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Asymmetric reduction of chloroacetophenones to produce chiral alcohols with microorganisms

Zhimin Ou*,,†, Jianping Wu*,***, Lirong Yang* and Peilin Cen***

*Institute of Biochemical Engineering, College of Material Science and Chemical Engineering, Zhejiang University, Hangzhou, 310027, China
**Pharmaceuticals College, Zhejiang University of Technology, Hangzhou, 310014, China
***The Key Laboratory of Industrial Biotechnology, Ministry of Education, Wuxi 214036, China
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Abstract−Four strains of yeast with reduction activity of chloroacetophenones were screened, in which *Saccharomyces cerevisiae* B5 showed best reduction activity and stereoselectivity. High optical purity (R)-2′-chloro-1-phenylethanol can be obtained with *Saccharomyces cerevisiae* B5 as biocatalyst. The influence of several co-substrates on the enantiometric excess (ee%) and yield of (R)-2′-chloro-1-phenylethanol was evaluated. 5% (v/v) ethanol is optimal co-substrate for (R)-2′-chloro-1-phenylethanol formation. The optimal bioconversion conditions of 2′-chloroacetophenone catalyzed by *Saccharomyces cerevisiae* B5 are as follows: pH 8.0, 25 °C and 24 h. The yield and the enantiometric excess of (R)-2′-chloro-1-phenylethanol can both reach more than 99% with 10.75 g/*l Saccharomyces cerevisiae* B5 (the cell dry weight) and 1 g/*l* 2′-chloroacetophenone used in the biotransformation.

Key words: Chloroacetophenones, 2′-Chloroacetophenone, *Saccharomyces cerevisiae* B5, (R)-2′-Chloro-1-Phenylethanol

INTRODUCTION

Chiral alcohols with additional functional groups are very important intermediates for the synthesis of enantiomeric pure pharmaceuticals and other chemicals. For example, optically active chloro-1-phenylethanols are required in the synthesis of chemotherapeutic drugs, chiral auxiliaries and other industrially useful materials [1]. (S)-3-chloro1-phenylpropanol and (R)-3-chloro-1-phenylpropanol are building blocks of (S)-fluoxetine and (R)-tomoxetine [2], which are antidepressant drugs. (R)-clorprenaline, which is effective for the treatment of diverse disease states such as bronchitis and asthma, can be prepared by (R)-2′-chloro-1-phenylethanol.

Stereoselective reduction of prochiral ketones is a useful method to afford chiral alcohols. Fig. 1 shows the mechanism of asymmetric reduction of chloroacetophenone compounds to produce chiral alcohols. In this area, asymmetric reduction with microbial cells is recognized as a valuable approach. Without enzyme isolation, the application of whole cell as the catalyst is a simple and costsaving process. The coenzyme regeneration can be realized by using the metabolic pathways inside the whole cell. Many methods have been adopted to improve the yield and the stereoselectivity: 1) screening suitable microorganisms [3-6] or constructing perfect gene engineering strains [7]; 2) addition of inhibitor to inhibit the enzymes which convert the substrate to undesirable configuration product [8]; 3) addition of co-substrates to increase the yield and control reaction stereoselectivity [9]; 4) selecting the optimum substrate as the difference of the group around the chiral carbon atom in substrate molecule structure may greatly influence the product configuration [10]; 5) preheating the microorganisms to control the stereoselectivity [10]; (6) changing the microorganism growth environment to gain desirable configuration product [11]; 7) different configuration products can be obtained by immobilized and free microorganisms [12-14]; and 8) alternating the reaction environment to gain ideal configuration products, such as water, organic solvents or water/organic two phase system. A different reaction environment can lead to different product configuration [15-17].

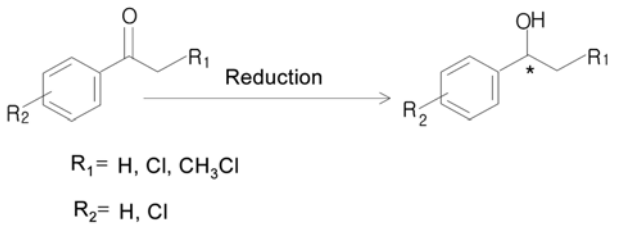


Fig. 1. Reduction of chloroacetophenone compounds.

In this paper, the stereoselective reduction of chloroacetophenones to the corresponding chiral alcohol with yeast cells is studied. The influence of the location of chlorine in phenyl ring on the reduction of series of chloroacetophenones is described. The effect of co-substrates and reaction condition on the yield will be investigated in detail.

EXPERIMENTAL

1. Chemicals

Acetophenone, 2-chloromethylacetophenone, 2′-chloroacetophenone, 3′-chloroacetophenone, 4′-chloroacetophenone, DL-1-phenylethanol were purchased from ACROS. (S)-3-chloro1-phenylpropanol and (R)-3-chloro1-phenylpropanol were from FLUKA. 2′-chloro-1-phenylethanol, 3′-chloro-1-phenylethanol and 4′-chloro-1-phenyletanol were synthesized by reduction of corresponding chloroacetophenones with $NaBH_4$ [18].

2. Microorganisms

†To whom correspondence should be addressed.
E-mail: oozzmm@163.com

Saccharomyces cerevisiae P2, *Saccharomyces cerevisiae* B5, *Candida pseudotropicalis* 104, *Candida utilis* 1257, *Candida utilis* 2.12, *Pichia membranaefaciens Hansen* 2.89, *Saccharomyces cerevisiae* 2.36, *Saccharomyces cerevisiae* 2.53, *Hansenula jadinii* 2.36, *Rhodotorula qlutinis* 2.102, *Candida famata* 2.270 were preserved in the Institute of Biochemical Engineering, Zhejiang University.

3. Cultivation of Cells

MYPG solid medium for preservation of strains contains 10 g/*l* wort, 3 g/*l* yeast extract, 5 g/*l* peptone, 10 g/*l* glucose, 20 g/*l* agar. Liquid medium for growth of strains contains 30 g/*l* glucose, 3 g/*l* yeast extract, 5 g/*l* $(NH_4)_2SO_4$, 0.5 g/*l* $MgSO_4 \cdot 7H_2O$, 1 g/*l* $K_2HPO_3 \cdot 3H_2O$, 1 g/*l* KH_2PO_3.

Each strain was inoculated to the sterilized liquid medium (100 ml) in 500 ml shaking flask with a shaking speed of 160 rpm at 30 °C. After 24 h, the seed culture (10 ml) was transferred into the liquid medium (100 ml), which was cultured at 160 rpm and 30 °C for another 24 h. The cell solution (10 ml) was collected, centrifuged, washed by distilled water and then suspended in 20 ml potassium phosphate (pH 8.0).

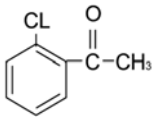
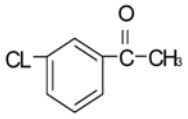
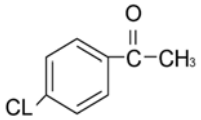
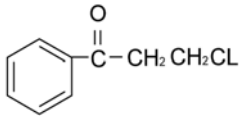
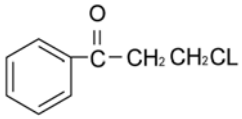
4. Reduction of Chloroacetophenones with Microorganisms

Substrate was added into a 50 ml shaking flask contained 20 ml potassium phosphate (pH 8.0) with cells. The flask was shaken at 160 rpm and 25 °C. The system was sampled at intervals. Then the cells were collected by centrifugation and discarded. Supernatant was extracted with 5 ml of ethyl acetate, and the ethyl acetate layer was used for GC analysis.

5. Analytic Method

The ethyl acetate layer was analyzed to determine the concentrations of chloroacetophenones and chloroacetophenols with a gas chromatograph (HP6890) equipped with a flame ionization detector: chromatograph column: HP Chiral 10% β-Cyclodextrin (30 m× 0.32 mm×0.25 μm). Nitrogen gas was used as carrier gas and a sample volume of 0.1 μL was injected by using a split ratio of 1 : 100. The flow rate was fixed at 1.0 mL/min at 130 °C for 30 min. The temperature of the injector and the detector was 250 °C.

Table 1. The stereoselectivity and yield of reduction of chloroacetophenones with yeast strains (0.5 g/*l* substrate concentration, 5% (v/v) ethanol as co-substrate, 25 °C, 24 h, 2.15 g/*l* cell dry weight)

Substrate	Strains	Yield (%)	ee (%)
	1	27	100
	2	>99	100
	3	>99	98
2'-CAP	4	45	97
	1	0	/
	2	24	100
	3	0.8	100
3'-CAP	4	17	89
	1	17	67
	2	29	64
	3	3	65
4'-CAP	4	28	14*
	1	30	74
	2	50	100
	3	48	100
2-CMAP	4	>99	33*
	1	0	/
	2	0	/
	3	0	/
AP	4	0	/

1, Saccharomyces cerevisiae P2; 2, Saccharomyces cerevisiae B5; 3, Candida pseudotropicalis 104; 4, Candida utilis 1257.

*The product configuration is in "S" forms, and the others are in "R" forms.

CAP, chloroacetophenone; CMAP, chloromethylacetophenone; AP, acetophenone.

RESULTS AND DISCUSSION

1. Screening of the Strains

The reduction results of five compounds by four strains with 5% (v/v) ethanol as co-substrate are listed in Table 1. *Saccharomyces cerevisiae* B5 shows its extensive ability in the reduction of 2′, 3′ and 4′-chloroacetophenone and 2-chloromethylacetophenone to (R)-alcohols with high activity and good stereoselectivity. So it will be investigated carefully in the following part. At the same time, *Candida utilis* 1257 can reduce 2-chloromethylacetophenone and 4′-chloroacetophenone to (S)-alcohols.

Reaction stereoselectivity and yield are related with the kind of reductases in microorganisms and the molecule structure of substrates. The location of chlorine substituted in a phenyl ring is very important for both enantiometric excess and yield. *Saccharomyces cerevisiae* B5 showed different activity on the reduction of various substrates in the following order: 2′-chloroacetophenone>2-chloromethylacetophenone>4′-chloroacetophenone>3′-chloroacetophenone >acetophenone. Carbonyl reduction is a nucleophilic reaction. Reduction is a process in which the organic molecules gain electrons or the electron cloud density of carbon atom is heightened by reducer. With higher oxidation state of carbon atoms in carbonyl, it is easier to gain electrons. The chloroacetophenones can be reduced more easily than acetophenone because of the electron-attracting inductive effect of chlorine atoms. Because of the coordination electron-attracting inductive effect and electron-excluding conjugative effect of chlorine on the phenyl ring, the electron cloud density of oxygen atom in carbonyl can be heightened and the oxidation state of carbon atom in carbonyl can be increased. The electron cloud density of oxygen atom in carbonyl in 2′-chloroacetophenone and 4′-chloroacetophenone are higher than that of oxygen atom in carbonyl in 3′-chloroacetophenone. As a result, 2′-chloroacetophenone and 4′-chloroacetophenone can be reduced more easily than 3′-chloroacetophenone. When a chlorine substitute occurs in the methyl group of 2-chloromethylacetophenone, the carbonyl group can also be reduced into hydroxyl group by yeast cells with high yield and enantiometric excess.

2. Influence of Co-substrate on 2′-Chloroacetophenone Reduction

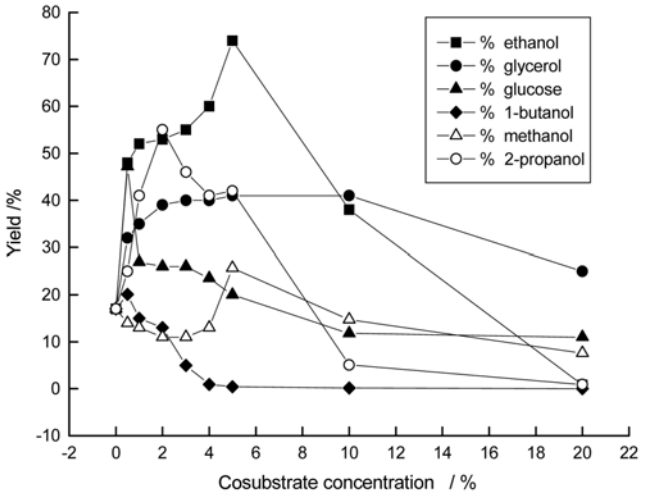


Fig. 2. Effect of the cosubstrates on the biotransformation of 2′-chloroacetophenone with *S. cerevisiae* B5 (1 g/*l* 2′-chloroacetophenone, 25 °C, 24 h, pH 8.0, 2.4 g/*l* cell dry weight).

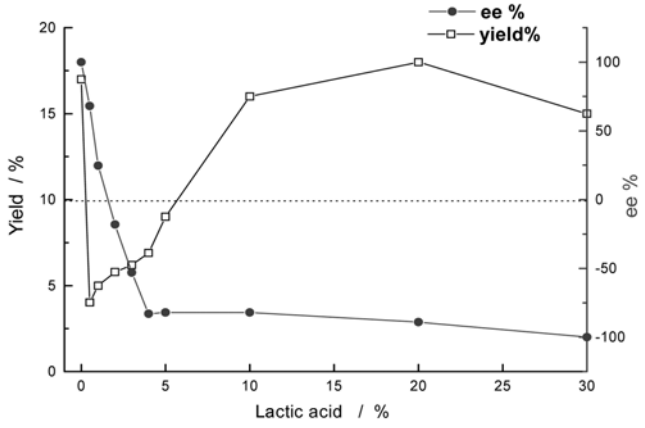


Fig. 3. Effect of lactic acid as co-substrate on the biotransformation of 2′-chloroacetophenone with *S. cerevisiae* B5 (1 g/*l* 2′-chloroacetophenone, 25 °C, 24 h, pH 8.0, 2.4 g/*l* cell dry weight).

It was reported that the addition of suitable co-substrate is of advantage to increase yield and can influence the stereoselectivity of reaction. Therefore, 2′-chloroacetophenone (1 g/*l*) was reduced by *Saccharomyces cerevisiae* B5 with methanol, ethanol, 2-propanol, 1-butanol, glucose, glycerol and lactic acid as co-substrates, respectively. The results are shown in Figs. 2 and 3. The enantiometric excess of (R)-2′-chloro-1-phenylethanol is 100% ee with methanol, ethanol, 2-pro-panol, 1-butanol, glucose and glycerol as co-substrates. When lactic acid was used as co-substrate, the enantiometric excesses of 2′-chloro-1-phenyl-ethanol varied with the different concentrations of lactic acid. And the yield of 2′-chloro-1-phenylethanol decreased with the increase of the lactic acid concentration. It is likely that lactic acid can inhibit the enzyme activity which converts 2′-chloroacetophenone to (R)-2′-chloro-1-phenylethanol. The yields were increased from 17% to 26%, 74%, 55%, 20%, 47% and 41% when the optimum concentrations of methanol, ethanol, 2-propaol, 1-butanol, glucose and glycerol were 5% (v/v), 5% (v/v),

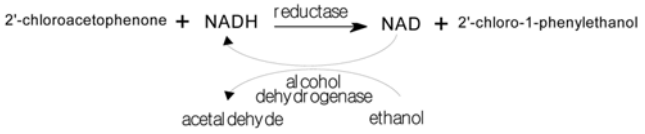


Fig. 4. Mechanism of the enzymatic 2′-chloroacetophenone reduction coupled with NADH regeneration.

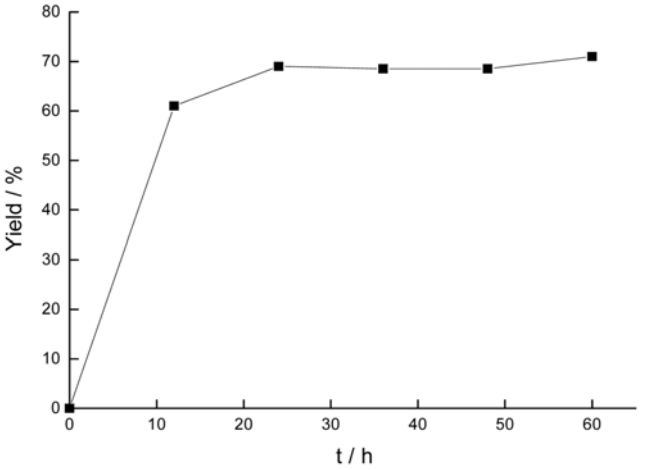


Fig. 5. Time course of *S. cerevisiae* B5 reduction reaction of 2′-chloroacetophenone (1 g/*l* 2′-chloroacetophenone, 2.15 g/*l* cell dry weight, pH 8.0, 25 °C, 5% (v/v) ethanol).

2% (v/v), 0.5% (v/v), 0.5% (w/v) and 5% (v/v) respectively.

The main function of co-substrate is for coenzyme regeneration. For enzymatic carbonyl group reduction, coenzyme NAD(P)H must participate in the coupled reaction to offer reduction power. Within yeast cells, there are abundant reductases, such as alcohol dehydrogenase. The alcohol dehydrogenase can oxidize ethanol into acetaldehyde and NAD^+ will be reduced to NADH. The mechanism of the enzymatic 2′-chloroacetophenone reduction coupled with NADH regeneration is shown in Fig. 4. If the bioconversion is performed without co-substrate addition, the reduction reaction can only use NAD(P)H accumulated during yeast cell cultivation, which limits the yield only about 17% (w/w). When co-substrate such as ethanol is added, the alcohol dehydrogenase inside the yeast cell can make NADH regeneration, so that the yield increases. However, if the concentration of co-substrate is too high, the co-substrate will probably inhibit the reduction process, which results in low yield. Another function of co-substrate is likely to change the permeability of cell wall, which will make the substrate easily transport into the cell. 5% (v/v) ethanol is selected as suitable co-substrate to further investigate in research.

3. Time Course of the Reaction and Reuse of *Saccharomyces cerevisiae* B5

1 g/*l* 2′-chloroactophenone, 5% (v/v) ethanol and the wet cell whose dry weight was 2.15 g/*l* were added into 50 ml flask that contained 20 ml potassium phosphate (pH8.0). The samples were analyzed every 12 hours. The results are shown in Fig. 5. The reaction yield reached 70% at 24 h and did not increase any more. So 24 h was regarded as the optimum time. The enantiometric excess of 2′-chloro-1-phenylethanol retained 100% ee during all the reaction time. After

Table 2. Results of addition of substrate consequently and the addition of substrate with co-substrate consequently

Sample	Yield (%)	ee (%)
1 g/*l* 2′-chloroacetophenone	2	100
1 g/*l* 2′-chloroacetophenone+0.5% (w/v)glucose	46	100
1 g/*l* 2′-chloroacetophenone+5% (v/v)ethanol	69	100

Table 3. Influence of cultivation condition of *S. cerevisiae* B5 and reaction condition on reduction (1 g/*l* 2′-chloroacetophenone, 2.15 g/*l* cell dry weight, pH 8.0, 25 °C, 5% (v/v) ethanol)

Culture condition	Reaction condition	Yield (%)	ee (%)
Anaerobic culture	Shaking	20	100
Anaerobic culture	Standing	12	100
Aerobic culture	Shaking	71	100
Aerobic culture	Standing	38	100

24 h, the cell was collected by centrifugation. The deposit was resuspended in 20 ml potassium phosphate (pH 8.0) containing 1 g/*l* 2′-chloroacetophenone, 20 ml potassium phosphate (pH 8.0) containing 1 g/*l* 2′-chloroacetophenone and 0.5% (w/v) glucose, 20 ml potassium phosphate (pH 8.0) containing 1 g/*l* 2′-chloroacetophenone and 5% (v/v) ethanol. The reactions were carried out for another 24 h. The results are shown in Table 2. After 24 h, *Saccharomyces cerevisiae* B5 still has reduction activity. When the co-substrates were added, *Saccharomyces cerevisiae* B5 gained the ability of coenzyme regeneration and the yield was still high, which showed *Saccharomyces cerevisiae* B5 can be reused well.

4. Effect of Culture Condition on the Reduction

After *Saccharomyces cerevisiae* B5 was cultured in anaerobic condition and aerobic condition, respectively, the wet cell whose dry weight was 2.15 g/*l* was dispersed in 20 ml potassium phosphate (pH 8.0) containing 1 g/*l* 2'-chloroacetophenone and 5% (v/v) ethanol. The reaction was carried out in shaking and standing condition. The results are shown in Table 3. A higher yield can be gained in aerobic culture and shaking reduction. The reductase activity in *Saccharomyces cerevisiae* B5 produced under aerobic culture was higher. A shaking reaction is advantageous for the diffusion of substrate into the cell and the release of product from the cell.

5. The Effect of Buffer pH on Reduction

A wet cell whose dry weight was 2.15 g/*l* was suspended in 20 ml potassium phosphate with different pH value. The reduction started with addition of 1 g/*l* 2′-chloroacetophenone and 5% (v/v) ethanol. The reaction carried out for 24 h at 25 °C. The results (Fig. 8) indicate that *Saccharomyces cerevisiae* B5 has great reduction ability from pH 5.0 to pH 10.0. The optimal pH was 8.0. There was no effect of pH on enantiometric excess of (R)-2′-chloro-1-phenylethanol and it remains 100%.

6. Effect of the Addition Amount of Biomass on the Reduction

The effect of addition amount of *Saccharomyces cerevisiae* B5 on reduction is shown in Fig. 6. The data indicated that the yield was increasing while more biomass was added. The yield reached

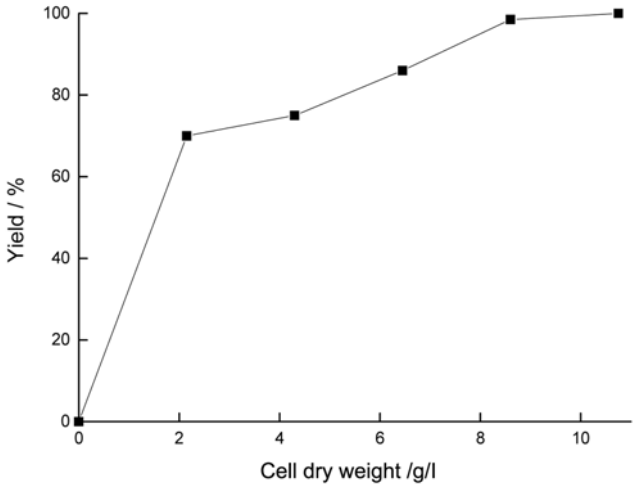


Fig. 6. Effect of cell concentration of *S. cerevisiae* B5 on the formation of (R)-2′-chloro-1-phenyl-ehtanol (1 g/*l* 2′-chloroacetophenone, pH 8.0, 25 °C, 5% (v/v) ethanol).

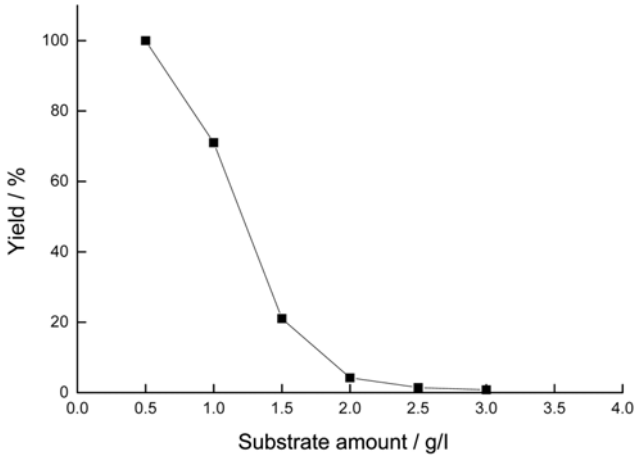


Fig. 7. Effect of the concentration of the substrate on the on formation of (R)-2′-chloro-1-phenyl-ehtanol (2.15 g/*l* cell dry weight, pH 8.0, 25 °C, 5% (v/v) ethanol).

>99% when the cell dry weight was 10.75 g/*l*. Obviously, the amount of coenzyme and redox enzyme both increased with more addition amount of cell. So the yield of (R)-2′-chloro-1-phenylethanol increased. The enantiometric excess of the product does not varied and retained 100% ee.

7. Effect of Substrate Concentration on the Reduction

The enantiometric excess of (R)-2′-chloro-1-phenylethanol was still 100% when different substrate amounts were added. The results are shown in Fig. 7. The yield went down sharply with the increase of substrate amount. As 3 g/*l* 2′-chloroacetophenone was added into the reaction, the yield of (R)-2′-chloro-1-phenylethanol only left to be 0.8%. It may be due to the fact that a large amount of substrate is toxic to microorganisms and then inhibits the activity of enzyme.

8. The Effect of Temperature on the Reduction

The effect of temperature on reduction is shown in Fig. 9. The yield was not changing greatly when the temperature was between 20-35 °C. As the temperature reached 40 °C, the yield decreased

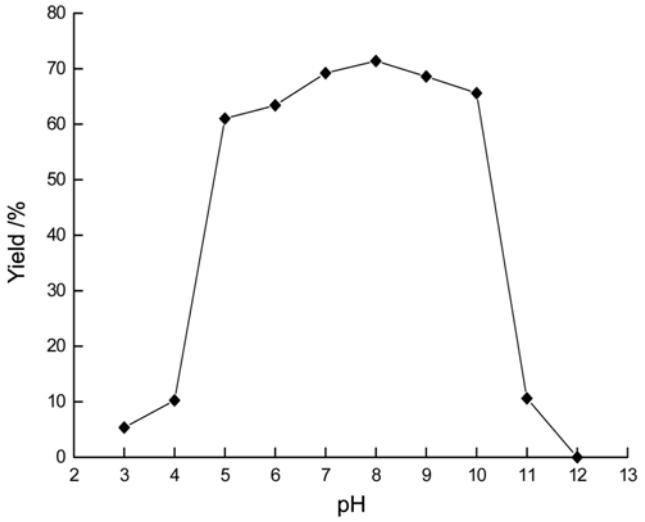


Fig. 8. Effect of pH on the formation of (R)-2′-chloro-1-phenyl-ehtanol (1 g/*l* 2′-chloroacetophenone, 2.15 g/*l* cell dry weight, 25 °C, 5% (v/v) ethanol).

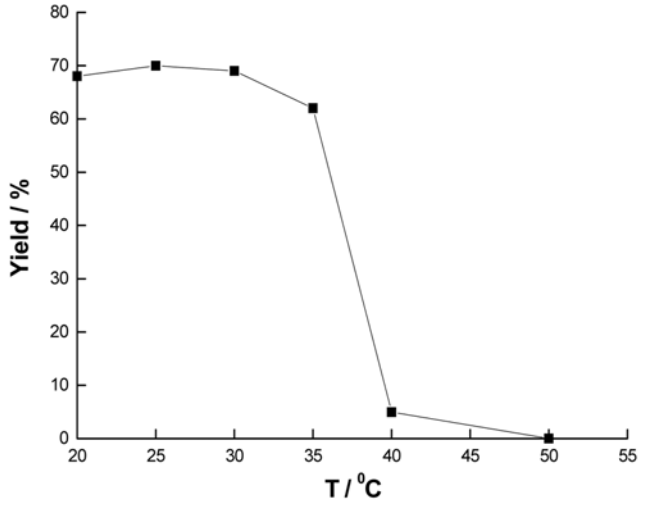


Fig. 9. Effect of temperature on the formation of (R)-2′-chloro-1-phenyl-ehtanol (1 g/*l* 2′-chloroacetophenone, 2.15 g/*l* cell dry weight, pH 8.0, 5% (v/v) ethanol).

sharply. The main reason was that the deactivated rate of enzyme was accelerated at high temperature. The optimal temperature was 25 °C. The enantiometric excess of (R)-2′-chloro-1-phenylethanol remained 100% ee at different temperature.

CONCLUSIONS

Four strains of microorganisms which have reduction activity of chloroacetophenones were screened, in which *Saccharomyces cerevisiae* B5 showed the highest activity and good stereoselectivity. 2′-chloroacetophenone was the most suitable substrate that can be reduced by *Saccharomyces cerevisiae* B5. *Saccharomyces cerevisiae* B5 showed different activity on the reduction of various substrates in the following order: 2′-chloroacetophenone>2-chloromethylacetophenone>4′-chloroacetophenone>3′-chloroacetohenone>acetophenone.

The influence of co-substrates on 2′-chloroacetophenone reduction with *Saccharomyces cerevisiae* B5 was investigated. Ethanol is the best co-substrate and the optimum concentration is 5% (v/v). The optimum reaction condition is 24 h, pH 8.0 and 25 °C. The yield of (R)-2′-chloro-1-phenylethanol decreases with the increase of substrate amount. The increase of biomass, aerobic culture and shaking reaction can help to raise the yield of (R)-2′-chloro-1-phenylethanol. When 10.75 g/*l* cell dry weight and 1 g/*l* 2′-chloroacetophenone were used, the yield reached >99%. The enantiometric excess of (R)-2′-chloro-1-phenylethanol remains nearly constant at all conditions and can reach 100% by the reduction of 2′-chloroacetophenone with *Saccharomyces cerevisiae* B5.

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