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Resolution of biotin intermediate lactone by enzyme-catalyzed stereoselective lactonization in organic solvent

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ABSTRACT

A strain of *Aspergillus oryzae* WZ007 producing intracellular lipase with preference for the formation of the D-biotin intermediate lactone ((3*aS*,6*aR*)-tetrahydro-1,3-dibenzylhexahydro-1*H*-Furo[3,4-*d*]imidazole-2,4-dione) **1** was screened from soil samples. The microbial cells fermented by the strain were used as biocatalysts after lyophilization, to resolve the racemic biotin intermediate lactone **1** by enantioselective lactonization in organic solvents. The enantioselective lactonization afforded optically active lactone **1** with high enantiomeric excess (e.e. $\geq$ 98%) and conversion ratio ($c \geq 40\%$) when the biotransformation was carried out in dichloromethane. The e.e. of product was up to 99% after purification by inducing crystallization in acetone and basically satisfied the industrial application. The biosynthesis has been employed to produce kilograms supplies of optically active lactone **1** with high optical purity.

1. Introduction

D-Biotin is an important member of the Vitamin B complex group, which is also called Vitamin H or Coenzyme R [1,2]. It has been extensively applied in medicine and health protection, particularly as the feedstuff additive with large commercial requirement. Since the Goldberg–Sternbach-Synthese [3–5] was accomplished in 1949, the total synthesis of D-biotin has made great progress [6]. In the overall synthesis, the long-standing problems are the control of the three adjacent stereogenic centers and the preparation of the key biotin intermediate, (3*aS*,6*aR*)-lactone **1** [7]. The routes to asymmetric synthesis of (3*aS*,6*aR*)-lactone have been numerously reported, but mostly using chemical methods. In these chemical methods, there are many disadvantages such as expensive chiral reagents, environmental pollution, and difficult separation [8–10]. The synthesis of (3*aS*,6*aR*)-lactone **1** which derived from pig liver esterase catalyzed asymmetric hydrolysis of *meso*-dicarboxylic esters and subsequently following by Grignard reaction was reported [11,12], but pig liver esterase was quite expensive.

In this paper, we described the novel enantioselective synthesis of the optically active (3*aS*,6*aR*)-lactone **1** through kinetic resolution by inexpensive microbial lipase. In the first step of the scheme (Scheme 1), acid **2** (1,3-dibenzyl-5-(hydroxymethyl)-2-oxo-4-imidazolidinecarboxylic acid) was obtained via chemical hydrolysis of racemic lactone **1** with sodium hydroxide. Subsequently, optically active (3*aS*,6*aR*)-lactone **1** was produced by dry microbial cells of *Aspergillus oryzae* WZ007 catalyzed-enantioselective lactonization of *rac*-**2**.

2. Materials and methods

2.1. Materials

Racemic lactone **1** was gift from Zhejiang Medicine Co. Ltd. (Zhejiang, China). All the organic solvents were obtained from commercial sources and used without further purification. Other chemicals were of analytical reagent grade.

2.2. Preparation the substrates rac-*2*

The substrate (1,3-dibenzyl-5-(hydroxymethyl)-2-oxo-4-imidazolidinecarboxylic acid) **2** was obtained via chemical hydrolysis of racemic lactone **1** with sodium hydroxide. Racemic lactone **1** was added to 1 M solution of sodium hydroxide and stirred at 200 rpm in a reciprocal shaker at 50 °C for 5 h. And then 1N hydrochloric acid was dripped to the above solution slowly until pH fell to about 7.0. The acid **2** was precipitated in acidic condition and obtained by filtration with busher funnel. The substrate sample was subsequently dried in oven for 6 h.

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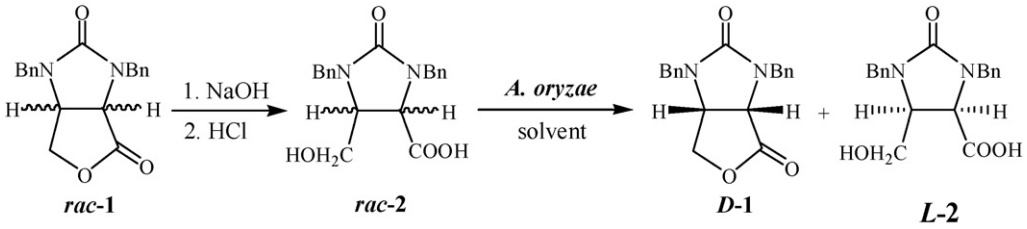

Scheme 1. Synthesis of (3*aS*,6*aR*)-lactone **1**.

2.3. Microorganisms and culture conditions

The strain of *A. oryzae* WZ007 was isolated from soil samples and deposited at China center for type culture collection with an accession number of CCTCC No. M206105. It was used throughout this study and routinely maintained on Czapek's medium slant ($NaNO_3$ 0.2%, K_2HPO_4 0.1%, KCl 0.5%, $FeSO_4$ 0.001%, $MgSO_4$ 0.05%, sucrose 3%, agar 1.5% mass ratio). The microorganisms were cultured in the liquid media (sucrose 5 g, peptone 15 g, yeast extract 15 g, olive oil 1 ml, NaCl 5 g, K_2HPO_4 2 g, $MgSO_4$ 1 g, $(NH_4)_2SO_4$ 5 g, dissolved in 1 l deionized water, 500 ml Erlenmeyer flasks containing 100 ml of medium and incubated for 48 h at 25 °C on a reciprocal shaker (180 rpm)). Cells were harvested by filtration at 4 °C and washed with distilled water. Microbial cells (50 g, wet weight) were added to 20 ml barbituric sodium-hydrochloric acid buffer (pH 7.0, 0.1 M) and lyophilized at −60 °C for 2 days.

2.4. Procedure of general biotransformation

Biotransformation was performed with dry microbial cells. Enzymatic lactonization were carried out in 50 ml screw capped test tubes by suspending dry microbial cells (5 g l^{-1}) in organic solvent (20 ml) with 0.5 g racemic acid **2**. The reaction mixture was incubated at constant temperature under shaking at 200 rpm in a reciprocal shaker. At appropriate intervals, the reaction was stopped by filtering off the dry microbial cells and unreacted substrate over filter paper. After drying over anhydrous Na_2SO_4, the filtrate containing the lactone was evaporated to dryness.

2.5. The method of water activity control

The water activity (a_w) of substrates and the biocatalyst was adjusted before starting the reaction by the following method: racemic acid **2** and dry microbial cells of *A. oryzae* WZ007 in powder form were incubated in the container containing a desired saturated salt solution or salt hydrate pairs for 7 days at 25 °C to equilibrate the materials for the desired a_w. The salts tested were: $CaCl_2{\cdot}H_2O/2H_2O$ (a_w 0.037), $Na_2CO_3{\cdot}H_2O$ (a_w 0.22), $CuSO_4{\cdot}5H_2O/3H_2O$ (a_w 0.30), NaCl (a_w 0.75), $Na_2SO_4/10H_2O$ (a_w 0.76), KCl (a_w 0.84). The initial a_w was determined by coulometric Karl Fischer titration (Metrohm 758 KF Coulometer, Switzerland).

After pre-equilibration, given amounts of substrates and the biocatalyst were transferred to 50 ml screw capped test tubes. In order to keep a_w constant during the reaction, the corresponding saturated salt solution and salt hydrate pairs as described above with the desired a_w were added directly to the reaction mixture. After 24 h the reaction was stopped and analyzed by HPLC.

2.6. Analytical methods

Molar conversions and enantiomeric excess of product were determined by chiral HPLC. Chiral HPLC analysis was performed on a HPLC (waters 2695, USA) equipped with chiral column (Chiral CD-Ph Shiseido, Japan) and UV detector at a wavelength of 225 nm. A mixture of acetonitrile and deionized water (70:30, v/v) was used as the eluent. Flow rate was 0.5 ml/min and retention times for D-lactone isomer and L-lactone isomer were 8.5 and 9.2 min, respectively.

The enantiomeric ratios (E) were calculated using the following equation [13], $E = \ln[1 - c(1 + \text{e.e.}_p)]/\ln[1 - c(1 - \text{e.e.}_p)]$, $\text{e.e.}_p = [([D] - [L])/([D] + [L])] \times 100\%$. Where c is the conversion ratio of reaction and e.e._p is the enantiomeric excess of the created lactone. The $[D]$ and $[L]$ are the area under the curve for D-lactone isomer and L-lactone isomer, respectively.

3. Results and discussion

Recently in organic synthesis, lipases are playing more and more important roles in catalyzing many reactions including esterifications, transesterifications and lactonization. Enzyme-catalyzed lactonization is the reversible reaction of hydrolysis, which generally occurs in low water content system. Considering that enzyme activity requires an amount of water content 'essential water layer' keeping the enzyme molecule configuration [14,15], we determined to choose the micro-aqueous phase system as the reaction system.

3.1. Optimization of the conditions of enzyme-catalyzed lactonization

In order to establish optimum reaction conditions, the effect factors of enzyme-catalyzed lactonization including organic solvents, buffer pH values, and temperature and a_w were studied.

3.1.1. Effect of solvent on enzyme-catalyzed enantioselective lactonization

The polarity of the solvent (expressed as log P) and the solubility of substrate often show great influence on enzyme activity and these factors must be taken into account [16,17]. Effect of different solvents on the enantioselective lactonization of **2** by dry microbial cells of *A. oryzae* WZ007 was first investigated in this study. As shown in Table 1, both the conversion rate and enantioselectivity were effectively influenced by the solvent employed. Although there was not a direct relationship between

Table 1
Effect of solvents on the conversion ratio, e.e._p and E value of enantioselective lactonization of **2**

Solvents	Log P	c (%)	e.e._p (%)	E
Acetonitrile	−0.33	27.4	12.2	1.3
Acetone	−0.23	28.0	50.4	3.7
Dichloromethane	−0.23	34.9	96.5	94.2
Toluene	2.5	30.4	91.7	34.2
Benzene	2.0	27.5	90.4	27.7
Chloroform	3.0	21.1	85.9	16.5

Reactions were carried out in different organic solvents with lyophilized microbial cells of *Aspergillus oryzae* WZ007 at 25 °C for 24 h.

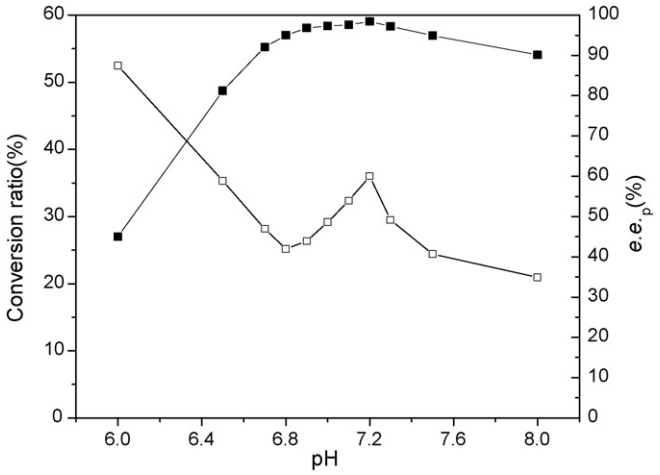

Fig. 1. Effect of pH value on the conversion ratio (□) and e.e.$_p$ (■) of enantioselective lactonization of **2**. Reactions were carried out in dichloromethane with lyophilized microbial cells of *Aspergillus oryzae* WZ007 lyophilized at different pH value at 25 °C for 24 h.

the catalytic activity of dry microbial cells and polarity of the solvents, hydrophobic solvents were more preferred compared to hydrophilic solvents in the biotransformation. Hydrophilic solvents might strip of essential water layer around the whole-cell biocatalyst and resulted in decrease in its activity. Among the solvents examined, dichloromethane proved to be the optimal reaction medium. Therefore, dichloromethane was selected as reaction medium for subsequent experiment.

Since the substrate acid **2** is insoluble in dichloromethane, the reaction system is a high solid–liquid ratio biocatalysis system containing two solid phases, an organic liquid phase and essential water layer. As the lactone had great solubility in organic solvent, the separation of product from the substrate and biocatalysts was convenient by common filtration.

3.1.2. Effect of buffer pH values on enzyme-catalyzed enantioselective lactonization

pH value had great effects on enzymatic ionization condition and conformation, therewith determined enzymatic activity and selectivity [18]. In this paper, we studied the effect of pH values by rendering pH memory to the microbial cells by lyophilized with barbituric sodium-hydrochloric acid buffer in the pH value range of 6.0–8.0.

Table 2
Effect of water activity on the conversion ratio, e.e.$_p$ and *E* value of enantioselective lactonization of **2**

Entry	a_w	*c* (%)	e.e.$_p$ (%)	*E*
Control	–	36.4	97.7	151.3
$CaCl_2{\cdot}H_2O/2H_2O$	0.037	9.5	91.5	24.8
$Na_2CO_3{\cdot}H_2O$	0.22	12.7	94.5	40.5
$CuSO_4{\cdot}5H_2O/3H_2O$	0.30	34.7	95.3	68.9
NaCl	0.75	35.4	97.1	116.0
$Na_2SO_4/10H_2O$	0.76	40.6	96.4	108.4
KCl	0.84	41.2	98.2	227.8

Reactions were carried out in different a_w with lyophilized microbial cells of *A. oryzae* WZ007 at 25 °C for 24 h. All items added 500 mg saturated salt solution or salt hydrates pair in reaction system.

The results shown in Fig. 1 indicated that it had relative good results in the pH range of 7.0–7.3 and the optimum of pH obtained for this reaction was 7.2. When pH value was under 6.8, the rate of reaction increased marginally but at the cost of activity and selectivity of enzyme. The reason lay in spontaneous lactonization of acid **2** in acid condition. When pH value was elevated in the range of 7.2–8.0, enzymatic activity and selectivity decreased at the same time.

3.1.3. Effect of temperature on enzyme-catalyzed lactonization

In organic phase reaction medium, thermal stability of enzyme was enhanced because of very little water inside. Reaction temperature is an important influencing factor of the enzyme catalytic activity and enantioselectivity by changing the structure and configuration of its active site.

Fig. 2 showed that the effect of reaction temperature on enantiomeric ratio and conversion rate in the range of 5–35 °C (dichloromethane boiling point 39.8 °C). When the reaction temperature was ranged from 5 to 25 °C, both of the activity and enantioselectivity increased but followed by a slight decrease at higher temperature. Thus subsequent experiments were carried out at 25 °C. When the reaction temperature further was elevated, the conformation of enzyme was possibly destroyed by heat-induced destruction of noncovalent interactions and consequently affecting enzyme activity and enantioselectivity [19].

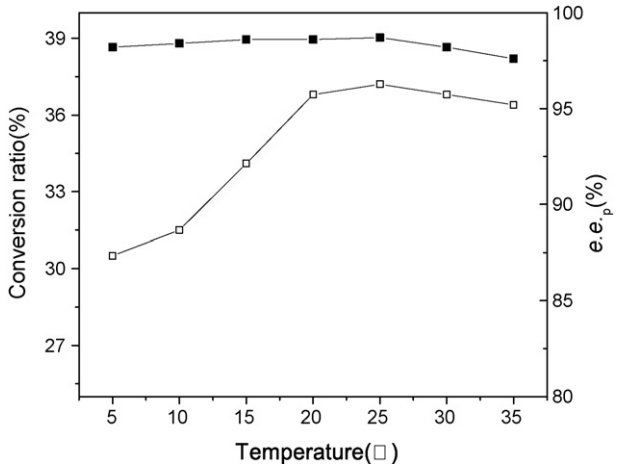

Fig. 2. Effect of temperature on the conversion ratio (□) and e.e.$_p$ (■) of enantioselective lactonization of **2**. Reactions were carried out in dichloromethane with microbial cells of *A. oryzae* WZ007 lyophilized at different temperature for 24 h.

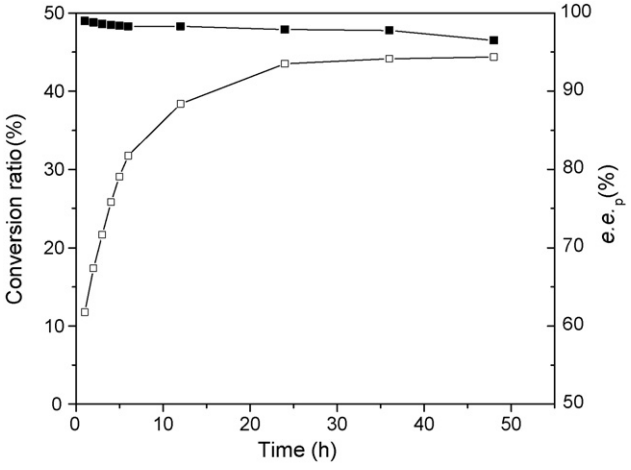

Fig. 3. Time course of the conversion ratio (□) and e.e.$_p$ (■) in enzyme-catalyzed lactonization. Reactions were carried out in dichloromethane with lyophilized microbial cells of *A. oryzae* WZ007 lyophilized at 25 °C for 24 h.

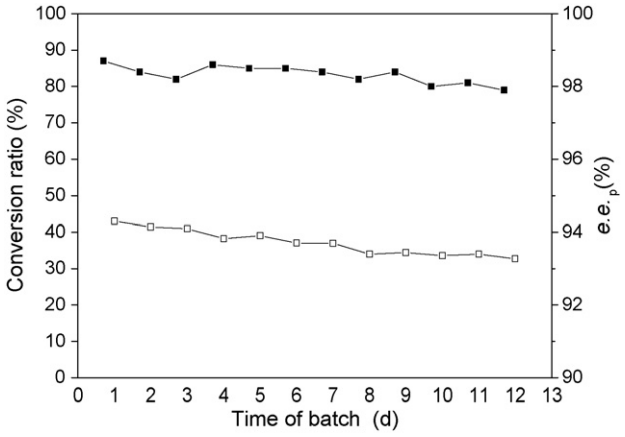

Fig. 4. Time course of enzyme-catalyzed lactonization in conversion ratio (□) and e.e.$_p$ (■). Reactions were carried out in dichloromethane with lyophilized microbial cells of *A. oryzae* WZ007 lyophilized at 25 °C for 24 h.

3.1.4. Effect of water activity on enzyme-catalyzed lactonization

In the enzyme-catalyzed lactonization system, water content plays key role as it influences dramatically the activity of the enzyme and the equilibrium of reaction course. As generally known to us, a_w describes accurately the effect of water in organic solvents. Many methods have been used to control a_w in organic media. Most of these methods are based on the fact that some salts have a fixed hydration state which yields a well-defined a_w for the saturated salt solution [20,21]. Such a saturate salt solution can be used to adjust a_w not only initially, but also during the reaction in which water is produced or consumed. Direct addition of saturated salt solution or salt hydrates pair is a simple and convenient way of 'buffering' optimal water conditions.

Table 2 demonstrated that a_w had great effects on the enzyme-catalyzed lactonization. As a_w ranging from 0.75 to 0.84, it had relative good conversion rate and enantioselectivity. The best result in the experiments was achieved by addition of the saturated potassium chloride solution and adjusting a_w of 0.84.

3.2. Time course of enzyme-catalyzed lactonization

In the preceding optimized reaction conditions, the time course of enzyme-catalyzed lactonization of **2** was shown in Fig. 3. Within 5 h, the conversion rate rapidly reached 25% with an e.e.$_p$ of 99.4%. Along with the reaction time increased, the conversion rate was gradually elevated but e.e. of produced lactone fell in some sort. At the reaction time of 24 h, the conversion ratio reached 41.5% and e.e. of produced lactone was 98.4%. After 24 h, the substrate conversion rate slowed down and the reaction probably had reached the equilibrium already.

3.3. The reusability of biocatalysts

Due to its insolubility in organic solvents, the whole cell biocatalysts can be very easily recovered for reuse. In order to improve the cost efficiency, microbial cells of *A. oryzae* WZ007 catalyzed enantioselective lactonization of **2** repeatedly by simple filtration and washing. The data in Fig. 4 showed that the lipase in microbial cells of *A. oryzae* WZ007 was highly stable and it retained about 75% of its original activity after 12 days. Furthermore, e.e. of produced lactone did not decrease significantly and kept in level of approximate 98% throughout 12 batches. The results indicated that resolution of chiral biotin lactone by the microbial cells of *A. oryzae* WZ007 could be applied in industrial production.

4. Conclusion

In summary, we developed an economical and convenient biocatalysis route to prepare optically active lactone **1** which is a key D-biotin intermediate. During the enzyme catalyzed reaction, dry microbial cells of *A. oryzae* WZ007 could act as an efficient biocatalyst with high activity and stability, particularly high enantioselectivity, which was crucial to the success of the asymmetric synthesis. The biocatalysis process also indicated its wide application in stereoselective resolution of the other chiral lactone.

Acknowledgements

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