

Toxicity of Aromatic Ketone to Yeast Cell and Improvement of the Asymmetric Reduction of Aromatic Ketone Catalyzed by Yeast Cell with the Introduction of Resin Adsorption

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Summary

Asymmetric reduction of the prochiral aromatic ketone catalyzed by yeast cells is one of the most promising routes to produce its corresponding enantiopure aromatic alcohol, but the space-time yield does not meet people's expectations. Therefore, the toxicity of aromatic ketone and aromatic alcohol to the yeast cell is investigated in this work. It has been found that the aromatic compounds are poisonous to the yeast cell. The activity of yeast cell decreases steeply when the concentration of acetophenone (ACP) is higher than 30.0 mmol/L. Asymmetric reduction of acetophenone to chiral *S*- α -phenylethyl alcohol (PEA) catalyzed by the yeast cell was chosen as the model reaction to study in detail the promotion effect of the introduction of the resin adsorption on the asymmetric reduction reaction. The resin acts as the substrate reservoir and product extraction agent *in situ*. It has been shown that this reaction could be remarkably improved with this technique when the appropriate kind of resin is applied. The enantioselectivity and yield are acceptable even though the initial ACP concentration reaches 72.2 mmol/L.

Key words: asymmetric reduction, chiral alcohol, acetophenone, *S*- α -phenylethyl alcohol, resin adsorption

Introduction

Because of safety, therapeutics and regulatory concerns, there has been increasing interest in the development of processes capable of producing enantiopure drugs (1). The enantiopure drugs are ordinarily synthesized from the chiral building blocks such as chiral alcohols and chiral amines. Chiral alcohols are the most important kind of chiral building blocks for many chiral pharmaceuticals due to their structural properties (2,3). They can be produced by the asymmetric reduction reaction of the corresponding prochiral ketone or resolution of the racemic alcohols. Biocatalysis that involves

either isolated enzymes or whole cells is becoming more and more attractive in the production of chiral alcohols due to the mild reaction conditions, high degree of stereoselectivity and environmentally friendly characteristics (4). The isolated enzymes used in chiral synthesis are mainly hydrolases, which can catalyze the asymmetric hydrolysis of racemic esters, belonging to resolution reactions, to produce the corresponding chiral alcohol. As the intrinsic disadvantage of the resolution reaction, the yield of the expectant enantiomer could not be more than 50 % if the other enantiomer was useless. On the contrary, the yield of the asymmetric reduction of the corresponding prochiral ketones catalyzed by oxidore-

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ductase could reach 100 % in theory. But these reactions require the presence of expensive cofactors such as NADPH or NADH (2). Living cell is an excellent alternative to the isolated oxidoreductase, since the oxidoreductase, cofactor and its regenerate system are all located within the living cell (5). The other advantages of using active cells are avoiding the expensive enzyme purification processes and making the oxidoreductase more stable in the living cells. However, the disadvantage of using a living cell as the catalyst is that the reaction cannot be carried out under high initial substrate concentration due to the toxicity of the substrate to the cell activity, which results in low space-time yield.

Optically active aromatic alcohols are the key chiral building blocks for many chiral pharmaceuticals such as L-chlorprenaline, S-fluoxetine, R-tomoxetine and R-denopamine. One of the most important routes of producing the enantiopure aromatic alcohols is by asymmetric reduction of the corresponding prochiral aromatic ketones catalyzed by active cells (5–8). It has been demonstrated that baker's yeast is the preferred candidate for this reaction because of its low cost and broad substrate types. Outstanding enantioselectivity of this reaction was achieved (8), but the space-time yield was low. The reason is that the initial substrate concentration must be low due to the toxicity of the substrate and product to the yeast cell (9,10). The space-time yield could be improved if the initial substrate concentration was increased, in which case the toxicity of the substrate to the active cell would be avoided (9).

Adsorbing resins have recently been used successfully for *in situ* product removal in fermentation to alleviate the product inhibition effect and the toxicity to the cell (11,12). Also, this technique may be efficient in the asymmetric reduction of prochiral aromatic ketone catalyzed by active cell. Recently, this technique has been successfully applied in asymmetric reduction of other kinds of prochiral ketones (13–15). The adsorbing resins act as the substrate reservoir and product extraction agent *in situ*, so the substrate amount can be remarkably increased, and the toxicity of the substrate and product alleviated, which makes the reaction more practicable.

In the present work, the reaction of asymmetric reduction of acetophenone (ACP) to chiral α -phenylethyl alcohol (PEA) catalyzed by active yeast cell is chosen as the model reaction. First, the toxicity of the substrate and product to the yeast cell are investigated, then the promotion effects of the introduction of resin adsorption to the asymmetric reduction reaction are studied in detail.

Materials and Methods

Reagents

Acetophenone (ACP) and benzaldehyde, of analytical purity grade, were purchased from Sinopharm Chemical Reagent Co., Ltd. (China). R- and S- α -phenylethyl alcohol (PEA), of lab reagent grade, were purchased from Acros Organics (New Jersey, USA). The others were of analytical purity grade and commercially available.

Inert macroporous adsorbing resins were used in this work. Amberlite™ XAD and XE series resins, which were macroporous aromatic resins, were purchased

from Rohm and Haas Company (USA). Resins ZG1 to ZG5, which were kindly given by Hangzhou ZhengGuang resin Co., Ltd. (China), were macroporous aliphatic resins with specific surface area from 200–900 m²/g.

The active baker's yeast was obtained from Meishan-Mauri Yeast Co., Ltd. (China).

Activation of dried yeast

The active baker's yeast was obtained as dried powder and it needed activation before use. A mass of 7.5 g of active dried baker's yeast was inoculated and incubated in 250 mL of activation medium in an orbital shaker at 170 rpm and 30 °C for 2 h. The activation medium was composed of 5 % glucose, 0.2 % (NH₄)₂SO₄, 0.1 % K₂HPO₄, 0.1 % CaSO₄, 0.1 % citric acid and H₂O. After activation, yeast cells were collected by centrifugation at 6400×g for 15 min and washed twice with KH₂PO₄-K₂HPO₄ buffer (0.05 mol/L, pH=7.0). The harvested yeast cells were kept at 4 °C for future use (14).

Resin pretreatment

The crude commercial resin was shipped as a water wet product imbibed with NaCl and Na₂CO₃ to retard bacterial growth, so it needed pretreatment before use. It was first washed twice with deionized water (10 mL for 1 mL of resin, 1 h) and then with ethanol (5 mL for 1 mL of resin, 1 h). Finally, the resin was dried at 60 °C under reduced pressure and preserved in a desiccator (14).

Determination of the toxicity of ACP and PEA to yeast cells

The method is based on the glucose consumption rate (16). In this method, a mass of 3.0 g of wet yeast cells was incubated in 30 mL of KH₂PO₄-K₂HPO₄ buffer (0.05 mol/L, pH=7.0) with ACP or PEA at a given concentration and a period of time which are given in the section Results and Discussion. Then the yeast cell was transferred into 30 mL of glucose solution containing 0.5 g of glucose. The glucose consumption rate was determined in the first 30 min. The retention activity of the yeast cell was expressed as relative activity. The relative activity was defined as the ratios of glucose consumption rate of the yeast cell incubated with ACP or PEA to that of the blank experiment (16).

Asymmetric reduction of ACP by yeast without resin adsorption

A mass of 7.0 g of activated wet yeast cells was suspended in 35 mL of KH₂PO₄-K₂HPO₄ buffer (0.05 mol/L, pH=7.0) in a 150-mL Erlenmeyer flask. A mass of 1.0 g of wet yeast equals 0.25 g of dry mass, which was determined by drying the wet yeast to constant mass at 120 °C. Then some ACP and 0.5 g of glucose as the co-substrate were added to the suspension. The asymmetric reduction reaction was processed in a thermostatic orbital shaker at 150 rpm and 30 °C for 30 h. After reduction reaction, the reactive mixture was extracted twice with 35 mL of ethyl acetate. The organic phase was dried with anhydrous Na₂SO₄. The concentration of ACP and PEA, and the enantiomeric excesses (e.e.) of S-PEA were determined.

Asymmetric reduction of ACP by yeast with resin adsorption

The typical experiment of asymmetric reduction of ACP by yeast cells accompanied by the introduction of resin adsorption was performed as follows: some resin and ACP were added into a 150-mL Erlenmeyer flask with 35 mL of $\text{KH}_2\text{PO}_4\text{-K}_2\text{HPO}_4$ buffer (0.05 mol/L, pH=7.0). The mixture was shook for 60 min to reach adsorption balance, then 7.0 g of wet yeast cells and 0.5 g of glucose as the co-substrate were added. The flask was placed in a thermostatic orbital shaker operating at 150 rpm and 30 °C to carry out the asymmetric reduction reaction for 30 h. After the reaction, the extraction and later treatment were the same as those of the reduction reaction without resin.

Analytical methods

The concentrations of ACP, R-PEA and S-PEA were determined with a gas chromatograph (Model 6820, Agilent Technologies Co., Ltd., USA) equipped with a chiral Cyclodex-B capillary column (0.32 mm×30 m, Agilent Technologies Co., Ltd., USA). Benzaldehyde was used as the internal standard substance. The conditions for gas chromatograph were: N_2 as the carrier gas with the flow rate of 3.5 mL/min, split ratio 50:1, flame ionization detector (FID), and programming temperature for the oven temperature. The temperature program was 80 °C for 4 min, ascending from 80 to 150 °C at the speed of 15 °C/min, and remaining at 150 °C for 3 min. The temperature of both injector and FID was 250 °C. The residence time of ACP, R-PEA and S-PEA was 8.973, 10.427 and 10.581 min, respectively.

The reaction degree and the enantioselectivity were indicated by yield (η , Eq. 1) and e.e. (Eq. 2) of S-PEA respectively, which were defined as:

$$\eta = \frac{c_{\text{PEA}}}{c_0} \times 100 \quad /1/$$

$$\text{e.e.} = \frac{c_{\text{S}} - c_{\text{R}}}{c_{\text{S}} + c_{\text{R}}} \times 100 \quad /2/$$

where c_0 is the initial ACP concentration, c_{PEA} is the final PEA concentration, c_{S} is the final S-PEA concentration, and c_{R} is the final R-PEA concentration.

Results and Discussion

Reaction properties of asymmetric reduction of ACP by baker's yeast

There are some oxidoreductases in the yeast cell, such as alcohol dehydrogenase, which can catalyze the asymmetric reduction reaction of the prochiral aromatic ketone, such as ACP, to chiral aromatic alcohol. The product of ACP asymmetric reduction catalyzed by the yeast cell is mainly S-PEA. The stereoselectivity of this reaction is very outstanding. The e.e. of S-PEA reaches about 99%. This reaction follows Prelog's rule. After 30 h, the reaction reached the balance, *i.e.* the concentration of ACP and PEA remained constant. This means not only that ACP can be reduced to PEA, but also that PEA can be oxidized to ACP catalyzed by the yeast cell. This was

also found in Gervais's report when simple aliphatic ketone was reduced in organic solvent (17).

The influence of the initial ACP concentration on the asymmetric reduction reaction was investigated. The experiments were carried out with 7.0 g of wet yeast and the initial substrate concentrations increased from 17.1 to 96.8 mmol/L. The results are shown in Fig. 1, where it is evident that the yield decreases with the increase of the concentration of ACP. The yield drastically decreased, especially when the concentration of ACP was higher than 27.0 mmol/L. The most plausible reason for the decrease of yield may be due to cell toxicity and substrate/product inhibition, as a result of increased substrate concentration (10).

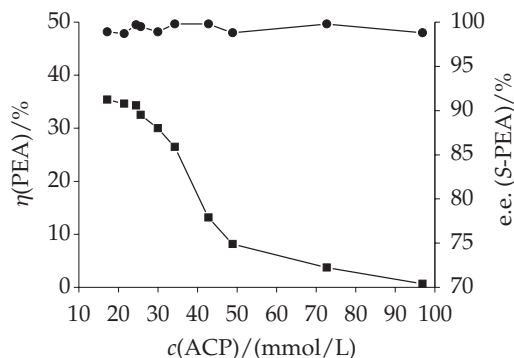


Fig. 1. Effect of the initial ACP concentration on the asymmetric reduction of ACP by baker's yeast: (■) PEA yield, (●) e.e. of S-PEA

Toxicity of ACP and PEA to yeast cell

It can be deduced from the above experiment that both the substrate and the product of this reaction are poisonous to the yeast cells. Generally, the compounds containing aryl group are pernicious to active cells. It is necessary to investigate the effect of ACP and PEA on the activity of the yeast cell. In these experiments, the concentration of ACP and PEA was increased from 0 to 68.0 mmol/L, and the incubation time was 6, 12, 24 and 30 h. Other conditions were described in the section Materials and Methods. The results are shown in Fig. 2.

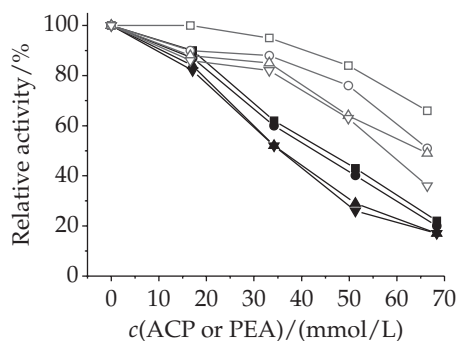


Fig. 2. Effect of ACP and PEA on the activity of baker's yeast: (■) incubation with ACP for 6 h, (●) incubation with ACP for 12 h, (▲) incubation with ACP for 24 h, (▼) incubation with ACP for 30 h, (□) incubation with PEA for 6 h, (○) incubation with PEA for 12 h, (△) incubation with PEA for 24 h, (▽) incubation with PEA for 30 h

As shown in Fig. 2, ACP and PEA are poisonous to the yeast cell, which is consistent with the previous findings that the substrate and the product inhibit the activity of the yeast cell. With the increase of the concentration of ACP and PEA and prolonging the incubation time, the toxicity of ACP and PEA to the yeast cell becomes more intense. Relative activity decreased steeply when the concentration of ACP was higher than 30.0 mmol/L, which is similar to the results of Rogers *et al.* (10) about acetophenone tolerance of yeast cell. It may also be concluded from Fig. 2 that ACP is more poisonous to the yeast cell than PEA. The result is consistent with the reduction reaction results of the above experiment.

Improvement of the asymmetric reduction of ACP by the introduction of resin adsorption

To avoid the toxicity of the substrate or product to the yeast cell, introducing resin adsorption may be a feasible technique. This method was successfully applied in our previous work of asymmetric reduction of β -oxo ester (14). The schematic diagram of asymmetric reduction reaction with introduction of resin adsorption is presented in Fig. 3.

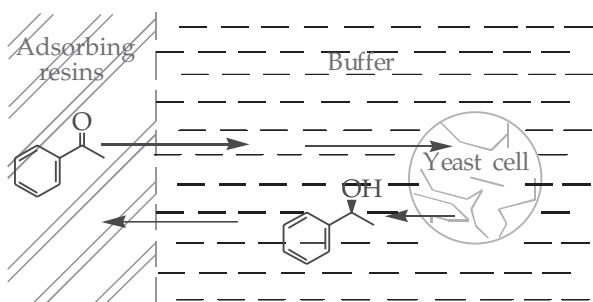


Fig. 3. Scheme of the process for asymmetric reduction of ACP by baker's yeast with the introduction of resin adsorption

As shown in the scheme, the reaction substrate (ACP) adsorbed in the resin can be slowly released to the aqueous phase. With the concentration gradient, ACP diffuses into the yeast cell and is reduced to PEA. The reaction product (chiral PEA) follows a reverse process of this, *i.e.* diffuses out of the cell and is adsorbed into the resin *in situ*. The reaction in the cell as the driving force breaks up the adsorption buffer balance of substrate and product between aqueous phase and resin phase, and pushes the process to the end. With this technique, the concentration of ACP and PEA in contact with the yeast cell may be controlled in a proper range, so that the toxicity of ACP and PEA to the cell can be avoided. The influence of various factors will be discussed in detail in the following paragraphs.

Effect of different kinds of resin on the asymmetric reduction of ACP by baker's yeast

Choosing the appropriate kind of resin is the key factor to this process. Based on literature reports (13,15) and our previous work (14), the inert macroporous adsorbing resins were chosen. Amberlite™ XAD and XE series resins and ZG1 to ZG5 resins were applied. The concentration of ACP and the mass of resin were 48.8

mmol/L and 1.0 g, respectively. Other conditions were described in the section Materials and Methods. The results are shown in Fig. 4.

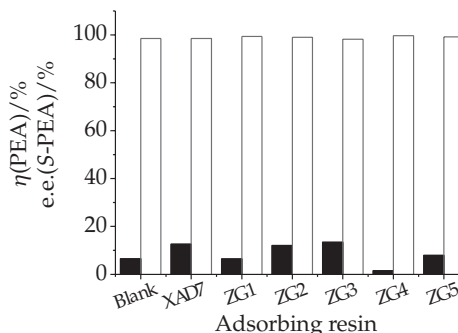


Fig. 4. Effect of the kind of resin on the asymmetric reduction of ACP with the introduction of resin adsorption: (■) PEA yield, (□) e.e. of S-PEA

It can be seen that the yield may be remarkably improved when ZG3 and Amberlite™ XAD7 resins were applied. The yield is decreased with the other resins, since the adsorbing ability of these resins to ACP is too strong to release ACP to aqueous phase and to be reduced. Fig. 5 gives the adsorption ability of these resins to ACP. The promotion effect was most remarkable with ZG3 resin because of its appropriate adsorbing ability to ACP, so in the following study the ZG3 resin was applied.

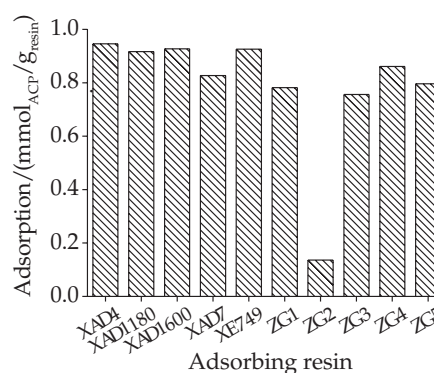


Fig. 5. Adsorption ability of the used resins to ACP

In the previous reports, XAD7 was adequate for promoting the asymmetric reduction of various kinds of prochiral ketone (13,15,18), which is in agreement with our results. Unfortunately, other kinds of resins were not investigated in these reports. Because the specific surface area of various resins is different, the adsorption ability of these resins to various substrates is different. So, not all resins are applicable to this process. The standard for the resin is that it must have suitable ability to adsorb to the reaction substrate. If the adsorption ability is too strong, the substrate could not be released to the aqueous phase and be reduced. On the contrary, if the adsorption capacity is too weak, the resin could not act as the substrate reservoir and product extraction agent *in situ*.

Effect of ACP concentration on the asymmetric reduction with resin adsorption

The influence of initial ACP concentration on the asymmetric reduction with resin adsorption was also investigated. The initial concentration of ACP was increased from 12.0 to 114.0 mmol/L. The resin mass was kept at 1.0 g, and other conditions were specified in the section Materials and Methods. The results are shown in Fig. 6.

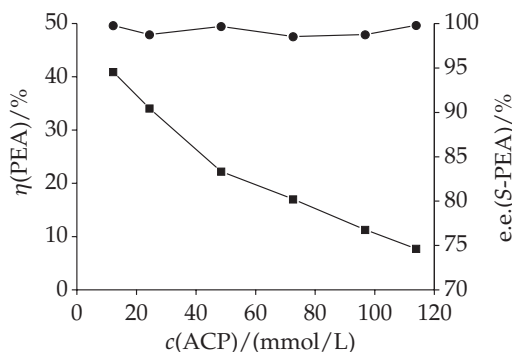


Fig. 6. Effect of the ACP concentration on the asymmetric reduction of ACP by baker's yeast with the introduction of ZG3 resin adsorption: (■) PEA yield, (●) e.e. of S-PEA

It is evident that the enantioselectivity and yield are acceptable even though the initial ACP concentration increases to 72.2 mmol/L. The yield drastically decreases when the initial concentration of ACP is just more than 27.0 mmol/L without resin adsorption, which can be concluded from Fig. 1. This indicates that this reaction can be remarkably improved with this technique. According to Roy *et al.* (18) as the substrate (1-acetonaphthone) concentration is increased, the conversion decreases very rapidly. Their experiment showed that the substrate concentration cannot be higher than 7.3 mmol/L (75 mg of 1-acetonaphthone in 60 mL of reaction buffer).

Effect of the mass ratio of resin on the asymmetric reduction of ACP by baker's yeast

To comprehensively understand this technique, the influence of the mass ratio of resin on the asymmetric reduction was investigated by using different resin and substrate ratio. The initial concentration of ACP was kept at

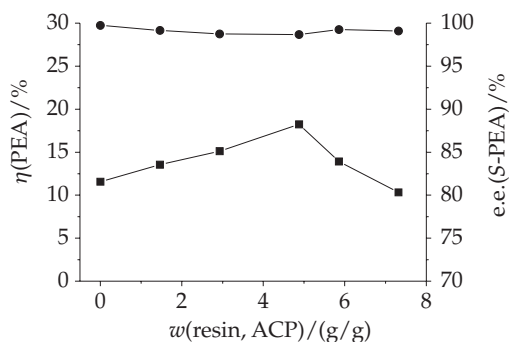


Fig. 7. Effect of the mass ratio of resin on the asymmetric reduction of ACP by baker's yeast with the introduction of ZG3 resin adsorption: (■) PEA yield, (●) e.e. of S-PEA

48.8 mmol/L and the ratio of resin to substrate $m(\text{resin})/m(\text{ACP})$ was increased from 0 to 7.3 g/g. Other conditions were described in the section Materials and Methods. The results are shown in Fig. 7, which shows that the optimum ratio of resin to substrate is about 4.8 g/g. This is similar to the results of Roy *et al.* (18) in their asymmetric reduction of 1-acetonaphthone by *Geotrichum candidum*. Their optimum ratio was about $m(\text{resin})/m(1\text{-acetonaphthone})=2$ g/g. The different ratio between our results and those of Roy *et al.* (18) is due to the difference between substrate and resin.

Conclusions

It was found that the initial substrate concentration could not be adequately high in the asymmetric reduction of aromatic ketones by ordinary yeast cell. The present work makes clear that the main reason is the obvious toxicity of aromatic compounds to yeast cells. Resin adsorption is applied to improve the asymmetric reduction reaction, which acts as the substrate reservoir and product extraction agent *in situ* to control the concentrations of substrate and product in the aqueous phase. The work indicates that this reaction can be remarkably improved with the appropriate kind of resin. With this technique, the initial ACP concentration can be increased to 72.2 mmol/L with acceptable yield. The method is very convenient, so it should be quite attractive in the practical application.

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