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Chemo-enzymatic synthesis of optically pure rivastigmine intermediate using alcohol dehydrogenase from baker's yeast

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ABSTRACT

An efficient and practical synthesis of (*S*)-rivastigmine intermediate was developed by employing a chemoenzymatic step toward the synthesis of chiral intermediate N-ethyl-N-methyl-carbamic acid-3-(1*S*-hydroxy-ethyl)-phenyl ester (**2**) using crude alcohol dehydrogenase from baker's yeast with reduced nucleotide adenosine dinucleotide (NADH) as proton donor has been demonstrated.

1. Introduction

Rivastigmine{(*S*)-3-[1-(dimethylamino)-ethyl]phenylethyl-(methyl)carbamate)} [1] is an active pharmaceutical ingredient that was developed for the treatment of patients with mild to moderate Alzheimer disease [2] and treatment of dementia caused by Parkinson's disease [3] and Lewy body [4].

Early researchers synthesized (*S*)-Rivastigmine by resolving racemic rivastigmine or intermediate by catalytic asymmetric hydrogen transfer based reaction on transition metals and chiral organic ligands [5–18]. Recently, development in enzymatic synthesis has lead toward synthesis of rivastigmine via chemoenzymatic route using lipases [12,13,19–21] as well as transaminases [22]. Various draw backs were observed in above said methods like complex operations, loss of yield, metal impurities in final active pharmaceutical ingredient, multiple purification procedures in case of lipase catalyzed resolution, three enzyme systems in transaminase reactions and multiple crystallization steps with chiral acids.

The route described herein aimed at using a chemoenzymatic approach (Scheme 1) is first of its kind where alcohol dehydrogenase from baker's yeast was used toward the synthesis of (*S*)-Rivastigmine [23]. An in-process purification of crude alcohol dehydrogenase from baker's yeast [24] has been adopted and used in enzymatic step. Various references are available toward the purification of alcohol dehydrogenase and stereoselective reduction reaction catalyzed by alcohol dehydrogenases [25–40]. This procedure includes in-process purification of crude alcohol dehydrogenase from baker's yeast and enzyme catalyzed stereoselective reduction as key step where alcohol dehydrogenase do stereoselective reduction by utilizing proton from NADH and glucose dehydrogenase do regeneration of NADH from glucose to gluconic acid, thereby shifting the equilibrium of the reaction to alcohol formation. This process can even overcome the limitation of getting 50% maximum yield of (*S*)-Rivastigmine.

2. Experimental

2.1. Enzymes

Alcohol dehydrogenase from Equine, alcohol dehydrogenase from *Saccharomyces cerevisiae*, aldehyde dehydrogenase from *S. cerevisiae*, alcohol dehydrogenase–agarose from baker's yeast, alcohol dehydrogenase from *Lactobacillus kefir*, yeast from *S. cerevisiae* Type I and yeast from *S. cerevisiae* Type II has been procured from Sigma–Aldrich. Locally available baker's yeast was procured from AB Mauri, UK, Blue Bird India Pvt. Ltd., India and Angel Yeast Co. Ltd., China. Glucose dehydrogenase CDX 901 was purchased from CDX-Codexis, USA.

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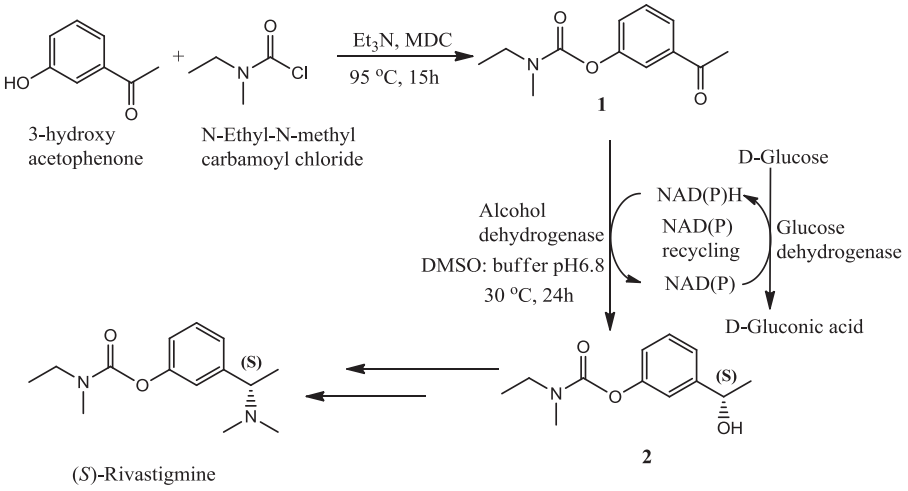

Scheme 1. Synthesis of (*S*)-Rivastigmine intermediate (2).

2.2. Chemicals and reagents

Nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate were procured from SISCO Research Laboratories Pvt. Ltd., Mumbai, India, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, methanol, toluene, methylene dichloride and dimethyl sulfoxide from Spectrochem Pvt. Ltd., India, triethyl amine, 3-hydroxy acetophenone, methylene dichloride, ethylenediamine tetraacetic acid disodium salt (EDTA.2Na), D-glucose, sodium sulfate anhydrous, and sodium hydroxide from Avra Laboratories Pvt. Ltd., Hyderabad, India and N-ethyl-N-methyl carbomyl chloride from Chemrich Fine Chemicals Pvt. Ltd., Hyderabad, India.

2.3. Synthesis of N-ethyl-N-methyl carbomyl acetophenone (**1**)

To a solution of triethyl amine (910 mL, 4.55 vol) was added, N-ethyl-N-methyl carbomyl chloride (182 g, 1.5 mol) and 3-hydroxy acetophenone (200 g, 1.47 mol). The mixture was stirred at 95 °C for 10–15 h. The reaction mixture was extracted with CH_2Cl_2 (1000 mL), washed with 0.5 N sodium hydroxide solution (2× 2000 mL), dried over Na_2SO_4 and distilled under vacuum to obtain product as brownish color. The product was analyzed by HPLC, ESI-MS and ^{1}H NMR oil (321.74 g 99% isolated yield, purity >98%, bp 337.9 °C) ESI-MS found 221.1 (M^+). ^{1}H NMR ($CDCl_3$, 300 MHz, ppm) δ 7.80–7.67 (m, 1H), 7.69 (s, 1H), 7.48–7.42 (m, 1H), 7.35–7.27 (m, 1H), 3.52–3.38 (m, 2H), 3.08–3.00 (s, 3H, 2 rotamers), 2.60 (s, 3H), 1.28–1.13 (m, 3H).

2.4. Screening for novel alcohol dehydrogenases toward preparation of N-ethyl-N-methyl-carbamic acid-3-[1S-hydroxy-ethyl)-phenyl ester (**2**)

Various alcohol dehydrogenases, 10 mg each was added in 2 mL buffer (containing 100 mM potassium phosphate, 5 mg NADP/NAD, 330 mM D-glucose, 2 U/mL glucose dehydrogenase, pH 7.0). A solution of N-ethyl-N-methyl carbamic acid-3-acetyl phenyl ester in dimethyl sulfoxide (10 mg in 0.1 mL) was added. The mixture was stirred at 30 °C for 24 h and monitored by HPLC. CH_2Cl_2 (5 mL) was added, the phases were separated. The obtained alcohol product N-ethyl-N-methyl-carbamic acid-3-(1*S*-hydroxy-ethyl)-phenyl ester (**2**) in the organic phase was analyzed by Chiral HPLC.

2.5. Screening for novel yeast from various sources toward preparation of N-ethyl-N-methyl-carbamic acid-3-[1S-hydroxy-ethyl)-phenyl ester (**2**)

A 40 mg of yeast from various sources (Table 1) were suspended in 5 mL buffer (containing 100 mM potassium phosphate, 5 mg NAD, 330 mM D-glucose, 2 U/mL glucose dehydrogenase, pH 7.0). A solution of N-ethyl-N-methyl carbomyl acetophenone in dimethyl sulfoxide (10 mg in 0.1 mL) was added. The mixture was stirred at 30 °C for 24 h and monitored by HPLC. CH_2Cl_2 (5 mL) was added the phases were separated. The obtained alcohol product N-ethyl-N-methyl-carbamic acid-3-(1*S*-hydroxy-ethyl)-phenyl ester (**2**) in the organic phase was analyzed by chiral HPLC (Table 1).

2.6. Preparation of cell free extract of yeast

The cell free extract of yeast from various sources was prepared by following procedure given by Buttler and Thelwall-Jonesa [45]. A 20 g of yeast was first grinded in a mixer and stirred in 40 mL of toluene at 40 °C for 1 h. It was then cooled to ambient temperature, mixed with 60 mL 1 mM EDTA.2Na solution and stirred slowly for 1 h at 5–10 °C. The mixture was first filtered through filter cloth and then passed through Hiflow super cell under gentle suction to obtain a cell free extract. Cell free extracts were evaluated for biotransformation of ketone (**1**) (Table 1).

2.7. Preparation of N-ethyl-N-methyl-carbamic acid-3-(1S-hydroxy-ethyl)-phenyl ester (**2**)

A 100 g of baker's yeast from Blue bird was first grinded in a mixer and stirred in 200 mL of toluene at 40 °C for 1 h [45]. It was then cooled to ambient temperature and mixed with 300 mL of 1 mM EDTA.2Na solution and stirred slowly for 1 h at 5–10 °C [44]. The mixture was filtered through filter cloth, followed by hiflow super cell under gentle suction. The result clear yellowish liquid (crude alcohol dehydrogenase extract) was then mixed with 650 mL of buffer (containing 0.25 mM potassium phosphate, 5 g NAD, 250 g D-glucose, 2.5 g glucose dehydrogenase, pH 6.8). A solution of N-ethyl-N-methyl carbamic acid-3-acetyl phenyl ester (**2**) in dimethyl sulfoxide (50 g in 150 mL) was added. pH 6.8 was maintained by adding 1 M NaOH using pH stat. The mixture was stirred at 30 °C for 24 h and monitored by HPLC. After the completion of reaction, methanol (300 mL) was added to reaction mixture and filtered through celite. Methanol was distilled off under reduced vacuum. The filtrate was extracted with 500 mL of CH_2Cl_2 twice.

Table 1
Screening for alcohol dehydrogenases.[a]

S. no.	Enzyme	Co-factor	Conversion by HPLC (%)	*S*-alcohol **2** (ee)
1	Alcohol dehydrogenase from Equine[a,b]	NADPH	95.4	99.35
2	Alcohol dehydrogenase from *Saccharomyces cerevisiae* [a,b]	NADH	99.8	99.21
3	Aldehyde dehydrogenase from *Saccharomyces cerevisiae* [a,b]	NADH	97.2	99.23
4	Alcohol dehydrogenase–Agarose from baker's yeast[a,b]	NADH	99.2	99.53
5	Alcohol dehydrogenase, from *Lactobacillus kefir* [a,b]	NADPH	98.2	99.01
6	Yeast from *Saccharomyces cerevisiae* Type I[a,c]	NADH	97.1	99.42
7	Yeast from *Saccharomyces cerevisiae* Type II[a,c]	NADH	98.1	99.41
8	Baker's yeast from AB Mauri, Peterborough[a,c]	NADH	97.2	99.5
9	Dry yeast from Blue Bird India Pvt. Ltd.[a,c]	NADH	98.2	99.4
10	Baker's yeast from Angel Yeast Co. Ltd.[c]	NADH	98.2	99.4
11	Cell free extract of baker's yeast from blue bird[d]	NADH	99.2	99.5
12	Cell free extract of yeast from *Saccharomyces cerevisiae* Type I[d]	NADH	99.2	99.5
13	Cell free extract of yeast from *Saccharomyces cerevisiae* Type II[d]	NADH	99.0	99.4

[a] % conversion from HPLC determined with respect to (**1**). (**1**) in dimethyl sulfoxide (10 mg in 0. 1 ml) in 2 ml of phosphate buffer pH 7.0 (containing 5 mg NADP/NAD, 330 mM D-glucose, 5 mg glucose dehydrogenase CDX901) in a 2 mL screw capped vial) with shaking at 30 °C for 24 h.
[b] 5 mg of each alcohol dehydrogenase enzyme in reaction mixture.
[c] 40 mg yeast.
[d] 20 g of Baker's yeast extracted into 40 mL toluene and 60 mL of 1 mM EDTA.2Na, 130 mL of 0.25 M phosphate buffer pH 6.8, 1 g NAD, 50 g D-glucose, 1 g glucose dehydrogenase CDX901 (**1**) in dimethyl sulfoxide (0.045 mol in 30 ml).

The organic layer was separated, combined, dried over sodium sulfate and distilled off to obtain colorless oil. The product was analyzed by chiral HPLC, RP-HPLC, ESI-MS, SOR and ^{1}H NMR (47.9 g, 95% isolated yield, purity >99%, ee: >99%, bp 337.3 °C). ESI-MS was found to be 223.1 (M^+). The $[\alpha]_{25}^{D}$ was found to −25.787 (c = 1.1, $CHCl_3$, 99.5% ee). ^{1}H NMR (DMSO, 300 MHz, ppm) δ 7.33–7.27 (t, J = 7.8 Hz, 1H), 7.17–7.15(d, J = 7.5 Hz, 1H), 7.06 (s, 1H), 6.96–6.93(m, 1H), 5.23–5.21 (d, J = 4.2 Hz, 1H), 4.75–4.67 (m, 1H), 3.49–3.38(m, 1H), 3.34–3.27(m, 1H), 3.01–2.89(s, 3H, 2 rotomers), 1.32–1.30(d, J = 6.3 Hz, 3H), 1.20–1.06 (m, 3H).

2.8. Analytical methods

2.8.1. High performance liquid chromatography

Reverse phase-high performance liquid chromatography analysis (RP-HPLC) of N-ethyl-N-methyl carbomyl acetophenone (**1**) and N-ethyl-N-methyl-carbamic acid-3-(1*S*-hydroxy-ethyl)-phenyl ester (**2**) were performed on Agilent Technologies high performance liquid chromatography instrument connected with UV detector at 210 nm using Inertsil ODS 3 V column (5 μm particle size, 150 mm × 4.6 mm length) eluted with 20 mM phosphate buffer pH 3.0: acetonitrile (70:30, v/v) at a flow rate of 1 mL/min. The retention time were found to be 4.0 min, 14.0 min and 9.0 min for 3-hydroxy acetophenone, N-ethyl-N-methyl carbomyl acetophenone (**1**) and N-ethyl-N-methyl-carbamic acid-3-[1*S*-hydroxy-ethyl)-phenyl ester (**2**) respectively.

Chiral HPLC [40] of N-ethyl-N-methyl-carbamic acid-3-(1*S*-hydroxy-ethyl)-phenyl ester (**2**) was performed on Agilent Technologies high performance liquid chromatography instrument connected with UV detector at 215 nm using Chiralpak AD-H (5 μm particle size, 250 mm × 4.6 mm length) column eluted with n-hexane:ethanol:trifluro acetic acid (95:0.5:0.1, v/v/v) 13. at a flow rate of 1 mL/min. The retention time were found to be 13.8 min and 15.5 min for (*R*)-isomer and (*S*)-isomer respectively of N-ethyl-N-methyl-carbamic acid-3-[1*S*-hydroxy-ethyl)-phenyl ester (**2**).

2.8.2. NMR spectra

^{1}H NMR spectra was recorded on a Bruker Avance 300 spectrometer. The spectra was recorded with DMSO-d^6 as internal standard for measuring chemical shifts to with ±0.01 ppm. A region from 0 to 10 ppm was scanned for all the samples.

2.8.3. Specific optical rotation

Specific optical rotation of isolated compounds were measured using Perkin-Elmer 243 polarimeter (Uberlingen, Germany).

2.8.4. Mass spectrometry

Electron spray ionization-mass spectra (ESI-MS) of isolated compounds were measured using Agilent 1100 LC/MSD Trap SL instrument.

3. Results and discussion

Synthetic route (Scheme 1) involved condensation of 3-hydroxy acetopheneone with N-ethyl-N-methyl carbomyl chloride to give (**1**) which in turn was stereoselectively reduced to (**2**) using alcohol dehydrogenase which can be further converted to Rivastigmine base via halogenation and finally condensation with dimethyl amine [41]. As a result, the use of alcohol dehydrogenase catalyzed reduction to produce the desired isomer was investigated.

3.1. Screening for novel alcohol dehydrogenases

A number of alcohol dehydrogenases from various sources like equine, *S. cerevisiae*, yeast from *S. cerevisiae* type I, yeast from *S. cerevisiae* type I, baker's yeast from AB Mauri, Peterborough, dry yeast from Blue Bird India Pvt. Ltd., baker's yeast from Angel yeast Co. Ltd., and cell free extract prepared from dry yeast, yeast from *S. cerevisiae* type I and yeast from *S. cerevisiae* type II, has been screened toward stereoselective reduction of ketone to alcohol (Table 1). The percentage conversion and enantioselective purity of (**2**) obtained with all the enzymes, yeast and cell free extracts were above 95% and ee above 98%. All enzymes were showed selectivity toward (*S*)-isomer. The alcohol dehydrogenase showed transfer of pro-R-hydride to the re-face of the carbonyl to give *S*-alcohol, as said in Prelog's rule [42,43].

3.2. Reaction profile

In presence of cell free extract of baker's yeast, the conversion showed an increase in formation of (**2**) from (**1**) during 0–24 h (Fig. 1). The rate of reduction was found to be 0.0019 mol h^{-1} g^{-1}.

3.3. Effect of baker's yeast concentration

A maximum conversion of 98% was achieved from cell free extract prepared from 2 g and 3 g of baker's yeast at 0.0045 mol of N-ethyl-N-methyl carbomyl acetophenone (**1**), 0.1 g of NADH, 0.1 g of glucose dehydrogenase CDX901 and 2.5 g of D-glucose with incubation upto 24 h (Table 2).

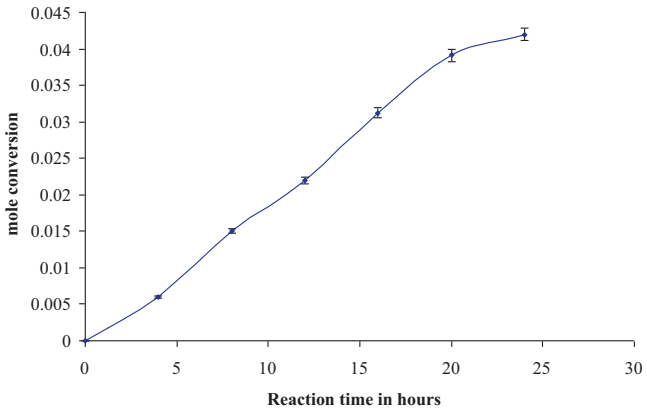

Fig. 1. Reaction profile. Reaction conditions: 0.045 mol of (**1**), 20 g of baker's yeast, 40 mL toluene, 60 mL of 1 mM EDTA.2Na, 130 mL of 0.25 M phosphate buffer pH 6.8, 1 g NAD, 50 g D-glucose, 1 g glucose dehydrogenase CDX901, 30 mL DMSO, with shaking at 30 °C for 24 h.

Table 2
Effect of baker's yeast.[a]

Yeast concentration [w/w,with respect to (**1**)]	% conversion
1 g	67.3%
2 g	98.2%
3 g	98.3%

[a] % conversion from HPLC determined with respect to (**1**) 4 mL toluene, 6 mL of 1 mM EDTA.2Na, 13 mL of 0.25 M phosphate buffer pH 6.8, 0.1 g NAD, 2.5 g D-glucose, 0.1 g glucose dehydrogenase CDX901, 0.0045 mol of (**1**) in 3 mL DMSO, with shaking at 30 °C for 24 h.

Table 3
Effect of glucose dehydrogenase concentration.[a]

Glucose dehydrogenase concentration CDX 901 [w/w with respect to (**1**)]	% conversion
0.05 g	14%
0.1 g	98.2%
0.15 g	98.2%

[a] % conversion from HPLC determined with respect to (**1**). 2 g baker's yeast, 4 mL toluene, 6 mL of 1 mM EDTA.2Na, 13 mL of 0.25 M phosphate buffer pH 6.8, 0.1 g NAD, 2.5 g D-glucose, 0.0045 mol of (**1**) in 3 mL DMSO, with shaking at 30 °C for 24 h.

3.4. Effect of glucose dehydrogenase concentration

Glucose dehydrogenase above 10% resulted in 98% conversion when cell free extract prepared from 2 g of baker's yeast, 0.0045 mol of N-ethyl-N-methyl carbomyl acetophenone (**1**), 0.1 g of NADH were used and incubation upto 24 h (Table 3).

3.5. Effect of NAD concentration

NAD above 0.1 g resulted in >98% conversion when cell free extract prepared from 2 g of baker's yeast, 0.0045 mol of N-ethyl-N-methyl carbomyl acetophenone (**1**), 0.1 g of NAD were used and incubation upto 24 h (Table 4).

Table 4
Effect of NADH concentration.[a]

NAD [w/w with respect to (**1**)]	% conversion
0.05 g	18%
0.1 g	98.2%
0.15 g	98.2%

[a] % conversion from HPLC determined with respect to (**1**). 2 g baker's yeast, 4 mL toluene, 6 mL of 1 mM EDTA.2Na, 13 mL of 0.25 M phosphate buffer pH 6.8, 2.5 g D-glucose, 0.1 g glucose dehydrogenase CDX901, 0.0045 mol of (**1**) in 3 mL DMSO, with shaking at 30 °C for 24 h

Table 5
Effect of buffer pH.[a]

0.25 M phosphate buffer pH	% conversion[a]
6.0	51.64
6.8	98.2
7.0	97.18
7.5	61.64

[a] % conversion from HPLC determined with respect to (**1**). 0.0045 mol of (**1**), 2 g baker's yeast, 4 mL toluene, 6 mL of EDTA.2Na, 13 mL of buffer, 0.1 g NAD, 2.5 g D-glucose, 0.1 g glucose dehydrogenase CDX901, 3 mL DMSO, with shaking at 30 °C for 24 h.

Table 6
Effect of buffer salt.[a]

Molar concentration pH 6.8	% conversion
0.1	94.7
0.2	97.8
0.25	98.2

[a] % conversion from HPLC determined with respect to (**1**). 0.0045 mol of (**1**), 2 g baker's yeast, 4 mL toluene, 6 mL of EDTA.2Na, 13 mL of 0.25 M phosphate buffer pH 6.8, 0.1 g NAD, 2.5 g D-glucose, 0.1 g glucose dehydrogenase CDX901, 3 mL DMSO, with shaking at 30 °C for 24 h.

3.6. Effect and buffer and buffer salt

Buffer of different pH in the range 6.0–7.5 (Table 5) and salt concentration in the range 0.1–0.25 mM (Table 6) were studied. A pH 6.8 at buffer concentration of 0.25 showed better conversion of 98%.

3.7. Effect of solvent

To improve the activity and selectivity of crude enzyme, reactions were studied in both miscible and non-miscible solvents. Addition of DMSO to reaction mixture showed improvement in conversion upto 98.2% against 75.6% conversion with only buffer (Table 7). However, solvents like methanol, N,N-dimethylformamide, tetrahedrofuran, dimethyl sulfoxide, isopropyl alcohol, ethanol, methanol, n-butanol, n-propanol and toluene showed no product formation.

3.8. Effect of temperature

An increase in temperature to 35 °C and 40 °C decreased the conversion by 16.1% and 23.1% respectively (Fig. 2). Decrease in activity was due to the denaturation of enzymes [46] as well as degradation of NADH [47] at higher temperature with the progress of reaction.

Table 7
Effect of co-solvent.[a]

Solvent	% conversion	*S*-isomer (% ee)
N,N-dimethyl formamide	No reaction	–
Tetrahydrofuran	No reaction	–
Dimethyl sulfoxide	98.2	>99%
No solvent	75.6	>99%
Isopropyl alcohol	No reaction	–
Ethanol	No reaction	
Methanol	No reaction	–
n-Butanol	No reaction	–
n-Propanol	No reaction	–
Toluene	No reaction	

[a] % conversion from HPLC determined with respect to (**1**). 0.0045 mol of (**1**), 2 g baker's yeast, 4 mL toluene, 6 mL of EDTA.2Na, 13 mL of 0.25 M phosphate buffer, 0.1 g NAD, 2.5 g D-glucose, 0.1 g glucose dehydrogenase CDX901, 3 mL of solvent with shaking at 30 °C for 24 h.

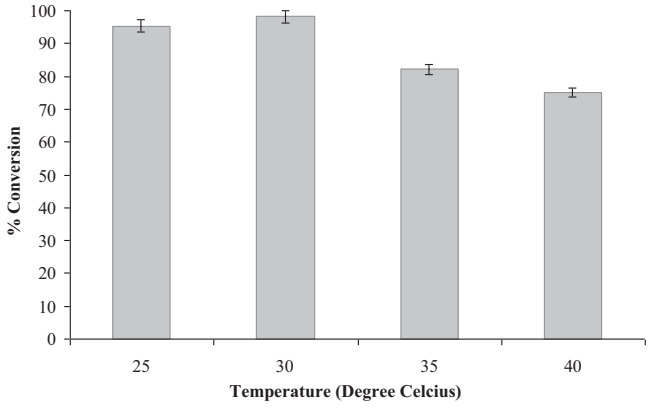

Fig. 2. Effect of temperature. % conversion from HPLC determined with respect to (**1**). 0.0045 mol of (**1**), 2 g baker's yeast, 4 mL toluene, 6 mL of EDTA.2Na, 13 mL of 0.25 M phosphate buffer, 0.1 g NAD, 5 g D-glucose, 0.1 g glucose dehydrogenase CDX901, 3 mL DMSO, with shaking for 24 h.

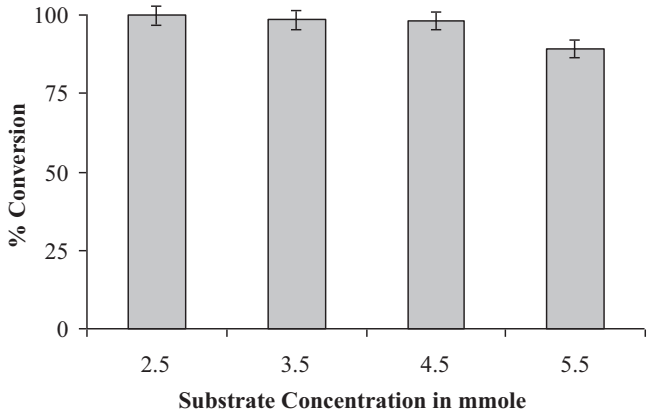

Fig. 3. Effect of substrate concentration. % conversion from HPLC determined with respect to (**1**). 2 g baker's yeast, 4 mL toluene, 6 mL of EDTA.2Na, 13 mL of 0.25 M phosphate buffer, 0.1 g NAD, 5 g D-glucose, 0.1 g glucose dehydrogenase CDX901, 3 mL DMSO, with shaking for 24 h.

3.9. Effect of substrate concentration

A maximum conversion of 99.2 has been obtained at 0.035 mol of N-ethyl-N-methyl carbomyl acetophenone (**1**) when cell free extract from baker's yeast at concentrations ranging from 2.5 to 4.5 mmol of N-ethyl-N-methyl carbomyl acetophenone (**1**), 0.1 of g NAD, 0.1 g of glucose dehydrogenase CDX901 with incubation upto 24 h were used (Fig. 3).

4. Conclusion

The above process is first of its kind. N-Ethyl-N-methyl carbomyl acetophenone (**1**) synthesized by condensation of N-ethyl-N-methyl carbomyl chloride with 3-hydroxy acetophenone has been screened for stereoselective reduction by enzyme alcohol dehydrogenase, NAD/NADP, glucose and glucose dehydrogenase of which all alcohol dehydrogenases yielded best result with a ee purity of 98.5%. Glucose dehydrogenase CDX901 in presence of D-glucose and NAD were used for regeneration of co-factor. Cell free extract of Yeast taken for optimization showed with 10% of NADP, 10% glucose dehydrogenase CDX901, 6.15 mol equivalent of D-glucose with respect to N-ethyl-N-methyl carbomyl acetophenone (**1**) in 3 volume of dimethyl sulfoxide can able to reduce stereoselectively to *S*-alcohol (**2**) with a yield of 95% with enantiomeric excess >99.0.

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