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Journal of Molecular Catalysis B: Enzymatic 1 (1996) 45–52

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

Review

Large-scale production of chiral alcohols with bakers' yeast

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Received 13 September 1995; accepted 24 November 1995

Abstract

Chiral alcohols were produced on a large scale by developing effective regeneration systems of nicotinamide coenzymes in bakers' yeast cells. In the asymmetric reduction of prochiral ketones, ethanol is used instead of carbohydrate as the energy source for NAD(P)H regeneration. We propose an overall scheme for NAD(P)H regeneration from $NAD(P)^+$ through the oxidative pathway of ethanol. In the oxidative resolution of a racemic alcohol, NAD^+ is effectively regenerated from NADH through the respiratory chain in the yeast cells. Thus, racemic 1,2-alkanediols were microbially resolved on a large scale by repeating the oxidative resolution and the asymmetric reduction.

Keywords: Bakers' yeast; Chiral alcohol; Asymmetric reduction; Oxidative resolution; Coenzyme regeneration

Contents

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SSDI 1381-117(95)00014-3

1. Introduction

Many synthetic organic compounds with chiral center(s) are used in the form of racemates in industry, as medicines, agricultural chemicals, and liquid crystals. For example, about a half of the synthetic medicines used world-wide (1327) have chiral center(s), but are sold as racemates (467) [1]. Recently, the side effects of an enantiomer with no physiologically desirable activity have been investigated. Thus, it has become important to provide optically pure compounds for medicines, the so called 'racemic switch'. Among the procedures to obtain chiral medicines, chiral building blocks as starting materials are widely applied. Therefore, it is very important to synthesize versatile chiral compounds on an industrial scale at low cost.

Enzymatic or microbial transformation of synthetic compounds is a convenient method for preparing chiral compounds. Lipase-catalyzed asymmetric hydrolysis of esters and bakers' yeast-mediated asymmetric reduction of prochiral ketones have been extensively studied due to the easiness of procedures and the mildness of experimental conditions. The applications of these biocatalysts have been recently reviewed in several reports [2–5]. Optical resolution by lipase is used in the synthetic processes of Diltiazem hydrochloride (a calcium channel blocker by Tanabe Pharmaceutical Co.) [6] and Etoc (an insecticide by Sumitomo Chemical Co.) [7]. Conversely, the bakers' yeast-mediated transformation has not been industrially applied.

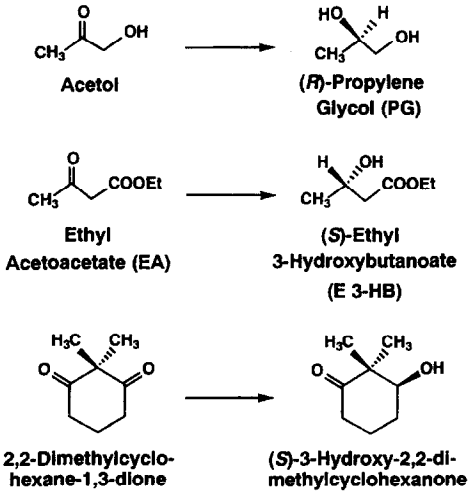

Fig. 1. The bakers' yeast-mediated bioreduction described in *Organic Syntheses*.

This review describes our efforts to produce chiral secondary alcohols by bakers' yeast in a large scale for industrial use. In yeast-mediated reduction and oxidation, regeneration of nicotinamide coenzymes is most important for successive transformation. Thus, effective regeneration systems coupled with yeast-mediated transformation were investigated.

2. Asymmetric reduction with bakers' yeast

2.1. Development of an effective procedure

Bakers' yeast catalyzes asymmetric reduction of prochiral ketones [8–14]. In *Organic Syntheses* (1943), the asymmetric reduction of acetol into (*R*)-propylene glycol (PG) with bakers' yeast is fully described (Fig. 1) [15]. In the 1980s, bakers' yeast-mediated reduction was taken up again in *Organic Syntheses* because of its importance as a synthetic procedure. The biotransformation of ethyl acetoacetate (EA) into (*S*)-ethyl 3-hydroxybutanoate (E 3-HB) [16] and of 2,2-dimethylcyclohexanone-1,3-dione into (*S*)-3-hydroxy-2,2-dimethylcyclohexanone [17] are reported (Fig. 1). Thus, asymmetric reduction mediated by bakers' yeast is recognized as a useful technique by synthetic organic chemists. However, this method has not been considered to be suitable for large-scale production of chiral alcohols. The reasons are as follows: (i) a relatively low concentration, (ii) tedious isolation and purification, and (iii) a relatively low optical purity [18–21]. In an effort to overcome these disadvantages, we have developed a new procedure based on a NAD(P)H regeneration system suitable for actual mass production.

Bakers' yeast-mediated bioreduction generally proceeds in an aqueous solution in the

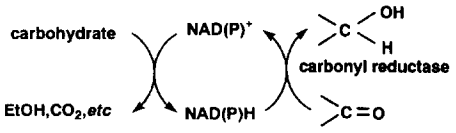

Fig. 2. Outline of the reaction mechanism by the conventional procedure using carbohydrate as the energy source.

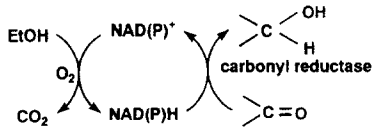

Fig. 3. Outline of the reaction mechanism by the new procedure using EtOH as the energy source.

presence of carbohydrate. Successive reductions are performed by regenerating the reduced form of nicotinamide coenzymes from the oxidized form through glucose oxidation (Fig. 2) [22]. However, the use of carbohydrate as the energy source for NAD(P)H regeneration is thought to be inadequate, because the amount of carbohydrate required for reduction is quite excessive compared with that of the substrate, and the metabolism of the carbohydrate is accompanied by the production of a vigorous foam of carbon dioxide and noxious by-products. Therefore, the efficiency of glucose as the energy source for NAD(P)H regeneration was assessed through extensive investigation of the EA reduction. In those experiments, we found that the specific consumption rate of glucose was about five times the specific reduction rate of EA and that the reduction proceeded even after disappearance of glucose under aerobic conditions. These results suggest that the yeast-mediated reduction proceeds by using ethanol as the energy source, which is in accord with our findings that reduction by using ethanol instead of glucose proceeded under aerobic conditions but hardly at all under anaerobic conditions. The specific consumption rate of ethanol was about twice the specific reduction rate of EA. The chemical yield and the optical purity of the product were similar to that generated by the conventional procedure. The asymmetric reduction of several carbonyl compounds by this new procedure also afforded similar results. The new procedure is summarized as shown in Fig. 3. It is clean and efficient, since no by-products, except carbon dioxide, were detected, and the energy source can be used effectively for coenzyme regeneration [23–25].

2.2. NAD(P)H regeneration system

In bakers' yeast-mediated reduction, NAD(P)H, which is the hydrogen donor in the reduction, must be successively regenerated from the corresponding oxidized form in the cells. Furthermore, the system responsible for the regeneration must be coupled with reduction of a substrate. The overall mechanism of yeast-mediated reduction, however, has not been studied before. Recently, the enzymes responsible for NADPH regeneration system coupled with asymmetric reduction of prochiral ketones in the conventional procedure using carbohydrate as the energy source has been characterized by Yamada et al. (Kyoto) (Fig. 4) [22].

In the new procedure using ethanol as the energy source, NAD(P)H is regenerated from $NAD(P)^+$ through the oxidative pathway of ethanol to carbon dioxide, and the system is coupled with reduction of a ketone. Studies using a cell-free extract of bakers' yeast and NAD(P)H confirmed that the reduction of acetol and EA is catalyzed by NADH- and NADPH-

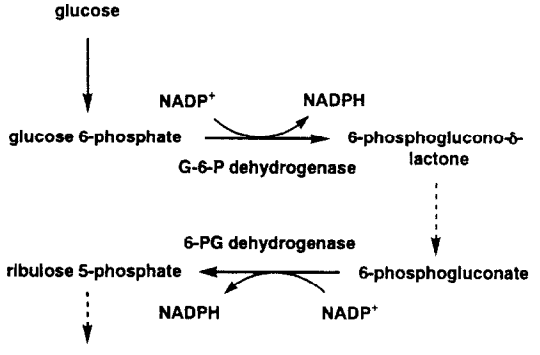

Fig. 4. NADPH regeneration system coupled with the asymmetric reduction of ketone by the conventional procedure using carbohydrate as the energy source.

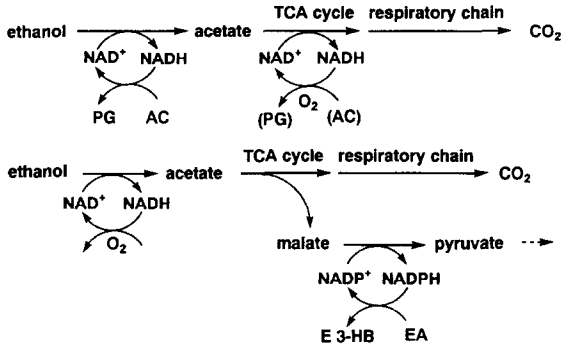

Fig. 5. Proposed scheme for the bioreduction of acetol and EA with bakers' yeast using EtOH as the energy source.

dependent carbonyl reductase, respectively. Therefore, we intensively examined the bioreduction of EA and acetol to define the NAD(P)H regeneration systems in bakers' yeast cells, using ethanol or acetate as the energy source under several sets of aerobic and anaerobic conditions. From these experiments, the overall scheme shown in Fig. 5 was proposed [26]. NAD^+ formed during the reduction of acetol is reduced to NADH through the oxidative pathway of ethanol to acetate, catalyzed by alcohol dehydrogenase and aldehyde dehydrogenase. On the other hand, $NADP^+$ formed in the reduction of EA is reduced to NADPH through the oxidative pathway of acetate to carbon dioxide. The NADPH is regenerated by coupled dehydrogenation of malate leaving the TCA cycle into pyruvate.

2.3. Optical purity

To use a chiral material as a building block, it is necessary to obtain it in optical purity of over 95%. The optical purity of (*S*)-E 3-HB obtained by the bakers' yeast-mediated reduction of EA was reported to be 83–90%ee [18]. In 1988, two NADPH-dependent oxidoreductases responsible for the enantioselective reduction of EA in bakers' yeast were purified and characterized [27]. One enzyme, with a K_m value for EA of 0.9 mM, catalyzes the reduction to give (*S*)-E 3-HB, and the other, with a K_m value for EA of 17.0 mM, to give (*R*)-E 3-HB. Thus, it was supposed that the former enzyme would mainly participate in the reduction at a low EA concentration, affording (*S*)-E 3-HB with high optical purity. Actually, the optical purity was dramatically improved by fed-batch operation to > 99% ee [28].

2.4. Large-scale production of (S)-E 3-HB and (R)-PG

Usefulness of the new procedure of the bakers' yeast-mediated reduction was demonstrated by preparing (*S*)-E 3-HB and (*R*)-PG on a large scale. (*R*)-PG is actually used as a chiral building block for preparation of (*S*)-Ofloxacin [29]. The large-scale production was effectively achieved using a bubble-column reactor, which has some advantages such as decrease in operating costs due to a low energy requirement [28,30]. Since the aeration rate is the most important factor in this reactor, its effect on the reduction rate was first examined. In the case of EA, the specific reduction rate increased according to the increase in the aeration rate, then reached to a constant. Conversely, the specific reduction rate of acetol was constant regardless of the aeration rate. These results were consistent with the reduction mechanism coupled with the regeneration system described in Section 2.2.

The results obtained by applying fed-batch operation to the new procedure and using the bubble-column reactor containing 10 l medium are summarized in Table 1. Compared with the results by the conventional procedure described in *Organic Syntheses*, the yield and the optical purity were markedly improved, and the procedure could be applied in an industrial scale.

2.5. Efficiency of ethanol

The relationship between a condition of oxygen supply, which is expressed in terms of a volumetric coefficient of mass transfer ($k_L a$), and the efficiency of ethanol in the bioreduction

Table 1
Comparison of results of the new procedure using a bubble-column reactor and the conventional method

	Present method (conventional)	
	EA reduction	Acetol reduction
Water	1,000 ml	1,000 ml
Bakers' yeast	112 g (77 g)	112 g (100 g)
Energy source	104 g of EtOH (192 g of sucrose)	18 g of EtOH (100 g of sucrose)
Substrate	60 g (15.3 g)	40 g (10 g)
Air	25 l/min (−)	3 l/min (−)
Reaction time	62 h (74 h)	38 h (72 h)
Temperature	30°C (r.t.)	30°C (32°C)
Recovery	40.5 g (10.6 g)	22 g (5.5 g)
Optical rotation	+44.3° (+37.2°)	−17.3° (−15.0°)
ee	99.3% (85%)	98.2% (90%)

[the specific reduction rate of a substrate (v_{sub})/the specific consumption rate of ethanol (v_{EtOH})] was examined. Fig. 6 shows saturated curves of v_{EtOH}, v_{EA}, and v_{acetol} with k_La as abscissa. Ethanol was consumed at almost the same specific rate regardless of the presence of a substrate (EA or acetol), and the efficiency of ethanol in the yeast-mediated reduction was evaluated [31].

In the bioreduction of EA coupled with NADPH regeneration, the efficiency decreased with increase in k_La and was about 0.5 at the actual operating condition of k_La of 100 [1/h]. As discussed in Section 2.2, 1 mol of NADPH is theoretically regenerated from 1 mol of ethanol. Therefore, half of the ethanol is wasted during respiration at a high k_La, though the specific reduction rate is fast. On the other hand, the efficiency of the bioreduction of acetol coupled with NADH regeneration had a maximal value of 1.9 at the actual operating condition of k_La of 20 [1/h]. Therefore, the bioreduction proceeds using about one third of the NADH regenerated through the oxidative pathway of ethanol to carbon dioxide.

The rate-limiting steps of the bioreduction of EA and acetol were estimated as follows. The maximum specific reduction rate of EA using ethanol as the energy source was 0.13 [mmol/h/g-wet-cell] at a k_La value of 100 [1/h], while the rate using glucose was 0.25 [mmol/h/g-wet-cell] under similar conditions. Values of v_{EtOH} and v_{EA} reached to a constant at k_La value above 100 [1/h] as shown in Fig. 6. Therefore, the rate-limiting step in the EA reduction using ethanol is probably the NADPH regeneration. On the other hand, the specific reduction rate of acetol using ethanol or glucose was the same and v_{acetol} reached to a constant at a k_La value of 40 [1/h] as shown in Fig. 6. Therefore, the rate-limiting step in the acetol reduction using ethanol is the reduction of acetol catalyzed by oxidoreductases.

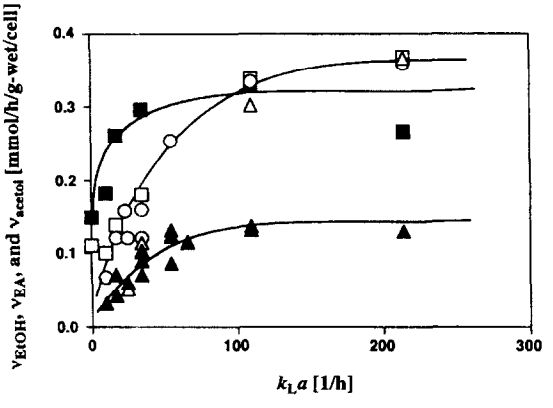

Fig. 6. Effect of k_La on the rates of ethanol consumption and reduction of EA or acetol. Symbols: ○, v_{EtOH} (absence of any substrates); △, v_{EtOH} (EA); □, v_{EtOH} (acetol); ▲, v_{EA}; ■, v_{acetol}.

3. Oxidative resolution with bakers' yeast

3.1. Preparation of chiral alcohol by oxidative resolution

Bakers' yeast is widely used as a 'reducing agent' in organic syntheses for preparing enantiomerically pure chiral compounds. One enantiomer can be conveniently prepared, but the other is essentially impossible to prepare with the same system. Although a method for controlling the enantioselectivity of yeast-mediated reduction has recently been reported by Ohno et al. (Kyoto) [32], it is not practical for large-scale production. Therefore, another method using

Table 2
Preparation of (*S*)-RCH(OH)CH_2OH from the racemic compound using bakers' yeast

1,2-Alkanediol	First treatment			Second treatment [a]	
R	$[\alpha]_D$ (°)	ee (%)	Yield [b] (%)	ee (%)	Yield [b] (%)
CH_3	+15.1	82.3	51	98.0	71
C_2H_5	−13.1	86.8	43	98.9	86
n-C_3H_7	−18.0	88.5	43	99.4	84
n-C_4H_9	−7.4	39.2	61	62.4	75
phenyl	+7.3	9.4	76	19.9	80

[a] Treatment of the product obtained by the first treatment with bakers' yeast.
[b] Isolated yield.

bakers' yeast, oxidative resolution, was developed to prepare the antipode of the reduction product in a large scale.

It was found that (*R*)-PG was easily oxidized to acetol with bakers' yeast under aerobic conditions, and (*S*)-PG, conversely, could not be oxidized under the same conditions. Treatment of racemic PG with bakers' yeast to oxidize (*R*)-PG to acetol followed by separation of the acetol affords (*S*)-PG [33,34]. The preparation of (*S*)-1,2-alkanediols in the same procedure is summarized in Table 2. Thus, a new procedure for the preparation of the antipode of the reduction product is shown in Fig. 7. The oxidation products, 1-hydroxy-2-alkanones, are also useful as substrates for bakers' yeast-mediated bioreduction.

3.2. Mechanism of bakers' yeast-mediated oxidation

In this biooxidation, NADH-dependent oxidoreductases participating in the asymmetric reduction of 1-hydroxy-2-alkanone catalyze enantioselective oxidation. NAD^+ is regenerated from NADH in the yeast cells for the successive oxidation. A preliminary experiment showed that the oxidation did not proceed under anaerobic conditions. Thus, an outline of the oxidation, especially in relation to a condition of oxygen supply, was examined by analyzing the transformation of (*R*)-PG to acetol [35].

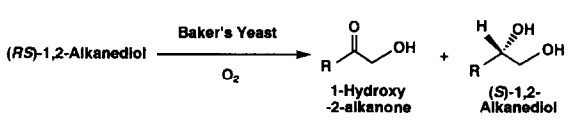

Fig. 7. Bakers' yeast-mediated biooxidation of 1,2-alkanediols.

The amounts of oxygen consumed and acetol produced according to the increase in increasing k_La, and the ratio was about 1.7 [acetol mmol/O_2 mmol]. Since 2 mol (*R*)-PG would be theoretically oxidized using 1 mol of oxygen as shown in Fig. 8, this result suggests that oxygen is effectively used during the biooxidation (85% efficiency).

The initial specific oxidation rate of (*R*)-PG increased according to the increase in k_La, then became constant at a k_La value above 100 [1/h] as shown in Fig. 9. This tendency was similar to that of v_{EtOH}. From this result, we estimated that the rate-limiting step of the oxidation of (*R*)-PG is the NAD^+ regeneration through the respiratory chain.

The kinetics of the biooxidation of (*R*)-PG by altering the substrate concentration and P_{O_2} were examined with a view towards the large-scale production of (*S*)-PG from racemic PG. For this experiment, NAD^+ concentration must be changed, but it is difficult. Therefore, P_{O_2} under constant k_La conditions was used as the

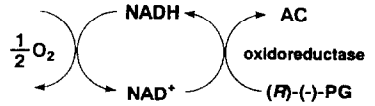

Fig. 8. Outline of the reaction mechanism of the yeast-mediated oxidation.

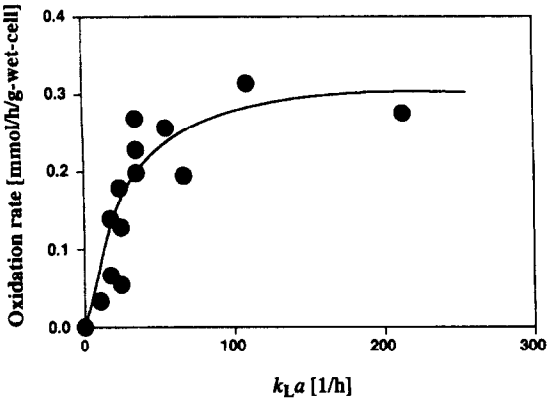

Fig. 9. Effect of $k_L a$ on the oxidation rate of (*R*)-PG.

apparent concentration of NAD^+. The Lineweaver–Burk plot shown in Fig. 10 shows that the oxidation reaction proceeds through an ordered bi–bi mechanism, indicating that product inhibition would be a practical problem for large-scale production. McGregor et al. have reported that the glycerol dehydrogenase (GDH)-catalyzed oxidation of 1,2-alkanediols proceeds through an ordered bi–bi mechanism [36]. Thus, it was suggested that there is an enzyme similar to GDH in bakers' yeast cells [37].

Lee and Whitesides (MIT) enzymatically prepared (*S*)-1,2-butanediol by the similar oxidative resolution of racemic 1,2-butanediol [38]. In their procedure, GDH (from *Enterobacter aerogenus* or *Cellulomonas sp.*) and glutamate dehydrogenase to regenerate NAD^+ from NADH were used for the resolution. In our process, enzymes catalyzing the oxidation and regenerating NAD^+ in bakers' yeast cells are effective in the oxidative resolution.

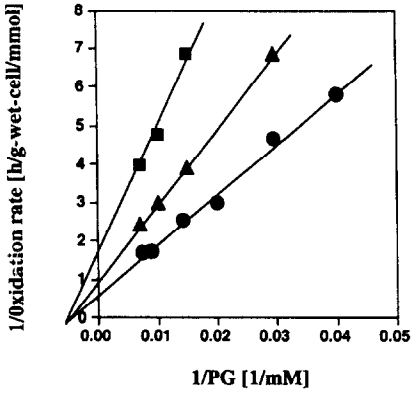

Fig. 10. Lineweaver–Burk plot for (*R*)-PG oxidation. Symbols (P_{O_2}): ■, 0.06 atm; ▲, 0.11 atm; ●, 0.21 atm.

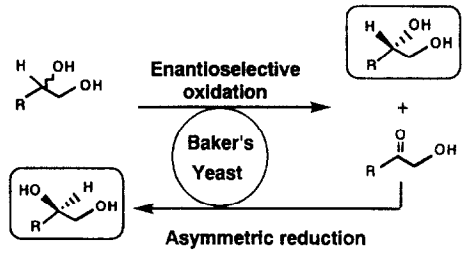

Fig. 11. Oxidative resolution of racemic 1,2-alkanediols with bakers' yeast.

3.3. Large-scale production of chiral 1,2-alkanediols

The oxidative resolution of racemic 1,2-alkanediols with bakers' yeast affords (*S*)-1,2-alkanediols and 1-hydroxy-2-alkanones that are easily separated due to a considerable difference in their volatilities. The asymmetric reduction of 1-hydroxy-2-alkanones with bakers' yeast affords (*R*)-1,2-alkanediols [13]. Thus, the optical resolution of racemic 1,2-alkanediols was conveniently accomplished using only bakers' yeast as summarized in Fig. 11.

The oxidative resolution and the asymmetric reduction have been achieved using the same bubble-column reactor on a large scale [35]. The specific oxidation rate is sufficiently fast and reaches the equilibrium within 24 h, affording (*S*)-1,2-alkanediols with 79% ee. The aqueous layers after removal of (*S*)-1,2-alkanediols are directly used for bakers' yeast-mediated bioreduction, affording (*R*)-1,2-alkanediols with > 98% ee.

4. Discussion

The enzymes in baker's yeast, especially the oxidoreductases for the asymmetric reduction or

enantioselective oxidation, are extremely versatile [8–14]. Nevertheless, the asymmetric reduction or the enantioselective oxidation has not been used in industry because of inefficiency as described in Section 2.1. To overcome this drawback, effective regeneration systems of nicotinamide coenzyme coupled with the asymmetric reduction or the enantioselective oxidation have been developed, and chiral alcohols have been produced in a large scale. Other species of yeast, *Candida*, *Hansenula*, and *Kloeckera*, also use ethanol as the energy source for NAD(P)H regeneration instead of carbohydrate. On the other hand, useful microbial oxidoreductases for preparation of chiral alcohols have been characterized, and their genes cloned [39]. Therefore, to combine the oxidoreductases with the effective regeneration system for nicotinamide coenzymes should result in useful microbial reduction and oxidation.

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