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Aldol Reactions by Lipase From *Rhizopus niveus*, an Example of Unspecific Protein Catalysis

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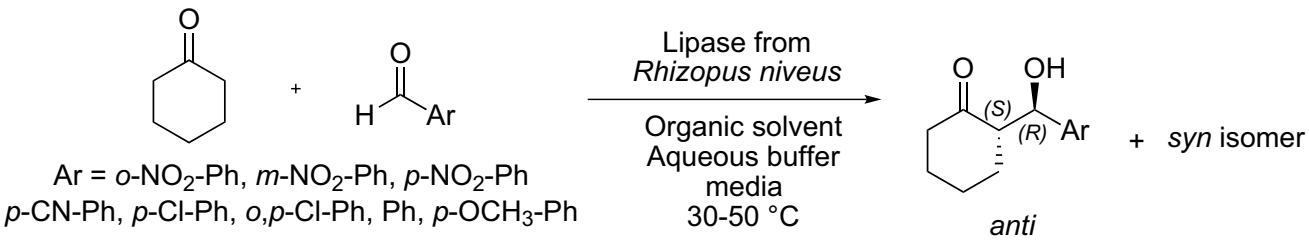
Abstract The lipase from *Rhizopus niveus* (RNL) catalyzed by unspecific protein catalysis the aldol reactions between cyclohexanone and aromatic aldehydes in organic solvents with water or aqueous buffer solution. The reactional conditions strongly influenced the yield (0–99%) and enantioselectivities in the *anti*-products (6–55% ee_{anti}). The aldol products with enantioselectivities in the *anti*-product were observed for inactive enzyme and in denaturing conditions. Therefore, the reactions in the evaluated conditions proceeded by unspecific protein catalysis with moderate enantioselectivities and not by promiscuous activity. These enzymatic studies identify a new green catalyst for aldol reactions and new possibilities for lipases, especially for the use of the lipase RNL, which has been scarcely reported in the literature and catalyzed the aldol reaction between different aromatic aldehydes and cyclohexanone.

Graphical Abstract



Keywords Aldol reaction · Enzymatic promiscuity · Hydrolase · Unspecific protein catalysis · Biocatalysis

1 Introduction

Enzymes are specific green catalysts, but usually have a secondary activity namely promiscuous, which are over the effect of evolution and natural selection [1, 2]. Aldolases are enzymes that carry out aldol reactions, however specificity and cost limit their employment in organic synthesis [3, 4]. An alternative for the use of aldolases are enzymes from other classes, which can display the promiscuous aldolase activity towards the formation of aldol compounds [5, 6].

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Branneby et al. [7] reported the first aldol reaction catalyzed by an enzyme, Cal-B. Further, the first asymmetric aldol reaction catalyzed by porcine pancreas lipase type II (PPLII) was reported [6], this enzyme was extensively studied for aldol reactions being employed with different substrates and reactional conditions [8–10].

Several lipases were also studied for aldol reactions, such as, bovine pancreatic lipase [11], *Mucor javanicus* lipase, *Burkholderia cepacia* lipase, *Candida rugosa* lipase, *Mucor miehei* lipase [9], *Rhizopus oryzae* lipase, hog pancreas lipase [12], Amano lipase A from *Aspergillus niger* [8], Amano lipase AK from *Pseudomonas fluorescens* and Amano lipase PS from *B. cepacia* [10].

Different classes of enzymes were also employed as biocatalysts for promiscuous aldol reactions such as protease from *Bacillus licheniformis* [13], protease from *Aspergillus usamii* [14], chymopapain [15], trypsin [16], Ficin [17], proteinase from *Aspergillus melleus* [18], nuclease P1 from *Penicillium citrinum* [19] and a thermophilic esterase from the archaeon *Aeropyrum pernix* K1 [20]. However, according to Lopez-Iglesias and Gotor-Fernández [21], it is important to distinguish the active site catalysis in a desired reaction from the unspecific protein catalysis, in which amino acid residues perform the aldol reaction. Since aldol reaction by protein, such as bovine serum albumin, without enantioselectivity was reported [22].

Lipase from *Rhizopus niveus* have a catalytic center analogous to neutral lipases and sheltered by an α-helix lid, opening the active site at oil–water interfaces [23]. This enzyme is commercially available and few catalysis examples were described in the literature, including the synthesis of aroma acetoin fatty acid esters [24], resolution of racemic unsaturated γ-lactones [25] and preparation of *S*-propranolol [26].

In this study, *R. niveus* lipase was screened and subsequently assessed for the aldol reaction between cyclohexanone and aromatic aldehydes, such as 4-nitrobenzaldehyde.

2 Experimental

2.1 Enzymes

The employed enzymes were lipases from porcine pancreas (100–500 U mg^{-1}), *R. niveus* (4.49 U mg^{-1}), *A. niger* (0.184 U mg^{-1}), *P. fluorescens* (≥20 U mg^{-1}), *Candida rugosa* (1176 U mg^{-1}), *C. cylindracea* (4.9 U mg^{-1}), *Burkholderia cepacia* (≥30 U mg^{-1}), *Thermomyces lanuginosus* (≥100 U mL^{-1}). Others employed enzymes were cellulase from *Trichoderma viride* (5 U mg^{-1}) and, cellulase (1.08 U mg^{-1}) and hemicellulase (1.5 U mg^{-1}) from *A. niger*. All the enzymes were obtained from Sigma-Aldrich.

2.2 Solvents and Reagents

4-Nitrobenzaldehyde (98%), 3-nitrobenzaldehyde (99%), 2-nitrobenzaldehyde (98%), 4-cyanobenzaldehyde (95%), 4-chlorobenzaldehyde (97%), 2,4-dichlorobenzaldehyde (99%), benzaldehyde (99%), 4-methoxybenzaldehyde (98%), cyclohexanone (99%), *p*-nitrophenyl butyrate (*p*-NPB, 98%), solvents (EtOAc, DMSO, MeOH, EtOH, MeCN and *i*-PrOH) and buffer solution reagents (citric acid monohydrate, sodium phosphate bibasic, sodium phosphate monobasic, imidazole and HCl) were obtained from Sigma-Aldrich.

2.3 General Procedure for Aldol Reaction Catalyzed by Enzymes

In a 25-mL round-bottom flask was added 100 mg of enzyme, 5.5 mL solvent, 4-nitrobenzaldehyde (1 mmol, 151 mg) and cyclohexanone (4 mmol, 415 µL). The resulting mixture was stirred for a specified time and temperature. Subsequently, the reaction was stopped with the addition of 30 mL of brine solution and 60 mL of ethyl acetate, filtered and a liquid–liquid extraction was performed. The organic phase was dried with anhydrous Na_2SO_4 and evaporated under reduced pression. The same procedure was employed for the others aromatic aldehydes. The obtained products were purified using flash column chromatography over silica gel eluted with *n*-hexane and ethyl acetate (7:3). The yield was determined and the products characterized.

2.3.1 (S)-2-[(R)-Hydroxy(4-nitrophenyl)methyl] cyclohexanone

White powder, $[\alpha_D^{25}]=+10.0$ (c 1.5, $CHCl_3$) ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.21 (2H, d, J = 8.5 Hz), 7.51 (2H, d, J = 8.5 Hz), 4.90 (1H, dd, J = 8.4, 3.0 Hz), 4.09 (1H, d, J = 3.0 Hz), 2.65–2.45 (2H, m), 2.36 (1H, m), 2.17–2.06 (1H, m), 1.87–1.78 (1H, m), 1.67–1.51 (3H, m), 1.45–1.31 (1H, m). Enantiomeric excess was determined by HPLC with a Chiralcell OD-H column (97:3 hexane:2-propanol), 30 °C, 254 nm, 1.0 mL min^{-1}; *rac-syn* enantiomers tr = 27.5 and 29.3 min; *anti* major (*S,R*)-enantiomer tr = 31.2 min, minor (*R,S*)-enantiomer tr = 39.2 min.

2.3.2 (S)-2-[(R)-Hydroxy(3-nitrophenyl)methyl] cyclohexanone [27]

1. White powder, $[\alpha_D^{25}]=+25.3$ (c 1.5, $CHCl_3$) ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.15–8.20 (2H, m), 7.67 (1H, d, J = 7.5 Hz), 7.52 (1H, t, J = 7.8 Hz), 4.90 (1H, d, J = 8.6 Hz), 4.15 (1H, d, J = 3.0 Hz), 2.68–2.58 (1H, m), 2.38–2.55 (2H, m), 2.16–2.07 (1H, m), 1.89–1.76

(1H, m), 1.31–1.71 (4H, m). Enantiomeric excess was determined by HPLC with a Chiralpak AD-H column (9:1 hexane:2-propanol), 30 °C, 225 nm, 1.0 mL min^{-1}; *rac-syn* enantiomers tr = 13.9 and 15.2 min; *anti* major (*S,R*)-enantiomer tr = 16.7 min, minor (*R,S*)-enantiomer tr = 20.6 min.

2.3.3 (S)-2-[(R)-Hydroxy(2-nitrophenyl)methyl] cyclohexanone [28]

Yellow powder, $[\alpha_D^{25}] = +5.3$ (c 1.5, $CHCl_3$) ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.84 (1H, d, J = 8.1 Hz), 7.77 (1H, d, J = 8.0 Hz), 7.63 (1H, t, J = 8.1 Hz), 7.43 (1H, t, J = 8.1 Hz), 5.45 (1H, d, J = 6.6 Hz), 4.10 (1H, br), 2.82–2.70 (1H, m), 2.50–2.30 (2H, m), 2.15-2.00 (1H, m), 1.90–1.50 (5 H, m). Enantiomeric excess was determined by HPLC with a Chiralcell OD-H column (95:5 hexane:2-propanol), 30 °C, 225 nm, 1.0 mL min^{-1}; *rac-syn* enantiomers tr = 10.6 and 12.3 min; *anti* major (*S,R*)-enantiomer tr = 15.0 min, minor (*R,S*)-enantiomer tr = 17.1 min.

2.3.4 (S)-2-[(R)-Hydroxy(4-cyanophenyl)methyl] cyclohexanone [27]

White powder, $[\alpha_D^{25}] = +14.0$ (c 1.5, $CHCl_3$) ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.65 (2H, d, J = 8.1 Hz), 7.45 (2H, d, J = 8.1 Hz), 4.85 (1H, dd, J = 8.1, 3.0 Hz), 4.13 (1H, d, J = 3.0 Hz), 2.60–2.40 (2H, m), 2.37 (1H, m), 2.17–2.06 (1H, m), 1.88–1.77 (1H, m), 1.72–1.47 (3H, m), 1.44–1.31 (1H, m). Enantiomeric excess was determined by HPLC with a Chiralpak AD-H column (80:20 hexane:2-propanol), 30 °C, 225 nm, 0.5 mL min^{-1}; *rac-syn* enantiomers tr = 15.8 and 17.8 min; *anti* minor (*R,S*)-enantiomer tr = 19.5 min, major (*S,R*)-enantiomer tr = 23.9 min.

2.3.5 (S)-2-[(R)-Hydroxy(4-chlorophenyl)methyl] cyclohexanone [27]

White powder, $[\alpha_D^{25}] = +10.6$ (c 1.5, $CHCl_3$) ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.29 (4H, dd, J = 20.2, 8.4 Hz), 4.75 (1H, dd, J = 8.7, 2.7 Hz), 4.00 (1H, d, J = 3.0 Hz), 2.60–2.40 (2H, m), 2.35 (1H, td, J = 12.9, 5.4 Hz), 2.15–2.05 (1H, m), 1.85–1.75 (1H, m), 1.70–1.50 (3H, m), 1.35–1.20 (1H, m). Enantiomeric excess was determined by HPLC with a Chiralpak AD-H column (90:10 hexane:2-propanol), 30 °C, 254 nm, 0.5 mL min^{-1}; *rac-syn* enantiomers tr = 15.8 and 18.4 min; *anti* minor (*R,S*)-enantiomer tr = 23.1 min, major (*S,R*)-enantiomer tr = 26.8 min.

2.3.6 (S)-2-[(R)-Hydroxy(2,4-dichlorophenyl)methyl] cyclohexanone [29]

White powder, $[\alpha_D^{25}] = +10.0$ (c 1.5, $CHCl_3$) ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.50 (1H, d, J = 8.4 Hz), 7.35 (1H, s), 7.29 (1H, d, J = 8.4 Hz), 5.29 (1H, dd, J = 7.5, 3.3 Hz), 4.10 (1H, d, J = 3.9 Hz), 2.70–2.55 (1H, m), 2.50–2.25 (2H, m), 2.15–2.05 (1H, m), 1.90–1.78 (1H, m), 1.75–1.50 (4 H, m). Enantiomeric excess was determined by HPLC with a Chiralcell AS-H column (90:10 hexane:2-propanol), 30 °C, 254 nm, 0.5 mL min^{-1}; *rac-syn* enantiomers tr = 9.5 and 10.5 min; *anti* minor (*R,S*)-enantiomer tr = 12.1 min, major (*S,R*)-enantiomer tr = 13.2 min.

2.3.7 (S)-2-[(R)-Hydroxy(phenyl)methyl]cyclohexanone [30]

White powder, $[\alpha_D^{25}] = +11.0$ (c 1.0, $CHCl_3$) ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.45–7.20 (5 H, m), 4.80 (1H, d, J = 9.0 Hz), 4.00 (1H, m), 2.70–2.50 (1H, m), 2.55–2.42 (1H, m), 2.34 (1H, td, J = 12.3, 5.4 Hz), 2.15–2.02 (1H, m), 1.88–1.70 (1H, m), 1.75–1.50 (3H, m), 1.40–1.20 (1H, m). Enantiomeric excess was determined by HPLC with a Chiralcell OD-H column (90:10 hexane:2-propanol), 30 °C, 254 nm, 0.5 mL min^{-1}; *rac-syn* enantiomers tr = 12.7 and 13.9 min; *anti* major (*S,R*)-enantiomer tr = 15.1 min, (*R,S*)-minor enantiomer tr = 17.8 min.

2.3.8 (R)-2-[(S)-Hydroxy(4-methoxyphenyl)methyl] cyclohexanone [29]

White powder, $[\alpha_D^{25}] = -10.0$ (c 1.0, $CHCl_3$) ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.22 (2H, d, J = 8.4 Hz), 6.88 (2H, d, J = 8.7 Hz), 4.74 (1H, dd, J = 8.8, 2.5 Hz), 3.92 (1H, d, J = 2.7 Hz), 3.80 (3H, s), 2.65–2.49 (1H, m), 2.52–2.43 (1H, m), 2.35 (1H, td, J = 12.9, 5.4 Hz), 2.18–2.03 (1H, m), 1.84–1.73 (1H, m), 1.70–1.45 (3H, m), 1.35–1.23 (1H, m). Enantiomeric excess was determined by HPLC with a Chiralpak AD-H column (95:5 hexane:2-propanol), 30 °C, 254 nm, 1.0 mL min^{-1}; *rac-syn* enantiomers tr = 17.6 and 20.8 min; *anti* minor (*S,R*)-enantiomer tr = 29.8 min, (*R,S*)-major enantiomer tr = 31.5 min.

2.4 Yield, Enantioselectivity and Diastereoselectivity Determination

NMR analyses: the reaction product was dissolved in 25 mL of ethyl acetate. Subsequently, 3 mL of this solution were evaporated under reduced pressure, dissolved in $CDCl_3$ and employed in ^{1}H-NMR analyses recorded on an Agilent Technologies 400/54 Premium Shielded spectrometer, with TMS as internal standard. The *anti* and *syn* ratio was determined by ^{1}H-NMR for the aldol products of

4-nitrobenzaldehyde (4.90, 5.46 ppm), 3-nitrobenzaldehyde (4.90, 5.45 ppm), 2-nitrobenzaldehyde (5.45, 5.95 ppm), 4-cyanobenzaldehyde (4.85, 5.43 ppm), 4-chlorobenzaldehyde (4.75, 5.35 ppm), 2,4-dichlorobenzaldehyde (5.29, 5.65 ppm), benzaldehyde (4.80, 5.39 ppm), 4-methoxybenzaldehyde (4.74, 5.32 ppm) with cyclohexanone.

HPLC analyses: yield determination by chromatographic analyses were performed only for the aldol product of 4-nitrobenzaldehyde with cyclohexanone. The yield was determined by HPLC employing water (A) and acetonitrile (B) as eluent, 35% B isocratic mode at 1.0 mL min^{-1} for 20 min using a SHIM-PACK CLC-ODS (M) of 25 cm. The retention times for the *anti*-product was 15.1 min and the *syn*-product 16.5 min.

In a round-botton flask, 1 mL of the initial solution was evaporated under vaccum and dissolved in 5 mL of isopropanol. Analytical curves for the *anti*- and *syn*-2-(hydroxyl(4-nitrophenyl)methyl)cyclohexan-1-one were constructed at 0.1, 0.7, 1.3, 1.9, 2.4 mg mL^{-1}. Linear equations were obtained for the *anti*- (c = 19,833x + 877,577) and *syn*-product (17,521x + 347,998). The yield was given by the equation $Y = (A_{syn}\text{-}877{,}577)/396{,}660 + (A_{anti}\text{-}347{,}998)/35{,}042)$.

2.5 Enzymatic Activity Determination

RNL activity was determined by the hydrolysis of *p*-nitrophenyl butyrate (*p*-NPB). The assay was monitored with a spectrophotometer (Hitachi U-200) at 400 nm, 30 °C and under magnetic stirring inside of a cuvette. The assay was carried out in 1450 µL of 20 mM pH 7.0 Tris-HCl buffer, 50 µL of 15 mg mL^{-1} comercial RNL or reaction sample and 15 µL of 70 mM *p*-NPB in acetonitrile [31].

3 Results and Discussion

3.1 Enzyme Screening with 4-Nitrobenzaldehyde and Cyclohexanone

Initially, a screening of lipases or cellulases for the aldol reaction between 4-nitrobenzaldehyde and cyclohexanone was performed (Scheme 1). The results are presented in Table 1.

The lipases from porcine pancreas (PPLII), *R. niveus* (RNL), *A. niger* (ANL), *P. fluorescens* (PFAL), *C. rugosa* (CRL), *C. cylindracea* (CCL) and cellulase from *T. viride* (TVC) (Entries 1–7, Table 1) catalyzed the aldol reaction. Whereas the aldol reaction product was not observed in significant amount for the experiments with lipases from *B. cepacia* (BCL), *T. lanuginosus* (TLL) and, cellulase (ANC) and hemicellulase (ANH) from *A. niger* (Entries 8–11, Table 1).

RNL was unsuccessfully employed for aldol reaction in previous studies using acetonitrile and H_2O as reaction medium [8, 10]. However, this enzyme catalyzed the aldol reaction in this study probably because of the use of DMSO and H_2O (9:1) as reaction medium with 35%

Table 1 Screening of enzymes for aldol reaction between 4-nitrobenzaldehyde and cyclohexanone

Entry	Enzyme	Yield (%)[a]	ee_{anti} (%)	*dr* (*anti:syn*)
1	PPLII	36	40	59:41
2	RNL	35	26	54:46
3	ANL	25	17	56:44
4	PFAL	15	0	55:45
5	CRL	9	29	55:45
6	TVC	20	30	56:44
7	CCL	13	22	55:45
8	BCL	Trace	Trace	Trace
9	TLL	Trace	Trace	Trace
10	ANH	Trace	Trace	Trace
11	ANC	Trace	Trace	Trace
12	Control[b]	Trace	Trace	Trace
13	RNL[c]	44	13	52:48
14	PPLII[c]	31	25	52:48
15	Control[c,d]	Trace	Trace	Trace

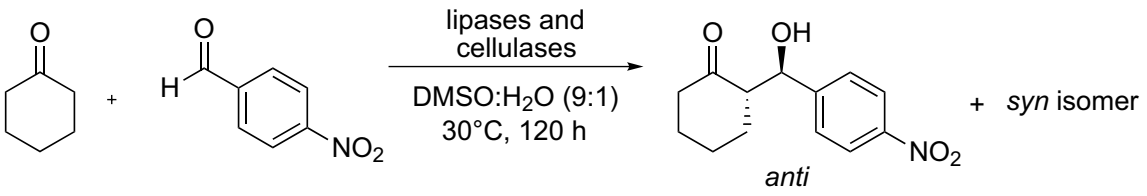
Reaction conditions: 4-nitrobenzaldehyde (151 mg), cyclohexanone (415 µL), enzyme (100 mg), 5.5 mL of DMSO and H_2O (9:1), 30 °C, 120 h, magnetic stirring

ee_{anti} enantiomeric excess of the (*S,R*)-anti-aldol product, *dr* diastereoisomeric ratio, *T* temperature

[a]Yield determined by HPLC-UV

[b]Control without enzyme

[c]Reaction carried out at 50 °C for 48 h



Scheme 1 Aldol reaction between 4-nitrobenzaldehyde and cyclohexanone by different enzymes

yield, 26% ee_{anti} and 54:46 *dr* in favour of the *anti*-isomer (Entry 2, Table 1).

The temperature increase to 50 °C promoted similar yields in shorter reaction time (48 h) when compared to reactions for 120 h at 30 °C. The yield was 36% for PPLII and 35% for RNL (Entries 1–2, Table 1), while at 50 °C for 48 h the yields were 31 and 44%, respectively (Entries 13–14, Table 1). None of the employed enzymes showed *ee* for the *syn* product and the aldol product was not observed in the control reaction at 30 °C (Entry 12, Table 1) and 50 °C (Entry 15, Table 1).

The results presented in Table 1 verified that PPLII (36%, Entry 1, Table 1) and RNL (35%, Entry 2, Table 1) provided the highest yield. PPLII has already been studied in the catalysis of the aldol reaction with results described in the literature [8]. Therefore, we decided to select RNL to be investigated as a biocatalyst of the aldol reaction between 4-nitrobenzaldehyde and cyclohexanone, since this enzyme is poorly studied in the literature.

3.2 Reaction Conditions Assessment for the Aldol Reaction Between 4-Nitrobenzaldehyde and Cycloehexanone

The increase of the water amount promoted an increase of the reactional yield from 44 to 97% (Entries 1–4, Table 2). An exception was observed for DMSO:H_2O ratio (1:9), in which 51% yield was determined (Entry 5, Table 2). At 48 h were obtained 96 and 97% of yield for reactions containing 30–70% water in DMSO (Entries 2–4, Table 2), showing the importance of the amount of water to maximize the yield. In reactions for 24 h with 30 and 50% water in DMSO, lower yields of 68 and 83% were obtained respectively (Entries 6–7, Table 2) when compared to 48 h-reactions (Entries 2–3, Table 2). Regarding enantioselectivity, no significant changes were observed since the selectivity was low.

The reaction at 30 °C (52% yield, 48 h) (Entry 9, Table 2) presented lower yield than at 50 °C (96% yield, 48 h) (Entry 2, Table 2), but with better enantioselectivities (16% ee_{anti} at 50 °C and 27% ee_{anti} at 30 °C).

The use of different water miscible solvents, including isopropanol, acetonitrile, ethanol and methanol in the aldol reaction was evaluated to increase reagent solubility and change polarity of the reaction medium (Table 3). The reaction did not occur in pure organic solvents such as isopropanol, acetonitrile and ethanol (Entries 1, 5 and 9, Table 3).

Showing that the presence of water in the reaction medium was essential, probably because it acts as molecular "lubricant" in the reaction, thus significant yields were observed in reactions that were employed at least 10% water (*v*/*v*) with these solvents (Entries 2–4, 6–8, 10–12, 14–16, Table 3). In addition, 32% yield for the experiment with only methanol as reaction medium was obtained (Entry 13, Table 3), showing that performing aldol reactions in organic solvent without water was possible, contrary to what had been observed for isopropanol, ethanol and acetonitrile.

The presence of higher amounts of water in the reaction medium promoted the increase of the reaction yield, as well as in the experiments with water and DMSO. Under the conditions employed in this work at 50 °C, it was possible to obtain a significant yield in reaction medium containing acetonitrile and water, a fact that was not observed in studies of the literature, in which this enzyme was also used [8, 10].

In 10% water in acetonitrile at 50 °C, a low yield of 16% was obtained, but with 52% ee_{anti} (Entry 6, Table 3). Whereas in 30% water in DMSO (*v:v*), 12% ee_{anti} was observed with 83% yield for 24 h (Entry 7, Table 2). The

Table 2 Aldol reaction between 4-nitrobenzaldehyde and cyclohexanone catalyzed by RNL in different DMSO and H_2O ratios

Entry	Time (h)	T (°C)	Solvent (DMSO:H_2O)	Yield (%)[a]	ee_{anti} (%)	*dr* (*anti:syn*)
1	48	50	9:1	44	13	52:48
2	48	50	7:3	96	16	52:48
3	48	50	5:5	97	11	51:49
4	48	50	3:7	96	10	53:47
5	48	50	1:9	51	16	55:45
6	24	50	7:3	68	17	53:47
7	24	50	5:5	83	12	51:49
8	24	30	7:3	31	28	55:45
9	48	30	7:3	52	27	56:44

Reaction conditions: 4-nitrobenzaldehyde (151 mg), cyclohexanone (415 µL), enzyme (100 mg), 5.5 mL of DMSO and H_2O, magnetic stirring

ee_{anti} enantiomeric excess of the (*S,R*)-*anti*-aldol product, *dr* diastereoisomeric ratio, *T* temperature

[a]Yield determined by HPLC-UV

Table 3 Aldol reaction between 4-nitrobenzaldehyde and cyclohexanone catalyzed by RNL in *i*-PrOH, MeCN, EtOH and MeOH and H_2O as co-solvent

Entry	Solvent	Yield (%)[a]	ee_{anti} (%)	*dr* (*anti:syn*)
1	*i*-PrOH	Trace	Trace	Trace
2	*i*-PrOH:H_2O (9:1)	14	24	60:40
3	*i*-PrOH:H_2O (7:3)	20	14	59:41
4	*i*-PrOH: H_2O (5:5)	47	11	57:43
5	MeCN	Trace	Trace	Trace
6	MeCN:H_2O (9:1)	16	52	61:39
7	MeCN:H_2O (7:3)	12	16	62:38
8	MeCN:H_2O (5:5)	34	15	60:40
9	EtOH	Trace	Trace	Trace
10	EtOH:H_2O (9:1)	34	16	58:42
11	EtOH:H_2O (7:3)	34	17	58:42
12	EtOH:H_2O (5:5)	62	12	59:41
13	MeOH	32	12	53:47
14	MeOH:H_2O (9:1)	31	10	57:43
15	MeOH:H_2O (7:3)	37	14	56:44
16	MeOH:H_2O (5:5)	70	11	54:46

Reaction conditions: 4-nitrobenzaldehyde (151 mg), cyclohexanone (415 µL), enzyme (100 mg), 5.5 mL of solvent, 50 °C, 24 h, magnetic stirring

ee_{anti} enantiomeric excess of the (*S,R*)-*anti*-aldol product, *dr* diastereoisomeric ratio

[a]Yield determined by HPLC-UV

results in aqueous medium with acetonitrile were interesting in terms of stereoselectivity, but it was decided to evaluate the reaction conditions in DMSO and water since the best yields were obtained in these conditions.

Significant yields were obtained in the use of solvents such as isopropanol, ethanol and methanol in the reactional medium with water for the aldol reactions using RNL (Table 3). Reactions containing water and acetonitrile, isopropanol, ethanol or methanol (5:5) had the yield of 34, 47, 62 and 70%, respectively (Entries 4, 8, 12, 16, Table 3), respectively, but were not higher than the yield achieved for 50% water in DMSO, in which 83% yield with similar selectivity was observed for 24 h (Entry 7, Table 2).

The aldol reactions can proceed at a significant speed without the presence of the biocatalyst, since these reactions are very susceptible to pH changes and promoted by acid or basic catalysis [11]. Therefore, the use of buffer solutions can be very important to control the enzymatic reactions and for obtaining a suitable enantioselectivity. Experiments were performed at pH 7 using 100 mM citrate (C), phosphate (P) or imidazole (I) buffers in DMSO, the results are shown in Table 4.

The enzymatic reaction in phosphate and citrate buffer significantly increased the yield when compared to the reactions with only water (Entries 1–2, Table 4). However, control experiments (without enzyme) showed that the reaction conditions in the two buffers promoted the aldol reaction without the need of RNL (Entries 4–5, Table 4), as observed for the citrate–phosphate buffer in the literature [11], which justifies the observed increase of yield and decrease of selectivity.

The highest reaction yield (82% yield and 4% ee_{anti}, Entry 1, Table 4) was obtained employing citrate buffer solution with DMSO (7:3) at 50 °C. However, the control experiment without enzyme showed that 20% of the yield at these conditions was not catalyzed by the enzyme

Table 4 Aldol reaction between 4-nitrobenzaldehyde and cyclohexanone catalyzed by RNL in different ratios of DMSO and buffers at pH 7.0

Entry	T (°C)	Solvent	Yield (%)[a]	ee_{anti} (%)	*dr* (*anti:syn*)
1	50	DMSO:C (7:3)	96	4	46:54
2	50	DMSO:P (7:3)	82	4	52:48
3	50	DMSO:I (7:3)	60	25	53:47
4	50	Control DMSO:C (7:3)	20	2	58:42
5	50	Control DMSO:P (7:3)	23	0	58:42
6	50	Control DMSO:I (7:3)	Trace	Trace	Trace
7	30	DMSO:C (7:3)	59	8	49:51
8	30	DMSO:P (7:3)	53	8	50:50
9	30	DMSO:I (7:3)	30	32	58:42
10	30	Control DMSO:C (7:3)	2	0	60:40
11	30	Control DMSO:F (7:3)	4	0	60:40
12	30	Control DMSO:I (7:3)	Trace	Trace	Trace

Reaction conditions: 4-nitrobenzaldehyde (151 mg), cyclohexanone (415 µL), enzyme (100 mg), 5.5 mL of solvent and H_2O, 24 h, magnetic stirring

ee_{anti} enantiomeric excess of the (*S,R*)-*anti*-aldol product, *dr* diastereoisomeric ratio, *C* citrate buffer, *P* phosphate buffer, *I* imidazole buffer, 0.1 M, pH 7.0, *T* temperature

[a]Yield determined by HPLC-UV

(Entry 4, Table 4). Similar results were obtained for the phosphate buffer solution (Entries 2 and 5, Table 4).

A reaction yield of 60 and 25% ee_{anti} was obtained in imidazole buffer solution (Entries 3, Table 4). This buffer did not catalyze the aldol reaction at 50 °C in the absence of the biocatalyst (Entry 6, Table 4), therefore imidazole buffer solution with DMSO was employed in the subsequent experiments.

Experiments at 30 °C were carried out and the contribution of the non-enzymatic-catalyzed reaction (Entries 10 and 11, Table 4) was smaller than in the experiments at 50 °C, thus, 2 and 4% yield for the citrate and phosphate buffer control were determined, respectively.

The temperature affected the reaction under these experimental conditions, thus the reaction in imidazole buffer solution with DMSO (*v*/*v*) showed twice the yield at 50 °C (60% yield) when compared to the reaction carried out at 30 °C (30% yield) (Entries 3 and 9, Table 4) with no significant change in stereoselectivity.

The DMSO and imidazole buffer ratio was assessed to evaluate differences between the employment of only water and imidazole buffer solution in the reaction medium (Table 5). The employment of imidazole buffer solution and DMSO, 5:5 (Entry 3, Table 5), showed 76% of yield and 13% ee_{anti}. A significant decrease of the reaction yield when compared with reactions carried out in water and DMSO (Entry 3, Table 2), which presented 83% yield and 12% ee_{anti} for 24 h.

As observed previously, increasing amount of aqueous solution (imidazole buffer) in the reaction medium favored a yield increase and a selectivity decrease (Entries 1–5, Table 5). It is noteworthy that the reaction can also be performed in imidazole buffer solution without the presence of organic solvents (61% yield, 10% ee_{anti}) (Entry 6, Table 5) and, the best obtained enantioselectivity was 24% ee_{anti} in 30% imidazole buffer in DMSO (*v*/*v*) (Entry 2, Table 5).

Experiments with DMSO and imidazole buffer solution (7:3) for different reaction times at 50 °C were performed in order to obtain the maximum reactional yield, which was 81% after 72 h of reaction with 18% ee_{anti} and 53:47 *dr* (Fig. 1). Experiments in milder conditions (30 °C) were also conducted to obtain better selectivities in DMSO and imidazole buffer solution (7: 3) using RNL, a yield of 78% after 120 h was observed with 30% ee_{anti} and 58:42 *dr* (Fig. 1).

For an increasing yield, the aldol reaction was carried out in DMSO and imidazole buffer (3:7, reduced amount of DMSO in relation to buffer solution) at 50 °C for 24, 48 or 72 h by RNL. 4-Nitrobenzaldehyde was completely converted in the aldol product and, 99% yield (96% isolated yield) with 6% ee_{anti} and 53:47 *dr* was obtained (Fig. 2a). Whereas at 30 °C, 55% yield with 22% ee_{anti} and 57% *dr* was determined (Fig. 2b). These experiments were performed to confirm that 80% of yield obtained in the previous experiments was not the reaction equilibrium.

It was also noted that different experimental conditions presented high reaction yields. Such as DMSO and water (5:5) that presented 97% yield and 11% ee_{anti} (Entry 3, Table 2) and DMSO and water (7:3) for 48 h at 50 °C (Entry 2, Table 2).

Table 5 Aldol reaction between 4-nitrobenzaldehyde and cyclohexanone catalyzed by RNL in different ratios of DMSO and imidazole buffer solution

Entry	Solvent (DMSO:I) (%)	Yield (%)[a]	ee_{anti} (%)	*dr* (*anti:syn*)
1	9:1	27	20	51:49
2	7:3	60	24	53:47
3	5:5	76	13	50:50
4	3:7	94	6	52:48
5	1:9	96	5	55:45
6	10:0	61	10	51:49

Reaction conditions: 4-nitrobenzaldehyde (151 mg), cyclohexanone (415 µL), enzyme (100 mg), 5.5 mL of DMSO and imidazole buffer, 50 °C, 24 h and magnetic stirring

ee_{anti} enantiomeric excess of the (*S,R*)-*anti*-aldol product, *dr* diastereoisomeric ratio, *I* imidazole buffer 0.1 M, pH 7.0

[a]Yield determined by HPLC-UV

3.3 Promiscuous Activity Evaluation

Experiments to distinguish between promiscuous activity and unspecific protein catalysis by RNL were performed.

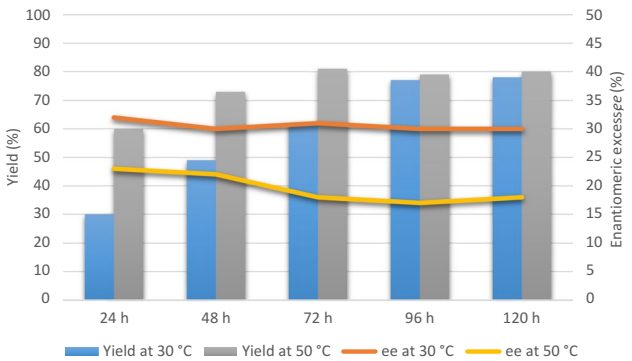
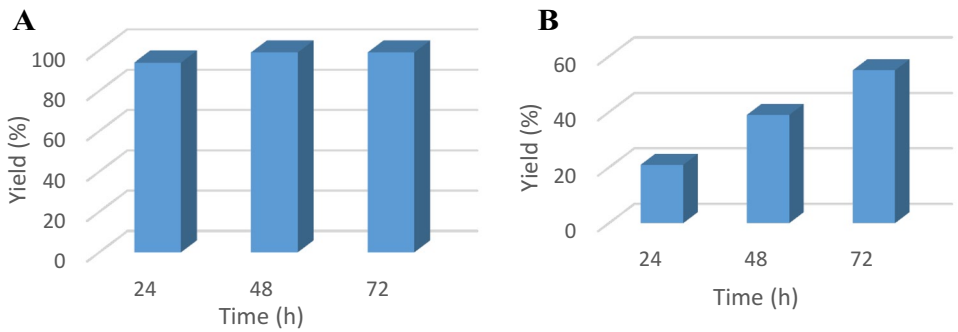


Fig. 1 Aldol reaction between 4-nitrobenzaldehyde and cyclohexanone catalyzed by RNL in DMSO and imidazole buffer solution (7:3) in different reaction times. Reaction conditions: 4-nitrobenzaldehyde (151 mg), cyclohexanone (415 µL), enzyme (100 mg), 5.5 mL of DMSO and imidazole buffer solution 0.1 M, pH 7.0 (7:3), 30 or 50 °C and magnetic stirring; yield determined by HPLC-UV; ee_{anti} enantiomeric excess of the (*S,R*)-*anti*-aldol product; diastereoisomeric ratio at 30 °C was constant 58:42 and at 50 °C was 53:47



ee_{anti}: 6% constant, *dr*: 53:47 constant[c]

ee_{anti}: 22% constant, *dr*: 57:43 constant

Fig. 2 Aldol reaction between 4-nitrobenzaldehyde and cyclohexanone catalyzed by RNL in DMSO and imidazole buffer (3:7) at **a** 50 °C and **b** 30°C. Reaction conditions: 4-nitrobenzaldehyde (151 mg), cyclohexanone (415 µL), enzyme (100 mg), 5.5 mL of DMSO and imidazole buffer solution 0.1 M, pH 7.0 (3:7), 50 °C for **a** and 30 °C for **b** and magnetic stirring; yield determined by HPLC-UV; reaction maximum yield was 99% determined by HPLC and 96% isolated yield after 72 h; ee_{anti} enantiomeric excess of the (*S,R*)-*anti*-aldol; *dr* diastereoisomeric ratio *anti:syn*

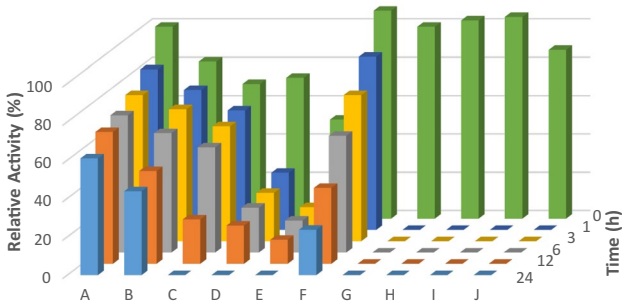


Fig. 3 Relative enzymatic activity of RNL in the hydrolysis of *p*-NPB after treatment in different conditions for different times. Control experiments were carried, the RNL enzymatic activity were determined with each substance in the analysis in relation to the value of 0 h enzymatic activity in phosphate buffer 20 mM pH 7.0, thus 100% was 0.215 U mL^{-1}. Experiments without enzyme were performed to determine the contribution of each substance that can act as base in the PNPB hydrolysis, such as imidazole. The non-enzymatic hydrolysis was subtracted of the determined activity when it was significant. Experiments were performed at 30 °C in: **a** phosphate buffer 20 mM pH 7.0. **b** Tris–HCl buffer 20 mM pH 7.0. **c** Imidazole–HCl buffer 100 mM pH 7.0. **d** DMSO. **e** DMSO and imidazole–HCl buffer 100mM pH 7.0 (7:3, *v/v*). **f** Phosphate buffer 20 mM pH 7.0 saturated with 4-nitrobenzaldehyde. **g** Phosphate buffer 20 mM pH 7.0 solution with 65 mg mL^{-1} cyclohexanone. **h** DMSO and imidazole–HCl buffer 100 mM pH 7.0 (7:3, *v/v*) solution with 25 mg mL^{-1} 4-nitrobenzaldehyde. **i** DMSO and imidazole–HCl buffer 100 mM pH 7.0 (7:3, *v/v*) solution with 65 mg mL^{-1} cyclohexanone. **j** Imidazole–HCl buffer 100 mM pH 7.0 (7:3, *v/v*) solution with 65 mg mL^{-1} cyclohexanone and 25 mg mL^{-1} 4-nitrobenzaldehyde

The enzymatic activity was measured in different conditions to evaluate the contribution of each component present in the reaction for enzymatic deactivation. RNL presented 61 and 44% of initial activity after 24 h in phosphate and Tris–HCl buffer, respectively, showing that this enzyme is unstable in buffer solutions (Fig. 3a, b). For Imidazole–HCl buffer (pH 7.0) the enzyme showed 23% of the initial activity after 12 h and no activity after 24 h, showing that the enzyme stability in this buffer is relatively low (Fig. 3c). The same pattern was observed in DMSO (Fig. 3d) and in DMSO and Imidazole–HCl buffer solution (7:3), in which 12 and 20% initial activity were observed after 24 h, respectively (Fig. 3e).

For 4-nitrobenzaldehyde in phosphate buffer, the enzyme activity was very similar to the activity determined for phosphate buffer, because of the insolubility of this compound in aqueous solutions (Fig. 3f). For cyclohexanone in phosphate buffer (Fig. 3g), DMSO and imidazole–HCl buffer solution (7:3) with 4-nitrobenzaldehyde (Fig. 3h) or cyclohexanone (Fig. 3i), or both (Fig. 3j), no enzymatic activity was observed after 1 h, showing that the employed experimental conditions completely deactivated the enzyme. Therefore, it is possible to conclude that the reaction proceeds by protein unspecific catalysis in these experimental conditions.

To prove that the aldol reaction by RNL occur due to unspecific protein catalysis, the enzyme was submitted to harsh conditions to evaluate if the enzymatic structure is important for the ee_{anti} values obtained in the previous experiments. The aldol product was observed even in denaturing conditions (Entries 1–4, Table 6) and in the presence of the irreversible hydrolase inhibitor phenylmethylsulfonylfluoride (PMSF) [8] (Entry 5, Table 6). In addition, urea (Entry 6, Table 6) and the inhibitor PMSF (Entry 7, Table 6) did not perform the aldol reaction. Thus, it is proved without any doubt that the aldol reaction catalyzed by RNL in this condition is an example of protein unspecific catalysis even with moderate enantioselectivity.

The presence of the PMSF inhibitor decreased the reaction yield (Entry 5, Table 6), probably because the acid-base catalysis also occurs by the serine, histidine or

Table 6 Aldol reaction between 4-nitrobenzaldehyde and cyclohexanone catalyzed by pretreated RNL

Entry	Experiment	Yield (%)[a]	ee_{anti} (%)	*dr* (*anti:syn*)
1	RNL pretreated at 100 °C in H_2O[b]	61	23	42:58
2	RNL pretreated in autoclave[c]	58	21	36:64
3	RNL pretreated at 100 °C in 1 mL of 0.83 M urea in H_2O[d]	95	11	51:49
4	RNL pretreated at 30 °C in 1 mL of 0.83 M urea in H_2O[e]	64	29	36:64
5	RNL inhibited with PMSF[f]	30	46	53:47
6	Urea[g]	Trace	Trace	Trace
7	100 mg PMSF[h]	Trace	Trace	Trace
8	BSA	49	1	43:57

Reaction conditions: 4-nitrobenzaldehyde (151 mg), cyclohexanone (415 µL), enzyme (100 mg), 5.5 mL of DMSO and imidazole buffer (7:3), 30 °C, 48 h and magnetic stirring

ee_{anti} enantiomeric excess of the (*S,R*)-*anti*-aldol product, *dr* diastereoisomeric ratio

[a]Yield determined by HPLC-UV

[b]RNL was pretreated at 100 °C in 1 mL H_2O for 48 h, evaporated under reduced pressure and employed for the aldol reaction

[c]RNL was pretreated in autoclave at 121 °C for 20 min in 1 mL H_2O, which was evaporated under reduced pressure

[d]RNL was pretreated at 100 °C in 1 mL 0.83 M urea in aqueous solution for 48 h, evaporated under reduced pressure and employed for the aldol reaction

[e]RNL was pretreated at 30 °C in 1 mL 0.83 M urea in aqueous solution for 48 h, evaporated under reduced pressure and employed for the aldol reaction

[f]RN was pretreated with 100 mg PMSF in 2 mL of THF at room temperature for 12 h. The THF was evaporated under reduced pressure and the reaction performed with the pretreated RNL

[g]The reaction was carried with 0.83 M urea, without enzyme

[h]The reaction was carried with 100 mg PMSF, without enzyme

aspartic acid residues of the catalytic triad. However, the active site of the enzyme is not responsible for the observed enantioselectivity, since the blockage of the catalytic center by PMSF increased the ee_{anti} to 46%, showing that the enantioselective catalysis occurs by a different part of the enzyme.

A control experiment with BSA in the same conditions applied to the aldol reaction by RNL was also performed. The reaction yield was 49% with 1% ee_{anti} and 43:57 *dr*, showing that this protein catalyzed the aldol reaction, but without significant enantioselectivity (Entry 8, Table 6).

3.4 Different Substrates

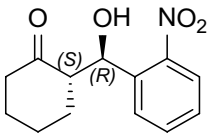
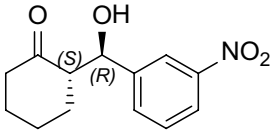
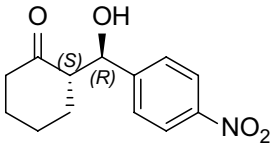
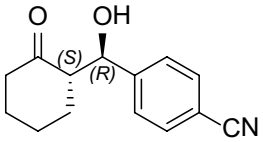
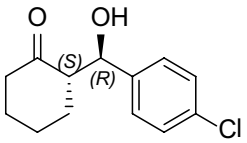
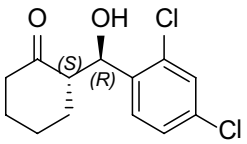
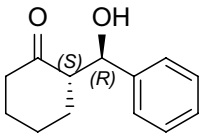
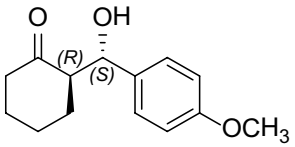
Experiments using *o*-nitrobenzaldeyde, *m*-nitrobenzaldehyde, *p*-nitrobenzaldehyde, *p*-cyanobenzaldehyde, *p*-chlorobenzaldehyde, *o,p*-dichlorobenzaldehyde, benzaldehyde and *p*-methoxybenzaldehyde with cyclohexanone were performed in the optimized conditions of the aldol reactions by RNL to evaluate the reactional scope (Table 6). The aldol products yields with *o*-NO_2, *m*-NO_2 and *p*-NO_2 substituents groups were 19, 41 and 81%, respectively, probably because of steric effect of *o*-NO_2 (lowest yield) and an increased electron withdrawing effect for *p*-NO_2 (highest yield) (Entries 1–3, Table 7). In terms of enantioselectivity, 13, 37 and 31% ee_{anti} were observed for *o*-NO_2, *m*-NO_2, *p*-NO_2, showing that the presence of a group in the *ortho* position was not an effective approach for the obtention of an increased enantioselectivity.

Moderate diastereoselectivities were observed for the *anti*-products for all employed substrates, i.e., 79:21, 61:39 and 74:26 diastereoisomeric ratios for *o*-NO_2, *p*-CN and *p*-OCH_3 aldol products, respectively (Entries 1, 4 and 8, Table 7). Whereas some substrates showed lower diastereoselective ratios, such as *p*-NO_2 and *o,p*-Cl that presented 58:42 and 56:44 (*anti:syn)* ratios, respectively (Entries 3–6, Table 7).

In general, the reaction yield increased according to the increasing electron-withdrawing characteristic of the aromatic aldehyde (*p*-methoxybenzaldehyde, *p*-chlorobenzaldehyde, *p*-nitrobenzaldehyde), i.e., 2, 23 and 81% of yield for *p*-OCH_3, *p*-Cl and *p*-NO_2 groups, respectively (Entries 3, 6 and 8, Table 7). Moderate enantioselectivities (31–55% *ee*, Entries 3–8, Table 7) of the *anti*-aldol products for aldehydes with *para* substituents groups in the aromatic ring were observed, showing that the reaction catalyzed by RNL was stereoselective.

The electron donating group *p*-OCH_3 (Entry 8, Table 7) promoted the formation of the aldol product (*R,S*)-*anti*, whereas the product (*S,R*)-*anti* was observed for the others aldol products. In general, RNL catalyzed the aldol

Table 7 Aldol reaction between aromatic aldehydes and cyclohexanone catalyzed by RNL in different DMSO and H_2O ratios

Entry	Product	Yield (%)[a]	ee_{anti} (%)	*dr* (*anti:syn*)
1		19	13	79:21
2		41	37	61:39
3		81	31	58:42
4		39	27	61:39
5		23	47	61:39
6		25	37	56:44
7		3	43	65:35
8		2	55	74:26

Reaction conditions: aromatic aldehyde (1 mmol), cyclohexanone (415 µL), enzyme (100 mg), 5.5 mL of DMSO and imidazole buffer (7:3), 30 °C, 48 h and magnetic stirring

ee_{anti} enantiomeric excess of the (*S,R*)-*anti*-aldol product, *dr* diastereoisomeric ratio

[a]Yield determined by HPLC-UV

reaction with different aromatic aldehydes and with moderate stereoselectivity.

4 Conclusion

The aldol reaction between cyclohexanone and 4-nitrobenzaldehyde was performed under different reaction conditions by RNL, including in buffer solutions without organic solvent (a more sustainable approach), in organic solvents in the presence of water and in buffer solution with organic solvent. However, suitable experimental conditions increased the yield and selectivity of the aldol reaction between cyclohexanone and aromatic aldehydes by RNL.

The aldol product was observed with inactive enzyme and even in denaturing conditions. Although ee_{anti} was

observed in the aldol product, the reaction proceeded by unspecific protein catalysis in these employed conditions. It is important to revaluate the increasing literature reporting the use of promiscuous enzymes (mainly hydrolases) in aldol reactions, since the employed solvents and even reagents deactivated the enzyme in a relatively short period of time and many of the reported enzymes may act by unspecific protein catalysis. Thought, this enzymatic study offers new possibilities for lipases, and specially to the use of the novel RNL, which has been scarcely reported in comparison with other well-known lipases and catalyzed the aldol reaction between different aromatic aldehydes and cyclohexanone.

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