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Evaluation of the reducing power effect on the enantioselective synthesis of methyl (*R*)-2-chloromandelate using *Saccharomyces cerevisiae*

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Abstract−The effect of the reducing power on the reduction of methyl-2-chlorobenzoylformate was evaluated by using carbon substrates with different reducing powers. Glucose, sorbitol, and gluconate regenerated 2, 3, and 1 NAD(P)H during its conversion to pyruvate, respectively. When sorbitol was used as the carbon substrate, complete conversion was achieved in 8 h while it took 12 h and 19 h when glucose and gluconate were used, respectively. The enantiomeric excess (ee) value was 96.7% when sorbitol was used.

Key words: Asymmetric Reduction, Reducing Power, Methyl (*R*)-2-Chloromandelate, *Saccharomyces cerevisiae*

INTRODUCTION

The enantioselectivity of drugs is important since impurities can cause serious side effects and the enantioselective synthesis of pharmaceutical intermediates is considered as a core process in drug production. Based on the high stereoselectivity of biocatalysts, their application in enantioselective product synthesis has been studied for decades.

Methyl (*R*)-2-chloromandelate is a key intermediate of the platelet aggregation inhibitor clopidogrel, which is used as an antiplatelet by inhibiting blood clot formation for patients who have heart attack or stroke risks. Since clopidogrel should be enantiopure, methyl (*R*)-2-chloromandelate is currently prepared via the fractional crystallization of its racemic mixture. Instead of fractional crystallization, biological synthesis of enantiopure chemicals has been studied because it is more straightforward and attractive [1-4].

The direct asymmetric reduction of methyl-2-chlorobenzoylformate to methyl (*R*)-2-chloromandelate has been studied using a purified enzyme or whole cell biocatalyst to replace the fractional crystallization process. When a carbonyl reductase-overexpressed recombinant *Escherichia coli* strain was used for the methyl-2-chlorobenzoylformate reduction, methyl (R)-2-chloromandelate was obtained at a 99% enantiomeric excess (ee) under NADP+ supplemented conditions [5]. When *Saccharomyces cerevisiae* (baker's yeast) was employed without NADP+ addition, methyl-2-chlorobenzoylformate was produced with an ee value of 96.1% [6].

Though biological transformation of ketones to optically active alcohols is an effective procedure [7,8], it requires reducing power which is generally supplied in the form of NADH and NADPH in living organisms. Therefore, the performance of ketone reduction is affected by the reducing power availability. In this study, the effect of reducing power on the synthesis of methyl (*R*)-2-chloromandelate was evaluated.

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MATERIALS AND METHODS

1. Preparation of Methyl-2-chlorobenzoylformate

2-chloromandelic acid (Sigma-Aldrich) was dissolved in 150 mL methanol and 3 mL of 11 M HCl was added. The mixture was stirred for 12 h at 80 °C and then cooled to room temperature. Methyl-2-chloromandelate was extracted using dichloromethane (DCM) and dried over Na_2SO_4. Purified methyl-2-chloromandelate was dissolved in 50 mL ethyl acetate and 90 mL 10% (w/v) NaOCl was carefully added. Then, 1 g of a phase transfer catalyst, benzyltriethylammonium chloride, was added to activate the reaction. The reaction mixture was stirred for 6 h at room temperature. The methyl-2-chlorobenzoylformate produced was extracted using ethyl acetate, dried over Na_2SO_4, and the solvent was removed in a rotary evaporator. The purity of product was analyzed by NMR.

2. Whole Cell Asymmetric Reduction and Analysis

Dried *S. cerevisiae* yeast (Sigma-Aldrich) was mixed with carbon substrates and methyl-2-chlorobenzoylformate in 5 mL of 100 mM phosphate buffer (pH 7). The initial concentrations of *S. cerevisiae*, the carbon substrate, and methyl-2-chlorobenzoylformate were 12.5 g/L, 100 g/L, and 25 g/L, respectively. The reaction mixture was incubated for 6 h at 30 °C and the pH was maintained at 7 by the addition of 1 M NaOH. Methyl (*R*)-2-chloromandelate was assayed by HPLC equipped with a Chiralcel OD-H column (0.46 cm ×25 cm) and n-hexane/2-propanol (97 : 3, v/v) was used as the mobile phase. The carbon substrates were analyzed by HPLC equipped with an Aminex HPX-87H column (300 mm×7.8 mm), which was eluted isocratically with 4 mM H_2SO_4. The reactions were performed at least three times.

RESULTS AND DISCUSSION

Considering its low cost, ease of use, and generally regarded as safe (GRAS) status, *S. cerevisiae* is considered an attractive biocatalyst and has been applied in a variety of enantioselective reductions [9-13]. Although it has been shown that methyl-2-chlorobenzoyl-

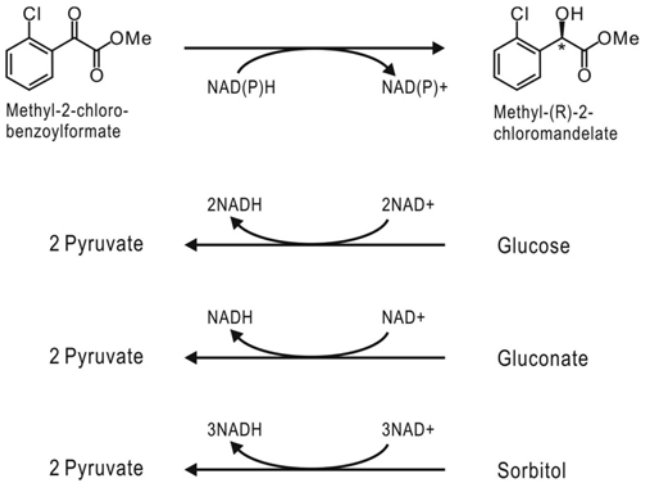

Fig. 1. Schematic diagram of the methyl 2-chlorobenzoylformate reduction with *S. cerevisiae* using various carbon substrates.

formate reduction can be efficiently facilitated by *S. cerevisiae*, further improvements are required for commercialization of the process. To improve the process, the limiting factor must be identified. There are several potential limiting factors including carbon substrate shortage, low biocatalyst activity, and insufficient reducing power. A previous study found that the carbon substrate and biocatalyst are not limiting factors since additional carbon substrate and biocatalyst did not provide any positive effect [6]. Therefore, we investigated the effect of the reducing power by using various carbon substrates with different redox states.

Sorbitol, gluconate, and glucose were used as the carbon substrates in this study. When one mole of glucose is converted to pyruvate through glycolysis metabolism, two moles of NADH are regenerated. Three and one moles of NADH are regenerated from each mole of sorbitol and gluconate, respectively (Fig. 1).

Although the consumption rates of the carbon substrates did not vary significantly (Fig. 2(a)), the ketone conversion time profiles show dramatic differences (Fig. 2(b)). When glucose was used, the exponential conversion period of the methyl-2-chlorobenzoylformate reduction reaction began after 6 h of incubation and 100% conversion was obtained after 12 h (Fig. 2(b)). When the carbon substrate was changed to sorbitol, which provides a 1.5-fold increase of the reducing power compared to glucose, the exponential period began after only 2 h of incubation and 100% conversion was achieved in 8 h. The gluconate-supplemented reaction time profile showed the longest lag period of 9 h and the maximum amount of product was produced after 19 h of incubation.

During the initial lag period, succinic acid was produced major byproduct, suggesting that dried *S. cerevisiae* activated its metabolism to adapt to the new environment [6]. The length of the initial lag period also varied depending on the carbon source. When a more reduced carbon substrate was used, the initial lag period was shortened (Fig. 2(c)). This result indicates that if more reducing power is supplied, cellular metabolism can be activated in a shorter time.

When gluconate was used, the ee value also decreased to 93.7% (Table 1). It was hypothesized that the low reducing power led to the less optimum condition causing long incubation time and the low ee value.

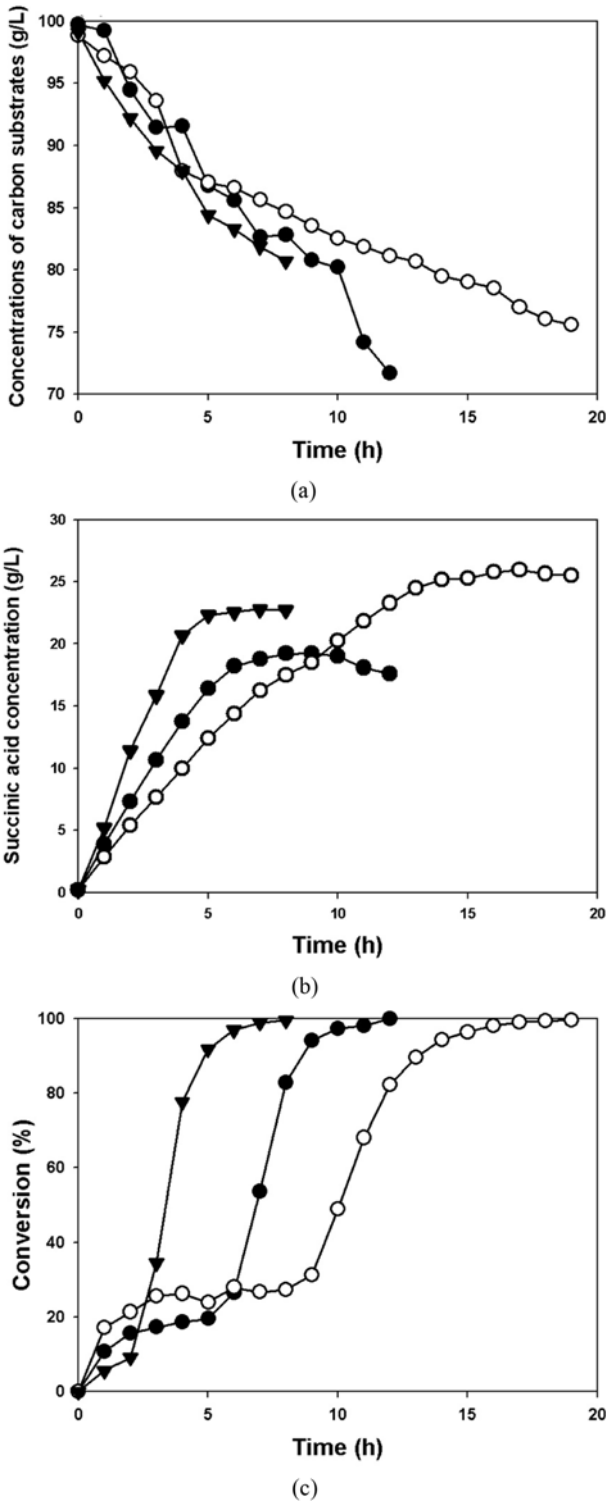

Fig. 2. Time profiles of the (a) carbon substrate concentration, (b) methyl 2-chlorobenzoylformate conversion, and (c) succinic acid concentration when glucose (●), gluconate (○), and sorbitol (▼) were used as the carbon substrate.

CONCLUSIONS

The effect of the reducing power on the reduction of methyl-2-chlorobenzoylformate to methyl (*R*)-2-chloromandelate was evalu-

Table 1. Effect of the carbon substrate on the reduction of methyl 2-chlorobenzoylformate with *S. cerevisiae*

	Carbon substrate			
	No	Glucose	Sorbitol	Gluconate
Incubation time (h)	24	12	8	19
Conversion (%)	57	100	100	100
Enantiomeric excess (%)	100	96.6	96.7	93.7
Consumed carbon substrate (g/L)	0	29.3	19.3	24.4

ated in this study. Three carbon substrates providing different reducing power were compared. When sorbitol was used as carbon substrate, complete conversion was achieved in 8 h with the ee value of 96.7%. The results clearly showed how the reducing power supply affects the performance of the biocatalyst mediated ketone reduction reaction. To provide more reducing power to the reaction system, several strategies can be considered. First, H_2 gas can be directly purged into the reaction mixture to help NAD(P)H regeneration. Second, a more reduced carbon substrate can be used to provide more reducing power, as demonstrated in this study. Third, acceleration of the carbon substrate metabolism will increase the NAD(P)H regeneration rate.

Considering the relatively low cost of the carbon substrate as compared to the product, the reaction time is a more important factor affecting process efficiency and cost. Therefore, evaluation of metabolically engineered microorganisms as whole cell biocatalysts is required.

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