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A Study and Application of Biocatalytic Synthesis of (S)-N-Boc-3-hydroxypiperidine

Ying Chen & Yi Xu*
School of Chemical and Environmental Engineering, Shanghai Institute of Technology, Shanghai, China

Jianbo Chen
College of Life and Environment Science, Shanghai Normal University, Shanghai, China

ABSTRACT: This paper first uses the environmental friendly whole cell of biocatalyst pichia pastoris SIT2014 to asymmetrically synthesized anti-tumor drug of chiral intermediate (S)-N-Boc-3-hydroxypiperidine.. Improve the final biocatalytic reduction yield to 85.4% based on the study of fermentation optimization and biocatalytic asymmetrical reduction system for pichia pastoris. The ee value of obtained reduction product (S)-N-Boc-3-hydroxypiperidine hits over 99%. The study of this article is a successful case where the biocatalyst is applied to the green synthesis of chiral intermediate of anti-tumor drug.

Keywords: pichia pastoris; biocatalyst; (S)-N-Boc-3-hydroxypiperidine; asymmetrical reduction

1 INTRODUCTION

Piperidines derivative has many pharmacological activities, such as anti-biosis, anti-tumor, senile dementia treatment and anesthesia. N-Boc-3-pipradrol is an important intermediate[1-2] of anti-tumor drug with important study value. Currently, there are few reports about the synthesis research on N-Boc-piperidines or other similar matters, neither in China. It is reported from foreign literatures that the synthesis of minor relevant similar drugs, such as N-Boc-piperidines-3-acetic acid, is conducted by high-temperature catalytic hydrogenation by pyridine-3-acetic acid under protection of Boc. The advantage of this synthesis method is that it contains less steps and requires just high-pressure reduction condition. It has several disadvantages, for example, it has a very high requirement on the devices, and its raw material costs much and is hard to be synthesized[3-4]. What mentioned above indicates in the study of compound of the same kind, many methods have demanding conditions and their values are hard to be realized. Whereas, the biocatalyst has many features[5], such as modest reaction condition, high catalytic efficiency, high selectivity and low pollution. It has many wide and important usages in drug synthesis[6-8]. The biocatalyst synthesis refers to the process of using organism, such as cell or cell organ, and taking enzyme as the catalyst to realize chemical conversion. While the process of using pure enzyme or organism to catalyze achiral or prochiral compound to be converted to chiral product is referred to as chiral synthesis of biocatalyst. The biocatalyst has the advantages of modest reaction condition and high catalytic efficiency. Most importantly, it complies with the current requirement of green chemistry[9-11]. It has a wide prospect of development and application.

The process of using carbonyl reductase in microbial cell to catalyze the substrate N-Boc-3-piperidone asymmetrical reduction to obtain the chiral N-Boc-3-piperidrol has many advantages, such as modest reaction condition, low pollution and economic process, and it is a green and environmental-friendly synthesis route with much competitiveness. This article first conducts a series of research on the selection and cultivation of biocatalyst to screen out the biocatalyst suitable for this asymmetrical reduction, and study the enzymatic reduction of N-Boc-3-piperidone by biocatalyst .

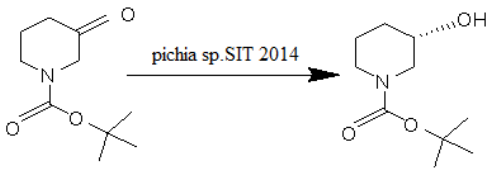

Figure1. Biocatalytic asymmetric synthesis of (*S*) -N-Boc-3-hydroxypiperidine

2 EXPERIMENTAL REAGENT AND ANALYSIS METHOD

Experimental reagent: glucose, peptone, yeast powder KH_2PO_4, NaCl, $MgSO_4 \cdot 7H_2O$, anhydrous sodium sulfate, ethanol, ethyl acetate, phosphate buffer solution, N-Boc-3-piperidones and (S)-N-Boc-3-piperidrol.

*Corresponding author: xuyi@sit.edu.cn

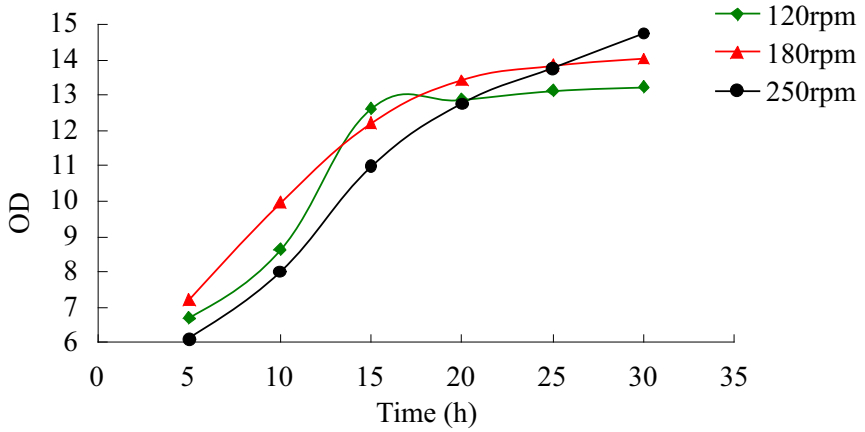

Figure 2. The growing curves of Pichia sp. in different shaking speed of shaker.

Analysis method: analysis on biological reduction yield by chiral gas chromatographic column.

Brand of column: RS-Supelco.

Model of column: Beta DEXTM 120 Capillary Column.

Specification of column: 30m*0.25mm*0.25um film thickness.

Analysis condition: the product reaction solution from reduction is extracted by ethyl acetate, and analyzed by gas chromatographic column after being dried, with sample size of 1ul.

The temperature of injector and detector is set as respectively 130 ℃ and 180 ℃.

The initial column temperature is 100 ℃, which will be increased to 160 ℃ by temperature programming. The steps of temperature programming are set as increasing to 130 ℃ as per 5 ℃ increment per minute within 100 ℃; keeping this temperature for 2 minutes and increasing to 160 ℃ as per 2 ℃ increment per minute and keeping this temperature for 2 minutes before it comes to an end. The N-Boc-3-piperidone (Boc-ketone for short) is kept in the gas chromatographic for 28.883 minutes, and (S)-N-Boc-3-piperidrol (S-Boc alcohol) is kept for 30.936 minutes.

Analysis of ee value by chiral liquid chromatographic: the product reaction solution from reduction is extracted and dried by ethyl acetate, and subject to liquid chromatographic analysis using isopropanol solvent sample, with sample size of 5ul.

The mobile phase is n-hexane: isopropanol =97:3, flow rate 0.8 ml/min; the detection wavelength is 210 nm; the temperature of column temperature phase is set as 27 ℃; chiral OD-H column 4.6 mm×250 mml; the detection time is 20 minutes. The method to calculate ee value of optical purity is as follows:

ee (%) =[S (configuration)-R(configuration)/S (configuration) +R(configuration)] ×100%

S (configuration): represents the peak area of S (configuration) detected in chiral liquid column

R (configuration): represents the peak area of R (configuration) detected in chiral liquid column

The obtained S-Boc alcohol is kept for 12.960 minutes; R-Boc alcohol for 13.861 minutes; Boc-ketone for 10.520 minutes.

3 OPTIMIZED SELECTION OF BIOCATALYST

As a catalyst, the microbe has many features such as simple cultivation and high accessibility. The yeast cell is a common microbe that takes itself as a catalytic precursor ketone to be reduced to chiral alcohol. A detailed fermentation optimization research on table turning speed, fermentation time and fermentation liquid pH for pichia pastoris kept in this laboratory in order to obtain a biocatalyst with high yield and efficiency. Main contents of culture media: glucose: 15(g/l); peptone: 5 (g/l); yeast powder: 5 (g/l); K_2HPO_4: 0.5(g/l); KH2PO4: 0.5 (g/l);) NaCl: 1(g/l); $MgSO_4 \cdot 7H_2O$: 0.5(g/l); the fermentation culture media is the same as seed culture media.

3.1 *Effect of table turning speed on cell growth*

Set the table turning speed as 120 rpm, 180 rpm and 250 rpm, and study the table turning speed most suitable for Aiqieer pichia cell with other cultivation conditions unchanged. The obtained relationship between table turning speed and cell growth is as shown in Figure 2.

It is known from the figure above that, the cell has a fastest fermentation and this process is completed within 18 h as the turning speed is 120 rpm, but the obtained concentration of the cell is not high. Whereas, as the turning speed hits 250 rpm, the obtained concentration of the cell reaches the highest level, but it has a slowest fermentation which will be completed in more than 30 h. So 180 rpm of turning speed is selected as the most proper turning speed in consideration of the balance of two affecting factors, that is, fermentation time and the concentration of cell.

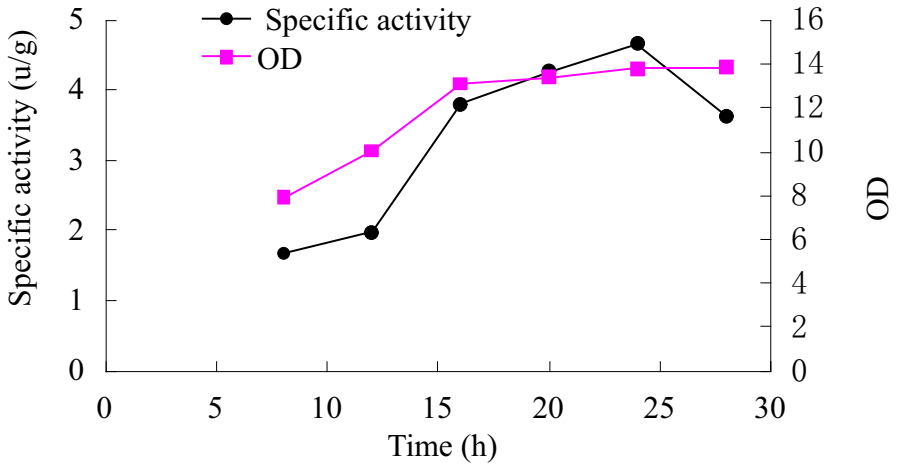

Figure 3. The optimization of fermentation time on Pichia pastoris cell

3.2 *Effect of fermentation time on cell growth*

As shown in the following Figure 3, with the increase of fermentation time, the growth of cell successively comes into logarithmic growth phase and balance phase. After 20 h of fermentation time, the cell starts to gradually stop its growth. Whereas, it is known from measuring the specific enzyme activity of cell in this period that its specific enzyme activity tends to increase and then decrease. As the fermentation time reaches 24 h, the enzyme activity hits the maximum value of 4.67 U/g. Therefore, it is summarized from the figure above that the optimal time for cultivation and fermentation of pichia pastoris cell is 24 h.

3.3 *Effect of pH value of fermentation liquid on cell growth*

The content of the seed culture media is the same as that of fermentation culture media in the cultivation process of the whole cell. After preparing the culture media, we use NaOH solution or concentrated phosphoric acid solution to adjust pH value to a specific level to ferment and cultivate the cell. After cultivation and centrifugal washing, the OD value of cell will be checked in ultraviolet spectrophotometer with its wave length of 600 nm. As shown in Figure 4, the growth of pichia pastoris in an environment with pH value between 4.5 and 7.0 tends to increase and then decrease. As shown in the data of Figure 4, the cell is suitable to grow in a culture media with fair acidity. OD value of the cell will be increased as pH value changes from 4.5 to 6.5; it will have the best condition of growth as pH at 6.5; while OD value will decline if pH continues to grow, which is harmful to the growth of cell.

Finally, determine the cultivation condition by optimizing the cultivation condition of biocatalyst. It is conducted as follows:

Contents of culture media: glucose: 15(g/l); peptone: 5 (g/l); yeast powder: 5 (g/l); K_2HPO_4: 0.5(g/l); KH_2PO_4: 0.5 (g/l); NaCl: 1(g/l); $MgSO_4 \cdot 7H_2O$: 0.5(g/l).

Adjust pH value to 6.5, and the seed culture media is the same as fermentation culture media. The pichia pastoris cell is taken to seed liquid by picking single colony with flat plate and cultivated under 30 ℃ and 180rpm for 15 h, and then sent to fermentation culture media to be cultivated for 24 h. Centrifuge the cell for spare after fermentation.

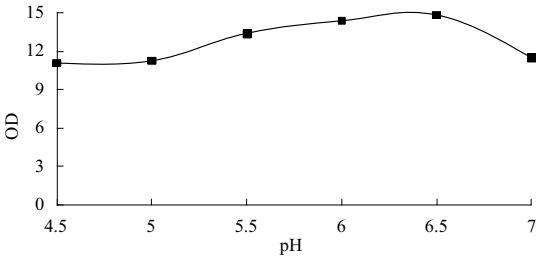

Figure 4. The OD value about cell under different pH value

4 ENZYMATIC REDUCTION OF N-BOC-3-PIPERIDONE

4.1 *Components of reaction solvent*

As for enzymatic reduction of N-Boc-3-piperidone, this article mainly conducts relevant research on the selection of reaction solvent. The components of reaction solvent are to be first explored and confirmed.

a. In pH=6.5 phosphate buffer, methanol co-solvent, adding 5% glucose to reaction system for direct reaction.

Table 1. Biological reduction reaction under different solvent system.

Entry	Sol.	Co-sol.	Glucose %	Final pH	Yield%	ee %	Conf.
a	PBS	CH_3OH	5	5.5	62.3	99	S
b	PBS	CH_3OH	0	6.5	70.8	99	S
c	PBS	CH_3OH	5	6.0	71.1	99	S
d	PBS	CH_3OH	10	6.0	70.9	99	S
e	A	CH_3OH	0	6.5	68.3	99	S
f	B	CH_3OH	0	7.0	68.1	99	S
g	PBS	IPA	5	6.0	66.7	99	S

b. In pH=7.0 phosphate buffer solution, methanol co-solvent, without adding any glucose to reaction system for direct reaction.

c. In pH=7.0 phosphate buffer, methanol co-solvent, adding 5% glucose to reaction system for reaction.

d. In pH=7.0 phosphate buffer, methanol co-solvent, adding 10% glucose to reaction system for reaction.

e. Take the culture media diluted for 5 times as the reaction solution for direct reaction A.

f. Take the culture media diluted for 10 times as the reaction solution for direct reaction B.

g. In pH=7.0 phosphate buffer, isopropanol co-solvent, adding 5% glucose to reaction system for reaction.

As shown in the data of following Table 1, the biocatalyst has an extremely high corresponding selectivity, and its reaction speed becomes very high in the previous three hours before decreasing and even coming to a halt. All the reaction time in the following table are 9 h. In the experiment, all the asymmetrically reduced N-Boc-3-piperidines in the solvents will obtain (S)-N-Boc-3-piperidrol with very high optical purity ee value (ee>99%), but the yield does not reach 100%. But it is known from comparing the experiment, the phosphate reaction system with glucose has a higher yield than that without glucose, which is because the added glucose fully complements the co-enzyme renewable cycling system in the cell and enables it to keep a relatively high enzyme activity. Whereas increasing the concentration of glucose does not have any obvious facilitation effect on the reaction (comparing the experiment a and d). By comparing the reaction solvent PBS and A, B, we know this biological reduction reaction still tends to be suitable for conduction in PBS. As shown in the data of experiment c and g, the methanol co-solvent has a better effect than isopropanol. So in the phosphate solvent with 5% of glucose and initial pH=7.0, and in the reaction system with 5% methanol co-solvent, the maximum conversion rate 71.1% will be finally obtained.

4.2 *Selection of pH value in the reaction process*

It is known from the experiment about contents in reaction solvent in the Table 1 above that, it has a better effect in the phosphate solvent with initial pH=7.0, so we conduct research on adjusting pH value to initial pH value in the reaction process.

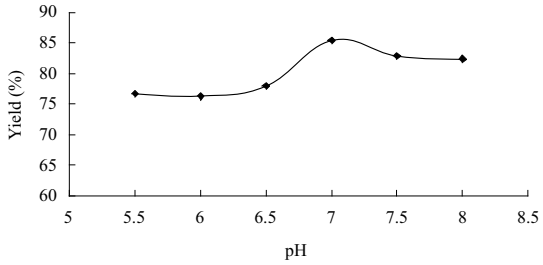

Figure 5. Effect of pH value of reaction system on the biocatalytic reduction

As shown in Figure 5, we can obviously improve the yield of this biological reduction reaction by controlling the pH value of reaction solution. As the pH value is controlled to 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0, the yield will be increased; as it becomes 7.0, the yield for 9h hits the maximum 85.4%, whereas the yield starts to decline when pH value continues to grow.

5 CONCLUSION

In this paper, we mainly research the growth enzyme of pichia pastoris and the way of carbonyl reductase in microbial cell, as a biocatalyst, to asymmetrically reduce N-Boc-3-piperidone to obtain the chiral (S)-N-Boc-3-piperidrol by enzyme method. Explore an efficient method with high yield to prepare the biocatalyst by studying the fermentation optimization of pichia pastoris in table turning speed, fermentation time and fermentation liquid pH, and use this biocatalyst to biologically catalyze and reduce

N-Boc-3-piperidone. It focuses on the effect of this reaction system on the whole reaction. In the experimental research above, the phosphate solvent with 5% glucose and 5% methanol co-solvent, as well as pH value controlled at 7.0 in the reaction process will have a yield rate of 85.4%, and optical purity value hits over 99%. Compared to the state before optimization, the yield is increased by 23.1%, and the optical purity value keeps above 99%. In this asymmetrical reduction reaction by enzyme method, the possible loss of enzyme activity is the main reason for incomplete reaction. To solve this problem, there are many issues that deserve to be studied. Meanwhile we may also find out the genetic sequence against the efficient enzyme of this reaction biocatalyst, and reform the wild bacteria into recombined bacteria to solve the loss of enzyme activity, and thus to realize the asymmetrical reduction reaction by enzyme method in order to finally achieve the goal of high productivity and efficiency and lay a solid foundation for future realization of industrialization.

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