing the alkyl esters, particularly the methyl ester of (±)-6-methoxy-α-methyl-2-naphthaleneacetic acid (Scheme 1). Another advantage is its cultivation to high cell density (>60 g dry cell mass L^{-1} in 14–16 h fermentation time).

The isolated organism was identified as a strain of *Trichosporon* sp. belonging to an imperfect yeast of the family *Cryptococaceae* and sub-family *Trichosporoidea*. The resting cells, as well as the ester hydrolase isolated from it, were found to have stable biochemical characteristics at temperatures in the range of 25–35°C and pH 6–9. The isolated enzyme in its lyophilized state in crude or cell free form retained its activity when stored at 4°C for 2 years. Under optimized fermentation conditions >60 g dry cell mass/L broth was obtained. It showed triglyceride (triacetin) hydrolysing activity of 45 U/g dry cell mass and 75 U/g solid for lyophilized cell free extract. After detailed experimentation an optimum enzyme to substrate ratio of 85 U of enzyme per gram of the substrate was established. Since we did not have a prior knowledge of the effectiveness of TSL with respect to various racemic alkyl esters of naproxen, for that reason several esters of (±)-6-methoxy-α-methyl-2-naphthaleneacetic acid were prepared to investigate its behaviour.

Table 1 summarizes the results of the kinetic resolution of various racemic alkyl esters using whole cell preparation of TSL. For the methyl ester the enzyme displayed a very high selectivity (ee >99%, $E \sim 500$) and a fairly good rate of hydrolysis to produce (*S*)-(+)-naproxen. However, the rate of hydrolysis as well as the selectivity were influenced by the size of alkyl group of the ester moiety. The lowest selectivity (ee ~20%) was observed when *n*-heptyl ester was used as a substrate. The chloroethyl ester group ($COOCH_2CH_2Cl$) on the other hand proved to be the least accepted substrate even though a selectivity of 80% was recorded.

2.1. Optimisation of reaction conditions

Since TSL showed both a high selectivity as well as a high conversion rate for the methyl ester of racemic naproxen, it was selected as the substrate for further optimization studies such as the influence of particle size of the substrate, substrate concentration, reaction temperature and pH as well as effect of co-solvents to improve overall time–space yields.



Scheme 1.

Table 1. Hydrolysis of racemic esters of (±)-6-methoxy-α-methyl-2-naphthaleneacetic acid using TSL (whole cell pellet)[a]

Entry (R)	Reaction time (h)	Conversion (%)	Product ee (%)	Enantioselectivity factor
CH_3	48	45	>99	500
C_2H_5	48	44	97	164
i-C_3H_7	96	45	96	118
CH_2CH_2Cl	96	11	80	10
$CH_2CH_2OCH_3$	96	28	73	8
n-C_4H_9	96	20	70	7
t-C_4H_9	96	34	80	13
n-C_7H_{15}	96	15	20	2
$CH_2C_6H_5$	96	25	94	44

[a] *Reaction conditions*: pH 8.0 (0.1 M phosphate buffer) temp. 28°C, particle size 300–150 μm (50–100 mesh), conc. of substrate: 40 g/L, enzyme 85 U/g of substrate, conversions and enantiomeric purities were determined by chiral HPLC analysis. Racemic acid and most of the racemic esters were resolved on a single chromatogram using a chiral HPLC Lichro Cart 250-4 (*S*,*S*)-Whelk-01 (5 μm) column.

2.2. Influence of the particle size of the substrate on the reaction kinetics

The particle size of the substrate proved to be very important in the overall kinetics of the reaction. The racemic methyl ester, being highly insoluble in an aqueous phase, was used in the form of a fine powder. It was observed that commercial samples of racemic methyl ester had wide variations in their particle size from 650 to 150 μm (20–100 mesh). As a result the rate of hydrolysis also varied from batch to batch. Therefore the effect of the substrate particle size reduction from 300 μm (50 mesh) to 150 μm (100 mesh) and further to 75 μm (200 mesh) was studied. As depicted in Figure 1, a direct relationship between the particle size of the substrate and the rate of the hydrolysis was observed which may be attributed to improved amalgamation and interactions between substrate and the enzyme.

2.3. Influence of substrate concentration on reaction kinetics

Keeping all other reaction parameters constant, the substrate concentration was varied from 40 g/L to 140 g/L. The average rate of product formation recorded was generally above or at 0.8 g/L/h for the first 24 h irrespective of the substrate concentration up to ~100 g/L (Fig. 2). However, the influence of a higher substrate concentration beyond 100 g/L became apparent when a significant drop in productivity was observed. This can be attributed to a combination of various factors such as higher viscosity and to a lesser extent product inhibition.

Marginally better reactor rheology was created when cell free extract in place of whole cell culture, was used. However the overall reaction rate, did not show any significant improvement (data not shown). Periodic addition of substrate and removal of the product through on line membrane filtration only marginally improved the reaction rate. It is apparent from the above studies that 80–100 g/L is the optimal concentration for TSL catalysed resolution.

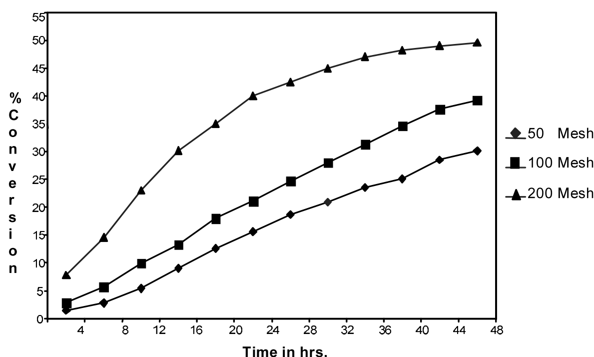


Figure 1. Influence of particle sizes on the rate of reaction at pH 8.0, temp 30°C, substrate concentration 50 g/L, enzyme 85 U/g of substrate.

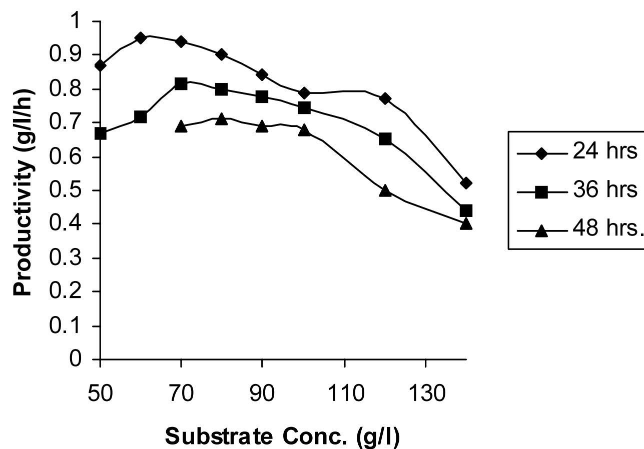


Figure 2. Effect of substrate concentration on the rate of product formation.

Table 2. Effect of temperature variation on enantioselectivity and hydrolytic activity of TSL[a]

S. No.	Temp. (°C)	Conversion (%)	Product ee (%)
1	25	35	99
2	28	45	99
3	30	45	99
4	32	45	99
5	35	45	97
6	42	47	80
7	45	43	60

[a] Conc. of the substrate 50 g/L, particle size 200 mesh, pH 8.0, time 30 h enzyme 85 U/g of the substrate.

2.4. Influence of temperature

Though live cells of TSL tolerated a wider range of cultivation temperature (25–35°C) and were able to withstand exposure to 45°C for 3 h, the optimum activity of the enzyme in terms of selectivity and conversion remained in a narrow range of 28–32°C (Table 2).

2.5. Influence of pH

Several buffers in the concentration range of 0.1–1.5 M were used as media but showed no significant improvement in the kinetics of the reaction. However at pH 7–8 an optimal reaction rate and selectivity was recorded. As can be seen from the Table 3, pH values below 7 are unfavorable in terms of both rate of hydrolysis and selectivity. An increase beyond pH 8 marginally improved the rate of the reaction but at the cost of selectivity. The marginal loss of ee at pH 9.0 can be attributed to competitive non-enzymatic hydrolysis in basic conditions as observed in a separate set of reactions carried out in the absence of enzyme where 3% hydrolysis with 0% ee was observed, thus signifying a retention of high selectivity by TSL even at higher pH.

Table 3. Influence of pH on the hydrolytic activity and enantioselectivity[a]

S. No.	pH	Conversion (%)	Product ee (%)
1	5.2–6.2	16	90
2	6.0	27	90
3	6.7	34	98
4	7.0–7.2	43	>99
5	7.8	45	>99
6	8.0	44	99
7	8.5	45	96
8	9.0	48	94

[a] Conc. of the substrate 50 g/L, particle size 200 mesh, time 30 h, enzyme 85 U/g of the substrate.

2.6. Influence of co-solvents

The use of co-solvents was also studied with methyl ester as the substrate. However, both non-polar and polar solvents (up to 10% v/v) showed significant decreases in reaction rates as well as in enantioselectivity (data not included).

2.7. Comparative hydrolytic activity of TSL vis-à-vis selected commercially available enzymes

The data of the hydrolytic activity and selectivity of four commercially available enzymes namely PPL, CCL, CRL, and PSL vis-à-vis TSL is presented in Table 4. While the PPL and PSL lipases failed to hydrolyse the methyl ester of (±)-6-methoxy-α-methyl-2-naphthaleneacetic acid under the experimental conditions, CRL and CCL displayed comparable enantioselectivity with respect to TSL. The latter however, showed distinct superiority with a far higher rate of hydrolysis.

2.8. Racemisation

Experiments were also carried out to racemise the enriched methyl ester of (*R*)-(−)-6-methoxy-α-methyl-2-naphthaleneacetic acid for its reutilization. Both sodium metal and sodium methoxide were found effective in completely racemising the ester (98% yield), the details are given in Section 3.

Table 4. Hydrolytic behaviour of TSL vis-à-vis selected commercial enzymes with (±)-6-methoxy-α-methyl-2-naphthaleneacetic acid methyl ester[a]

Enzyme	Reaction time (h)	Conversion (%)	ee (%)	Configuration
PSL	60	–	–	–
PPL	60	–	–	–
CCL	60	14.3	99	*S*
CRL	60	7	99	*S*
TSL	**24**	**45**	**>99**	***S***

[a] Substrate conc. 40 g/L, pH 8.0 (0.1 M phosphate buffer), temp. 32°C, particle size ∼200 mesh, enzyme 85 U/g of substrate, for commercial enzymes ratio of enzyme (dry powder):substrate 1:3.

3. Experimental

3.1. General

All the reagents used in the study were of analytical grade. The authenticity of racemic esters prepared during the study was confirmed by spectroscopic analysis including NMR, LC/MS and of the resolved enantiomers by optical rotation and chiral chromatographic methods. Melting points recorded by capillary method are uncorrected. The optical rotations were measured on a Perkin–Elmer 241 polarimeter with chloroform as the solvent. HPLC analyses were carried out on a Lichro Cart 250-4 (*S*,*S*)-Whelk-01 (5 μm) column with a mobile phase of hexane:isopropanol:acetic acid (90:10:0.5%) and a flow rate of 1.2 mL/min using a diode array detector (254 nm). Reactions were routinely monitored on 0.25 mm silica gel plates (E. Merck) using UV light for detection of the spots. The commercial lipases like *Porcine pancreatic* lipase (PPL, enzyme activity 0.23 U/mg solid with triacetin), *Candida rugosa* lipase (CRL enzyme activity 0.190 U/mg solid with triacetin) and *Candida cylindraceae* lipase (CCL, enzyme activity 0.33 U/mg solid with triacetin) were purchased from M/s Sigma Chemical, USA. *Pseudomonas* sp. lipase (PSL, enzyme activity 0.25 U/mg solid with triacetin) was obtained as free gift form M/s. Amano, Japan. The procured lipases were used as such without purification. Metrohm pH Stat-718 was used for monitoring the pH. Standard sieves of 50, 100 and 200 mesh were employed for obtaining the required particle size of the substrate. For cell disintegration, a Kinnomed Konsolt-AB (Sweden) disintegrator was used.

3.2. Cultivation of *Trichosporon* sp. (TSL)

A culture medium, comprising of 1.5% glucose, 0.05% potassium dihydrogen phosphate (KH_2PO_4), 1.0% corn steep liquor and 0.3% of urea (pH 6.8 before sterilization and pH 6.5 after sterilization) was prepared and dispensed in shake flasks (200 mL each) and in a 10 L stainless steel fermenter (SS 304S, working volume 7.5 L) and autoclaved. The pre-culture of the strain *Trichosporon* sp. (DSMZ 11829) was prepared in the shake flask by inoculating a loopful of the culture prepared on solid agar medium and incubating the flask at 30°C on a rotary shaker. The 24 h old pre-culture thus produced was inoculated (5% v/v) into the culture medium and fermentation carried out at 500 rpm, 0.5 volume/volume/min aeration rate and kept at a constant temperature of 30°C for 14–18 h. The culture was thereafter centrifuged to collect the yeast cells. The cell pellet was washed twice with distilled water and the wet cell mass of TSL thus obtained was lyophilized at −40°C to obtain a dry powder that was found stable for 2 years when stored at 5–8°C.

3.3. Preparation of cell free extract and crude enzyme

The wet cell mass prepared as described above was either sonicated (at 40% w/v solid suspension) for 5 min or milled with glass beads of size ϕ 0.5 mm for 10 min

at 0–4°C. The sonicated or glass milled mixture was centrifuged at 10,000*g* for 10 min at 0–4°C. For the larger quantities (>500 g cell pellet) cell free extracts were prepared in a continuous glass mill using a cell disintegrator[52] (volume 150 mL, glass beads, size ϕ 0.1 mm). A yeast cell suspension of 40% (w/v) was prepared in a phosphate buffer 0.1 M at pH 7.0 keeping a flow rate of 450 mL/h at 0–4°C. The glass-milled mixture was centrifuged at 10,000*g* for 10 min at 4°C. The cell free extract thus obtained was lyophilized at −40°C with the resulting solid showing a hydrolysing activity of 0.075 and 0.145 U/mg, respectively, using triacetin and *p*-nitrophenyl acetate as assay substrates (one unit of enzyme corresponds to liberation of 1 μmol of organic acid from triacetin or hydrolysis of 1 μmol of *p*-nitrophenyl acetate in 1 min). Protein estimation was made according to Lowry's method using BSA as the reference protein.[53]

3.4. Preparation of alkyl esters

3.4.1. Method A. In a typical procedure, a solution of (±)-6-methoxy-α-methyl-2-naphthaleneacetic acid (150 g, 650 mmol) in methanol (520 mL) was placed in a three-necked round bottom flask fitted with a reflux condenser. Concentrated sulfuric acid (10 mL) was added with constant stirring while maintaining the temperature at 60–70°C until the addition was complete (70 min). The contents were then heated on a water bath for 20 min, cooled and solid filtered. The filtrate was concentrated to one fourth of its original volume and cooled again. The two crops of the solid were pooled together, washed thoroughly with water and dried to give the racemic methyl ester (146 g, yield 92%) mp 75°C.

3.4.2. Method B. In an alternative process the racemic ester was prepared by treating the dichloromethane solution of the acid chloride of (±)-6-methoxy-α-methyl-2-naphthalene acetic acid (160 mmol) with dry *n*-butanol (200 mmol). The resulting ester after normal work up was purified by column chromatography on silica gel using EtOAc:CH_2Cl_2 (9:1) as the eluant to furnish the corresponding butyl ester (40.7 g, yield 93%) mp 52–53°C.

3.5. Process for the kinetic resolution of the methyl ester of (±)-6-methoxy-α-methyl-2-naphthalene acetic acid

Two different methodologies depending upon the scale of the reaction were adopted for the downstream process [separation of (*S*)-(+)-naproxen from enriched (*R*)-(−)-ester after the completion of the reaction].

3.5.1. Method A (suitable up to 500 g scale). *Trichosporon* sp. (TSL, 280 g lyophilised powder, ~85 U/g of substrate) was added to a suspension of racemic methyl ester (150 g, 200 mesh, substrate conc. 90 g/L) in a sodium phosphate buffer (0.1 M, pH 8.0, 1650 mL). The contents were stirred at 30°C and the pH maintained by a pH stat using 1 M NaOH solution. Periodically aliquots were drawn and analysed on chiral HPLC. The reaction was terminated after 48 h (conversion ~41%) by adding dilute HCl. The acidified contents (pH 3) were extracted with ethyl acetate (5×600 mL). The combined organic layer was then extracted with 5% sodium hydroxide solution (4×125 mL). The aqueous alkaline portion was washed with ethyl acetate:*n*-hexane (1:9, 3×100 mL) to remove the traces of the enriched (*R*)-ester and thereafter acidified with dilute hydrochloric acid. The resulting white precipitate of the (*S*)-(+)-acid was filtered, washed with distilled water and dried at 50°C to give (*S*)-(+)-naproxen as a white powder (53.8 g) $[\alpha]_D = +65.6$ (*c* 1, $CHCl_3$) ee ~99% (chiral HPLC Lichro Cart 250-4 (*S*,*S*)-Whelk-01 (5 μm) column). The organic layer and ethyl acetate:*n*-hexane washings were pooled, washed with distilled water (3×50 mL), dried over sodium sulfate and concentrated to furnish enriched (*R*)-ester (86.5 g).

3.5.2. Method B (suitable for multi-kg scale). The methyl ester of (±)-6-methoxy-α-methylnaphthaleneacetic acid (5.0 kg, 200 mesh), was added to an aqueous suspension, comprising of lyophilized cells of TSL (9.0 kg, ~85 U/g of substrate) in distilled water (44 L) and the contents stirred at 600 rpm at a temperature 30±1°C while maintaining the pH at 8.0 (substrate concentration approx. 80 g/L) with a pH stat using a 2 M NaOH solution. Aliquots were regularly drawn to monitor the progress of the reaction on a chiral HPLC. The reaction was terminated after 48 h (43% conversion) with the pH adjusted to 8.5, the contents centrifuged and the cell mass washed thoroughly with 5% NaOH solution (6 L) and re-centrifuged. This process was repeated twice and the combined aqueous solution passed through a filter membrane followed by extraction with *n*-hexane:ethyl acetate (9:1, 2×3.5 L). The resolved (*S*)-acid from the aqueous solution was precipitated by adding 15% sulfuric acid (6 L), filtered, washed with distilled water and dried in an air oven at 50°C to furnish the (*S*)-naproxen (1674.5 g) mp 152–52.5°C $[\alpha]_D = +66.6$ (*c* 1, $CHCl_3$,), ee >99% (HPLC). The organic solvent washings containing small amounts of the (*R*)-ester were concentrated (40.5 g) and pooled with the cell mass left after centrifugation. The cell mass holding the enriched (*R*)-ester, with some small amounts of the (*S*)-acid left unextracted after the removal of bulk acid, was then partially dried at 50°C (air oven). The dried mass (approx. 12 kg) was then placed in an extractor (Dean Stork type) containing toluene (40 L). Boiling and distillation of toluene (5 L) removed traces of water from the cell mass and the contents were refluxed at 110°C to dissolve the ester and the acid adsorbed on it. The organic layer was drained out and the solid mass extracted twice with boiling toluene (2×20 L). After cooling, the combined toluene extracts were extracted with 5% sodium hydroxide solution (6 L) and the alkaline aqueous portion washed with ethyl acetate:*n*-hexane (1:9, 1.5 L) and then acidified to pH 4.0 with dilute hydrochloric acid. The precipitate was filtered, washed with distilled water and dried to give a white solid of (*S*)-(+)-naproxen (164 g). The combined toluene layer [estimated to contain 2.71 kg of enriched (*R*)-ester] was washed with distilled water (2×2 L), dried over anhy-

drous calcium chloride and the solution used directly for racemisation reaction.

3.6. Racemisation of (*R*)-enriched ester

A toluene solution comprising of enriched (*R*)-(−)-methyl ester of 6-methoxy-α-methyl-2-naphthalene acetic acid (approx. 2.7 kg, ~11 mol) was initially concentrated (5 L toluene distilled over) to remove the last traces of water. To the remaining solution was added either freshly cut sodium metal (40 g) or freshly prepared sodium methoxide (prepared from 40 g sodium metal and dry methanol). The contents were then refluxed and reaction monitored by chiral HPLC. After complete racemisation (2.5–3 h), the mixture was cooled, neutralized with concentrated sulfuric acid (50 mL) and washed with water until pH 7 was attained. The organic layer was dried over anhydrous sodium sulfate and concentrated at reduced pressure to furnish the racemised ester (2.6 kg, 98% yield). The racemic ester thus obtained was decolourised by activated carbon prior to micronisation and reuse for kinetic resolution

4. Conclusion

Trichosporon sp. (TSL) whole cells or its cell free ester hydrolase selectively hydrolyses (±)-6-methoxy-α-methyl-2-naphthaleneacetic acid alkyl ester to yield (*S*)-naproxen. The yeast organism, or the enzyme derived from it, depicts a very high enantioselectivity (ee >99%, $E=500$) in resolving the racemic ester into the free acid and unhydrolysed ester. The rate of hydrolysis increased significantly with a decrease in substrate particle size from <50 to 200 mesh. The enzyme to substrate ratio of 85 U of enzyme per gram of the substrate was optimal with the rate of hydrolysis not much influenced up to a substrate concentration of 100 g/L. Temperature in the range of 28–32°C and pH between 7 and 8 were found to be optimum for the best time space yield and enantioselectivity. The use of a buffer or an aqueous solution had no direct effect on the rate of the hydrolysis or enantioselectivity. The use of co-solvents also did not show any advantage. A racemisation method using sodium metal or sodium methoxide for enriched (*R*)-(−)-ester has also been optimized with a >98% yield. With this step, a practical and semi dynamic process has been developed for the conversion of (±)-naproxen ester to (*S*)-naproxen.

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