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Enantioselective synthesis of (*S*)-1-Boc-3-hydroxypiperidine using enzymes and whole cell biocatalysts

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ABSTRACT

Preparation of (S)-1-Boc-3-hydroxypiperidine is an important stereoselective step in the synthesis of many anti-cancer drugs like tyrosine kinase inhibitors. Our study involved the synthesis of (S)-1-Boc-3-hydroxypiperidineusing Baker`s yeast, ketoreductase enzyme and microbial cells. The novel approach using Baker`s yeast yielded a cost-effective, environment-friendly and enantioselective process. The ketoreductase enzyme provided a high chiral selectivity. The time course of the reaction and percent enzyme used was significantly reduced. Synthesis of (S)-1-Boc-3-hydroxypiperidine was also studied using microbial cells. The investigational studies done in this article will surely aid in improving the existing processes in many aspects as discussed above.

INTRODUCTION

Applications of enzymatic biocatalysis and whole cell biotransformations have had a great impetusfor the production of pharmaceutical key intermediates. Enzymes offer many competitive advantages over conventional chemical catalysts [1]. The most important being regioselectivity and stereoselectivity that eliminate multiple protection and deprotection steps [2]. Our objective was to utilize the stereoselectivity of enzymes and whole cell biocatalysts for the production of an intermediate,(*S*)-1-Boc-3-hydroxypiperidine. (*S*)-1-Boc-3-hydroxypiperidine is a key intermediate in the synthesis of many drugs like tyrosine kinase inhibitors [3].

(*S*)-1-Boc-3-hydroxypiperidine has been commercially synthesized from *N*-1-Boc-3-hydroxypiperidine by catalytic hydrogenation to give racemic 1-Boc-3-hydroxypiperidine [4]. The racemic mixture is resolved by chiral resolving agents like *L*-camphorsulfonic acid [5]. The catalytic hydrogenations are performed athigh temperatures and pressures. Moreover, the chemical resolution results in less than 50 % yield.

Baker's yeast has long been used for the reduction of prochiral ketones, β-keto esters [6], β-α-oximino esters [7], unsaturated aldehydes [8], etc. The baker's yeast mediated reduction offers many advantages such as low cost, easy availability[9], nonpathogenic, enantioselective and regeneration of cofactors [10]. Many authors havereported stereoselective reduction reactions catalyzed by ketoreductases [11]. Mutagenesis techniques have been employed to make tailor-madeketoreductase that show tremendous chiral selectivity [12] and broad substrate range [13].Fungal[14], [15]and bacterial [16], [17] species are also reported to cause reduction of prochiral ketones to the corresponding chiral alcohols.

MATERIALS AND METHODS

2.1 Enzymes

ES-KRED-8000 kit was purchased from Syncozymes (Shanghai) Co., Ltd. KRED 110 and NAD^+ were purchased from Enzyme Works Inc. Baker's yeast was purchased from Blue Bird Foods (India) Pvt. Ltd.

2.2 Chemical Reagents

N-1-Boc-3-piperidone was purchased from Novel Organic Synthesis Pvt. Ltd. Ethanol and dimethyl sulfoxide from Spectrochem Pvt. Ltd., India. Ethylenediamine tetraacetic acid disodium salt, D-glucose, sodium sulfate anhydrous, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium bicarbonate, sodium chloride, potassium chloride and sodium hydroxide from Merck, India.

2.3 Screening of baker's yeast for the Preparation of (S)-1-Boc-3-hydroxypiperidine

In a 2L four necked round bottomed flask was added 500 mL water, 250g baker's yeast, 20g glucose. 20g N-1-Boc-3-piperidone was dissolved in 10mL DMSO and added. The reaction was stirred at room temperature. The pH of the reaction was maintained at pH 7.00 ± 0.5 with 7% $NaHCO_3$. The reaction was stirred at room temperature for 8 – 10 days. The progress of the reaction was monitored by TLC(ethyl acetate and n-hexane in the ratio of 1:1, ninhydrin). After completion of the reaction, the reaction mass was passed through celite. The filtrate was extracted with 1.5 L ethyl acetate. The extract was dried over Na_2SO_4 and evaporated under vacuum to yield brown oily residue (16 g, 95 % yield, and purity 77.68 %). The crude residue was purified by column chromatography using 3% ethyl acetate in n-hexane to obtain > 98% purity. The product was analyzed by GC, chiral HPLC, ESI-MS, SOR and ^{1}H NMR. ESI-MS was202(MH^+). The chiral purity was found to be 90% and $[\alpha]_{25}^{D}$ was +17.3 (c = 0.9, ethanol).^{1}H NMR ($CDCl_3$, 300 MHz, ppm) 1.43-1.55 (11H), 1.73-1.78 (1H), 1.87 – 1.89 (1H), 3.03 – 3.13 (2H), 3.73-3.78 (1H), 4.11-4.13 (2H).

2.4 Screening of ketoreductases preparation of (S)-1-Boc-3-hydroxypiperidine

To 1mL of phosphate buffer saline (PBS) pH 7.0 was added 5mg enzyme, 5mg NADH, 10mg glucose dehydrogenase and 10mg glucose. 10 mg of *N*-1-Boc-3-piperidone dissolved in 100 µL of ethanol was added.The reaction was stirred at 250 rpm and 25 °C for 24 hours. The progression of the reaction was monitored by TLC (ethyl acetate and n-hexane in the ratio of 1:1, ninhydrin).

2.4.1 Preparation of (S)-1-Boc-3-hydroxypiperidine using KRED 110

50mL of 200mM triethanolamine HCl buffer pH 7.5 was charged intoa250mL four neck RB flask. The temperature was set at 35-40°C.150 mg KRED 110 and 25mg NAD^+ was added. 5g *N*-1-Boc-3-piperidone dissolved in 3mL isopropyl alcohol was added slowly. The reaction was monitored by TLC (ethyl acetate and n-hexane in the ratio of 1:1, ninhydrin). After completion of the reaction, the reaction mass was filtered through celite and the filtrate was extracted with 150 mL of ethyl acetate. The ethyl acetate layer was separated, dried over sodium sulfate and evaporated under vacuum to obtain yellow to brown oily residue (4.7 - 4.8 g,95% yield, and 95% purity). The crude residue was purified by high vacuum distillation to obtain >99% purity. The final product was analyzed by GC, chiral HPLC, ESI-MS, SOR and ^{1}H NMR. ESI-MS was 202(MH^+).The chiral purity was found to be 100 % and $[\alpha]_{25}^{D}$ was +22.69 (c = 0.9, ethanol).^{1}H NMR ($CDCl_3$, 300 MHz, ppm)1.46-1.54 (11H), 1.73-1.80 (1H), 1.87-1.88 (1H), 1.89 (1H), 3.02– 3.16 (2H), 3.51 (1H), 3.72 -3.77 (2H).

2.5 Screening of isolated colonies isolated from effluent treatment plant for the preparation of(S)-1-Boc-3-hydroxypiperidine

2.5.1 Collection of sample from the Effluent treatment plant

A grab sample was taken from the effluent treatment plant (ETP). 5 mL sample was collected in a 15 mL falcon tube. The sample was diluted 100 times before being used for inoculation in sterile media.

2.5.2 Isolation of pure cultures

100 µL of the collected sample was inoculated into a 25 mL of sterile fluid thioglycolate media, pH 7.00 ± 0.5. The culture was incubated at 37ºC and 200 rpm. After 24 hours of incubation, yellowish-brown colored growth was observed.

Pure cultures were isolated by streak plating after serial dilutions. 1 mL of the broth was diluted up to 10^{-5} and streaked on fluid thioglycolate agar medium using a sterile wire loop. The plates were incubated at 37 °C for 24 hour [18].

2.5.3 Screening of isolated colonies for the preparation of (S)-1-Boc-3-hydroxypiperidine
25 mL of 29 g/L Fluid thioglycolate media was adjusted to pH 7.00 ± 0.5 and autoclaved. The media was inoculated with a single isolated colony and incubated at 37 °C and 220 rpm for 24 hours. After 24 hours, 100 mg of *N*-1-Boc-3-piperidone in 100 µL of ethanol was added and incubation was continued for 72 hours. The reaction was sampled at regular intervals. The progress of the reaction was monitored by TLC(ethyl acetate and n-hexane in the ratio of 1:1, ninhydrin). After completion of the reaction, the reaction mass was passed through celite and the filtrate was extracted with 50 mL of ethyl acetate. The extract was dried with Na_2SO_4 and evaporated under vacuum to obtain yellow to brown oily residue. The product was given for ESI-MS and SOR.

2.6 Analytical Methods
2.6.1 Gas chromatography
Gas chromatographic analysis of (*S)*-1-Boc-3-piperidone was performed on Agilent Technologies Gas chromatography instrument using Rtx-5 Amine (30 m × 0.53mm, 3.0 µm) capillary column.The elution was carried outwith nitrogen as carrier gas and the eluents were detected by Flame ionization detector. The retention time was 14.04 min for (*S*)-1-Boc-3-hydroxypiperidine and 14.04 min for *N*-1-Boc-3-piperidone.

2.6.2 Chiral HPLC
Chiral HPLC of (*S*)-1-Boc-3-hydroxypiperidinewas performed on Waters High performance liquid chromatography instrument connected with UV detector at 210 nm using Chiralpak IC (250 × 4.6 mm, 5µm) column eluted withmobile phase of 5% IPA and 95% n-hexane (v/v) at a flow rate of 1 mL/min. The retention times were found to be 12.60 min and 13.69 min for (*R*)-isomer and (*S*)-isomer respectively [19]**.**

2.6.3 Specific optical rotation
Specific optical rotation was measured using Perkin-Elmer 243 polarimeter. The specific optical rotation of the compounds were measured at the sample concentration of 0.9% in ethanol at 25°C [20].

2.6.4 Mass Spectrometry
Electron Spray Ionization-Mass spectra (ESI-MS) was measured using Agilent 1100 LC/MSD Trap SL instrument.

2.6.5 NMR spectra
^{1}H NMR spectra was recorded on a Bruker Avance 300 MHz spectrometer. The spectra was recorded with CDCl3as solvent and trimethylsilane (TMS) as an internal standard for measuring chemical shifts. A region from 0 – 10 ppm was scanned for all the samples [19].

RESULTS AND DISCUSSION

3.1Screening of baker's yeast for the preparation of(S)-1-Boc-3-hydroxypiperidine
The reaction mixture contained 40g/L N-1-Boc-3-piperidone in 10 mL DMSO, 12.5 X baker's yeast, and 1 X glucose. The pH of the reaction dropped and was required to be maintained at 7.00 using 7 % $NaHCO_3$. The reaction was stirred at room temperature for 8 – 10 days. The progress of the reaction was monitored by TLC. The analysis was done by GC, Chiral HPLC, ESI-MS, SOR and H^1NMR. The chiral purity was found to be 90% and $[\alpha]_{25}^{D}$ was +17.3 (c = 0.9, ethanol). ^{1}HNMR was complying with that in reported literature.

3.1.1 Effect of pH
The reaction was carried out at three different pH values, viz., 6, 7 and 8. However, the pH 7 was found to be optimal for the activity of baker's yeast (Table 1).

Table 1 Effect of pH on the activity of baker's yeast

S. No.	Substrate (g/L)	Baker's yeast	Glucose (g/L)	pH	Reaction time (days)
1	40	12.5 X	40	6 ± 0.5	12
2	40	12.5 X	40	7 ± 0.5	11
3	40	12.5 X	40	8 ± 0.5	12.5

40g/L N-1-Boc-3-piperidone in 2mL ethanol, 25 g of baker's yeast, 2 g glucose, and 50 mL of 0.1 M phosphate buffer at 30 ^{o}C; pH maintained at ± 0.5 using 7 % $NaHCO_3$.

3.1.2 Effect of temperature

Three different temperatures were chosen for temperature optimization: 25°C, 30ºC and 37°C. Though there was no significant difference in the rate of the reaction at three different temperatures, 30°C being optimal for yeasts was chosen for further optimization of the reaction conditions.

3.1.3 Effect of solvents

Three solvents dimethyl sulfoxide, ethanol and methanol were tried for the dissolution of *N*-Boc-1-piperidone. In fact, *N*-1-Boc-3-piperidine had higher solubility in dimethyl sulfoxide than ethanol and methanol. Thus, the rate of reaction was found to be higher with dimethyl sulfoxide (Table 2).

Table 2 Effect of co-solvent on the activity of baker's yeast

S. No.	Solvent	Solubility of *N*-1-Boc-3-pirperidine at 25ºC	Reaction Time (days)
1	2 % DMSO	5 mg/mL	10
2	2 % Ethanol	3 mg/mL	12
3	2 % Methanol	2 mg/mL	12

40g/L N-1-Boc-3-piperidone, 25 g of baker's yeast, 2 g glucose, and 50 mL of 0.1 M phosphate buffer at 30 ^{o}C; pH maintained at ± 0.5 using 7 % $NaHCO_3$.

3.1.4 Effect of glucose concentration

Utilization of glucose was found to have a profound effect on the growth and activity of the baker's yeast. However, the consumption of glucose caused sharp decrease in pH up to 3 - 4 which was required to be adjusted.

3.1.5 Effect of metal ions

The effect of metal ions like Mg^{2+}, Ca^{2+}, Fe^{2+}, Cu^{2+}, and Co^{2+}on the activity of the baker's yeast was investigated using EDTA as the control. There was no effect of metal ions and EDTA on the activity of the baker's yeast.

3.2Screening of ketoreductases for the preparation of (S)-1-Boc-3-hydroxypiperidine

A set of 6 ketoreductase (KRED) enzymes viz., KRED 107, 108, 109, 110, 111, 112 from ES-KRED-8000 kit was screened for the enantioselective reduction of *N*-1-Boc-3-piperidone to (*S*)-1-Boc-3-hydroxypiperidine. The screening kit had 5 mg of each ketoreductase enzyme in 1 m1 of phosphate buffer pH 7.0 containing 5mg NADH, 10mg D-glucose, 10mg glucose dehydrogenase. 10 mg of *N*-1-Boc-3-piperidone in 100 µL of ethanol was added. The reaction mixture was stirred at 250 rpm under controlled temperature of 30 ºC for 24 hours. The reaction progression was monitored by TLC. Out of the 6 ketoreductase enzymes screened, KRED 110 was found to have a complete conversion. KRED 110 was selected for further optimization at gram scale level.

3.2.1Preparation of (S)-1-Boc-3-hydroxypiperidine using KRED 110

The reaction mixture contained 10g/L *N*-1-Boc-3-piperidone dissolved in IPA, 3% KRED 110, 10mM NAD^{+}, and 200mM triethanolamine HCl buffer pH 7.5. The reaction was stirred at 230 rpm at 35 – 40 ºC for 3 - 4 hours. The reaction progression was monitored by TLC. The analysis was done by GC, Chiral HPLC, ESI-MS, SOR and H^{1}NMR. The chiral purity was 100% with the chromatographic purity of>99%. SOR in 0.9%ethanol at 25 ºC was 22.69. ^{1}HNMR was complying with that in reported literature.

3.2.1.1 Effect of temperature

The rate of reaction was found to increase with increase in temperature up to 40°C. At 45 °C the rate of reaction started to decrease. 35 - 40 °C was found to be optimal for the activity of the enzyme. There was no effect on the chiral selectivity of the enzyme with change in temperature(Figure 1).

The objective of this study was to use minimum amount of enzyme for the reduction of *N*-1-Boc-3-piperidone. The percentage of enzyme was reduced down to 3% with the reduction in the time course of the reaction up to 3 hours.

3.2.1.2 Effect of pH
The effect of pH was studied from 6.8 to 8.0. There was no significant effect of pH on the rate and chirality of the reaction.

3.3 Screening of microbial cultures for preparation of(S)-1-Boc-3-hydroxypiperidine
After 24 hours of incubation, isolated colonies were observed on the surface of fluid thioglycolate agar medium.10 distinct colonies were identified and designated as A, B, C, D, E, F, G, I, and J.

Each colony was screened for the ketoreductase activity in the conversion of *N*-1-Boc-3-piperidone to (*S*)-1-Boc-3-hydroxypiperidine. Most of the cultures gave predominantly positive SOR values which corresponds to (*S*)-selectivity. Of the 10colonies screened,3 colonies gave negative SOR values which indicates (*R*)-selectivity (Table3). The identification and characteristics of the isolated colonies is being explored.

Table 3 Screening of the 10 different colonies isolated for the ketoreductase activity

S. No.	Colony	*N*-1-Boc-3-piperidine*	Reaction time (hours)	SOR
1	Colony A	Absent	72	14.34
2	Colony B	Present	72	-
3	Colony C	Present	72	-
4	Colony D	Present	72	-
5	Colony E	Absent	72	15.17
6	Colony F	Absent	72	-2.46
7	Colony G	Absent	72	10.37
8	Colony H	Absent	72	4.29
9	Colony I	Absent	72	-2.325
10	Colony J	Absent	72	-1.556

100 mg of N-1-Boc-3-piperidone in 100 µL of ethanol and 25 mL of 29 g/L Fluid thioglycolate media pH 7.00 ±0.5 at 37 °C and 220 rpm.
**Based on TLC (ethyl acetate and n-hexane in the ratio of 1:1, ninhydrin).*

CONCLUSION

Our investigation demonstrates that the baker's yeast has an ability to reduce prochiral cyclic ketone to the corresponding (*S*)-1-Boc-3-hydroxypiperidine. This process offers a new strategy for the preparation of (*S*)-1-Boc-3-hydroxypiperidine, thus eliminating the use of costly, toxic and hazardous reducing agents. Baker's yeast is inexpensive, readily available, nonhazardous and environment friendly. We were able to perform reactions up to 20 g scale at 40g/L titer with the overall yield of 90 - 95 %; however, the chemical processes give maximum theoretical yield of only 50 %.

Though the preparation of (*S*)-1-Boc-3-hydroxypiperidine using ketoreductase enzymes has been reported in the literature, we were able to improve the process to reduce the enzyme concentration down to 3 %. Our temperature optimization studies shortened the time course of the reaction to 3 – 4 hours. The reduction in the percentage of the enzyme and time course of the reaction leads to lesser operational costs.

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