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# Effects of hydroxypropyl-β-cyclodextrin on steroids 1-en-dehydrogenation biotransformation by *Arthrobacter simplex* TCCC 11037

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ABSTRACT

Cyclodextrins (CDs) have been shown previously to improve the steroids biotransformation. In this study, the biotransformation of cortisone acetate (CA) to prednisone acetate (PA) by *Arthrobacter simplex* TCCC 11037 (ASP) was selected as a model system for evaluating the effects of hydroxypropyl-β-cyclodextrin (HP-β-CD) on steroids 1-en-dehydrogenation process. By comparing the kinetic characteristics of CA dehydrogenation in HP-β-CD free or containing medium it was confirmed that this reaction followed the kinetics of produce inhibition, and the low solubility of CA was the limiting step of this bioconversion process at high substrate concentrations. However, the inclusion complex induced by HP-β-CD could overcome this problem. At 32 °C, the solubility of CA and PA in 45 mg mL$^{-1}$ HP-β-CD containing medium expressed 36.7- and 19.8-fold higher than that in HP-β-CD free medium and the product inhibition could be effectively alleviated by HP-β-CD. The favorable biocompatibility between HP-β-CD and *A. simplex* $\Delta^1$-dehydrogenase was also an unnegligible contribution to the enhancement of this biotransformation.


## 1. Introduction

1-en-Dehydrogenation of steroids catalyzed by *Arthrobacter simple* is known to be the key process in the synthesis of anti-inflammatory and anti-allergy glucocorticoid agents [1]. As a rule, the dehydro derivatives have better physiological activity than their precursors, at the same time, have fewer side effects. However, the steroids involved in bioconversions have a remarkable hydrophobic nature, which results in a poor bioavailability of substrate in bioconversion process [2]. As a result, a lot of efforts have been devoted to improve the steroids accessibility to biocatalysts. Methods such as addition of the water-soluble organic co-solvent [3] or surfactant [4] and organic–aqueous biphasic system [5] are wildly developed, but there is also a main disadvantage the organic solvent toxicity on the activity and stability of biocatalyst [6]. In whole-cell system, the organic solvent might lead to the disruption of membrane, denaturation of membrane bound enzymes, even the cytolysis [7]. Therefore, the key issue for steroids 1-en-dehydrogenation is to provide a proper concentration of substrate, which is available for catalytic cell. This is also the key problem in hydrophobic compounds biotransformation.

Cyclodextrins (CDs) are known to form inclusion complexes with many types of organic molecules for their intermolecular hydrophobic cone-like cavity. They have received considerable attention in pharmaceutical application because of its effective role in improving water solubility, chemical stability and bioavailability of various drugs through the inclusion complexes formation [8]. Additionally, CDs is inert to microorganisms and has a benefit to respiratory-chain activity [9]. This useful combination could prompt their use in microbial transformations of hydrophobic compounds, as shown by Bar and co-workers [10–12] and Roglič and Plazl [13]. HP-β-CD is a common and typical kind of β-CD derivates. It not only maintains β-CD's inclusion complex property but also improves the solubility of β-CD; furthermore, it has a relatively low price. HP-β-CD thus has a practical application prospect. Up to now, the researches on the enhancement of steroids 1-en-dehydrogenation by HP-β-CD are very limited, and the present explanation for the enhancement mechanism induced by CDs is mainly attributed to the improvement of the steroids solubility, which is unilateral.

In present work, the bioconversion of CA to PA by *Arthrobacter simplex* TCCC 11037 (Fig. 1) was selected as a model for evaluating the influences of HP-β-CD on steroids 1-en-dehydrogenation reaction. During the experiment, the kinetic characteristics of CA dehydrogenation in presence or absence of HP-β-CD were

*Abbreviations:* CA, cortisone acetate; PA, prednisone acetate; CDs, cyclodextrins; HP-β-CD, hydroxypropyl-β-cyclodextrin; *ASP*, *Arthrobacter simplex* TCCC 11037; $R_a$, initial reaction rate for incubated cell (mg mL$^{-1}$ h$^{-1}$); $R_0$, initial reaction rate for fresh cell (mg mL$^{-1}$ h$^{-1}$); $K_S$, stability constant of the complex; $S_0$, initial CA concentration; $P_0$, initial product concentration; $V'$, initial reaction rate in addition of PA; $V_0$, initial reaction rate without additional PA; E, cell concentration; $T_m$, measured time; $T_c$, calculated time.

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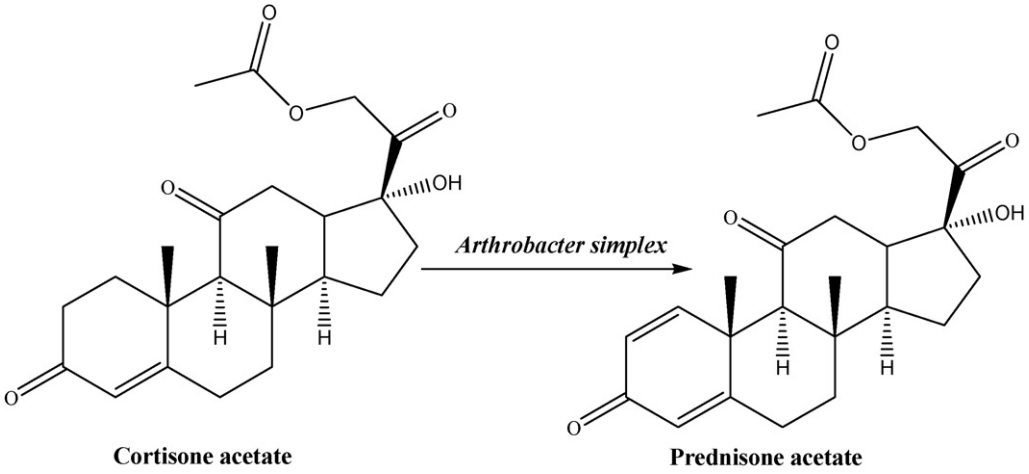


**Fig. 1.** $C_{1,2}$-dehydrogenation of cortisone acetate catalyzed by *Arthrobacter simplex*.

investigated. Some parameters relating to the kinetics were determined, such as $K_m$ and $V_{max}$. The influences of HP-β-CD on $\Delta^1$-dehydrogenase stability, steroids solubility, especially the alleviation of product inhibition were investigated systematically.

## 2. Materials and methods

### 2.1. Microorganism and medium

*A. simplex* TCCC 11037 stored in our laboratory was maintained at 32 °C on slant consisting of glucose 10 g $L^{-1}$, yeast extract 10 g $L^{-1}$ and agar 20 g $L^{-1}$ (pH 7.5). Seed medium consists of glucose 10 g $L^{-1}$, corn slurry 10 g $L^{-1}$, peptone 5 g $L^{-1}$ and $KH_2PO_4$ 2.5 g $L^{-1}$ (pH 7.2). Substrate (CA) was supplied by Tianjin Pharmaceutical Company (99.4% purity). Standard CA and PA were purchased from Sigma–Aldrich Co., HP-β-CD from Xi'an Deli Biology & Chemical Industry Co. Ltd (31.7% degree of substitution, 1523 average relative molecular mass).

### 2.2. Cultivation and preparation of biocatalyst

The *ASP* cells were prepared in two consecutive cultivation steps (18 h for seed culture and 27 h for cell cultivation, respectively) in shake flasks. The whole *ASP* cells grew in 500 mL shake flasks containing 100 mL culture media using 5% (v/v) of seed culture on a rotary shaker (160 rpm) at 32 °C. 0.01% (m/v) CA used as an inductor of $\Delta^1$-dehydrogenase was added into cell cultivation medium after 16 h growth. The cells were centrifuged at 4500 × *g* for 15 min, and washed with 0.05 mol $L^{-1}$ $KH_2PO_4$–NaOH buffers two times (pH 7.2). The buffers used in this experiment were all the same. Washed cells were resuspended in buffer and kept cell concentration (*E*) at 2.0 mg dry weight $mL^{-1}$.

### 2.3. Steroid transformation

Thirty millilitres of cell suspension was added into a 250 mL shake flask and pre-incubated at 32 °C, 180 rpm for 10 min to keep temperature equilibration. Then a described content of CA and HP-β-CD was added. 100 μL samples were drawn at various time intervals, while continuously being shaken.

### 2.4. Steroid analysis

The samples were withdrawn and extracted by chloroform, and dried in vacuum then the solid extracts were redissolved in eluent (dichloromethane:ether:methanol, 86:12:2, v/v/v) and filtered through a 0.2 μm filter. It was assayed by HPLC (Agilent 1100, USA), measuring absorbance at 240 nm. Analysis was performed on a Kromasil 100-5SIL, 250 × 4.6 mm column with a flow rate of 0.8 mL $min^{-1}$. The concentration of CA and PA were determined from calibration curves, making from eluent solutions of standard CA and PA.

### 2.5. Effect of HP-β-CD on biocatalytic stability

Equivalent whole *ASP* cells were incubated for different times in phosphate buffer in presence or absence of HP-β-CD (45 mg $mL^{-1}$). Subsequently, the HP-β-CD was removed by centrifugation (4500 × *g*, 15 min). After washing the cells twice with phosphate buffer, they were resuspended in phosphate buffer to a 2.0 mg dry weight $mL^{-1}$. Then the remaining enzymatic activity assay was determined by substrate conversion method ($S_0$ = 0.5 mg $mL^{-1}$), and expressed in terms of relative activity that was obtained from the specific value of $R_a$ (initial reaction rate for incubated cell (mg $mL^{-1}$ $h^{-1}$)) and $R_0$ (initial reaction rate for fresh cell (mg $mL^{-1}$ $h^{-1}$)). To ensure that the remaining activity was measured in initial stage of the reaction, the conversion ratios at different times were investigated preliminary. The results showed that a linear increase of CA conversion ratio was observed with time extended, when CA conversion ratio was no higher than 40%. The initial reaction rate was estimated from the slope of conversion curve when CA conversion ratio was no higher than 30%.

### 2.6. Effect of HP-β-CD on steroids solubility

Phosphate buffer and various concentrations of HP-β-CD were added to a final volume of 30 mL in 250 mL shake flasks. After adding an excess of CA or PA, the shake flasks were incubated under the condition identical to those employed in bioconversion (32 °C, 180 rpm). The flasks were tightly sealed to avoid evaporation. After 24 h, 1 mL aliquot of the slurry was withdrawn and filtered through a 0.2 μm filter. The filtrate was dried in vacuum and redissolved in eluent, and then analyzed by HPLC.

## 3. Result and discussion

### 3.1. Effect of HP-β-CD on process of dehydrogenation

As shown in a previous research [14], the use of HP-β-CD facilitated the steroids biotransformation process conspicuously. Process curves for different HP-β-CD concentrations were shown in Fig. 2. CA conversion ratios were 50% higher after 2 h conversion and 90%

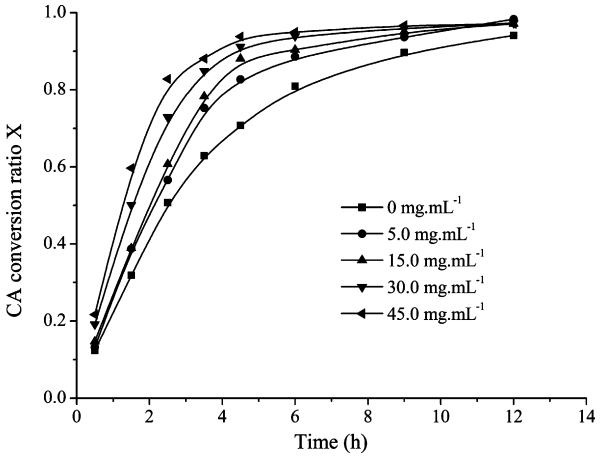


**Fig. 2.** Effects of HP-β-CD concentrations on the CA bioconversion process ($S_0$ = 10.0 mg mL$^{-1}$, $E$ = 2.0 mg mL$^{-1}$).

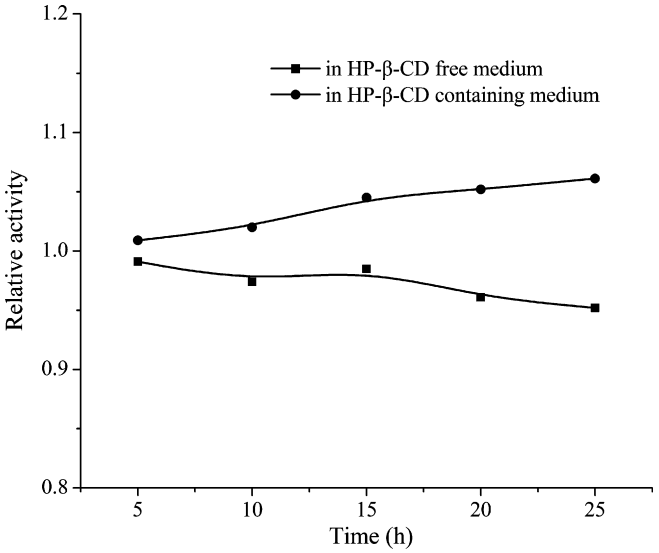


**Fig. 3.** Stability investigation on $\Delta^1$-dehydrogenase from *A. simplex* TCCC 11037.

higher after 7 h in presence of HP-β-CD, which were obviously higher than that of control group. All the final CA conversion ratios in HP-β-CD containing medium could reach 97% or higher, while it was below 95% in HP-β-CD free medium after a longer biotransformation period. The initial reaction rates also gradually increased with the increasing content of HP-β-CD. The time for reaching final conversion ratios, 6, 9, 11, 12 and 20 h, were determined for HP-β-CD 45, 30, 15, 5 and 0 mg mL$^{-1}$, respectively. The reasons for this enhancement induced by HP-β-CD could be explained from following aspects.

### 3.2. Effect of HP-β-CD on biocatalytic stability

Biocatalytic stability is an important parameter for the biocatalyst. However, the lost of biocatalytic stability is generally inevitable for the introduction of organic co-solvent. For evaluating the effect of HP-β-CD on biocatalytic stability, the *ASP* $\Delta^1$-dehydrogenase activity stability in HP-β-CD medium was investigated and the results were presented in Fig. 3. Evidently, the *ASP* $\Delta^1$-dehydrogenase activity kept stable in phosphate buffer and no noteworthy decrease in activity was observed after 24 h of storage under the condition the same as CA 1-en-dehydrogenation bioconversion. Interestingly, 45 mg mL$^{-1}$ HP-β-CD did not do any harm to the $\Delta^1$-dehydrogenase activity stability but exhibited a slight enhancement of catalytic activity, contrary to general organic co-solvent. Therefore, HP-β-CD had a perfect biocompatibility with *ASP* $\Delta^1$-dehydrogenase. The reason for the slight enhancement of activity observed in this experiment could be ascribed to the interaction between CDs and cell membrane or cell wall by virtue of either their complexing ability and/or surface activity as mentioned by Donova et al. [15]. These properties of CDs could result in the facilitation of outer-surface lipid and protein leakage from the cell wall, which might slightly promote the steroids membrane permeability.

### 3.3. Effect of HP-β-CD on steroids solubility

Since this biotransformation involves water-insoluble substrate CA and $O_2$ (final acceptor of electrons and hydrogen atoms), the rate of CA 1-en-dehydrogenation in HP-β-CD free or containing medium may be not determined by the intrinsic kinetics of the bioconversion, but by dissolution rate of either the crystalline steroids or gaseous $O_2$. The maximal CA 1-en-dehydrogenation

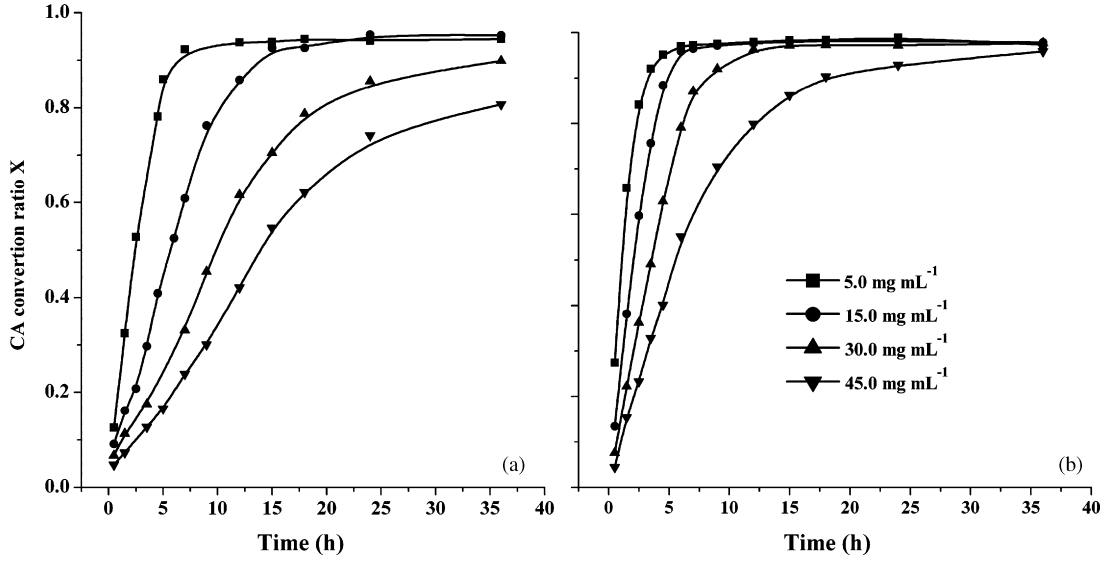


**Fig. 4.** Effect of initial substrate concentrations on conversion process (a) in HP-β-CD free medium and (b) in HP-β-CD containing medium ($C_{HP\text{-}\beta\text{-}CD}$ = 45 mg mL$^{-1}$).

**Table 1**
Solubility of CA and PA in different concentrations of HP-β-CD solution.

| HP-β-CD (mg mL$^{-1}$) | CA (mg mL$^{-1}$) | PA (mg mL$^{-1}$) |
|---|---|---|
| 0 | 0.039 | 0.048 |
| 5 | 0.107 | 0.098 |
| 15 | 0.241 | 0.201 |
| 30 | 0.831 | 0.614 |
| 45 | 1.47 | 1.00 |
| $K_S$ (M$^{-1}$) | 1890 | 945 |

32 °C in 0.05 mol L$^{-1}$ $KH_2PO_4$–NaOH phosphate buffer.

rate obtained from this experiment was 4.7 mg mL$^{-1}$ h$^{-1}$, as shown in Fig. 4, and this would require an equivalent $O_2$ transfer rate of 0.187 mg mL$^{-1}$ h$^{-1}$. This low demand could be easily satisfied by the 250 mL shake flask under the CA transformation condition (described in Section 2.3) [16]. Therefore, the limiting step of this biotransformation process could be safely attributed to the substrate solubility and dissolution rate, which was in according with previous steroids biotransformation researches [17,18].

The solubilities of CA and PA in different concentrations of HP-β-CD medium were investigated at 32 °C the same as bioconversion temperature. The results were list in Table 1. It was clearly seen that the solubilities of CA and PA were enhanced with the increase of HP-β-CD, while the solubilization effect on CA was more obvious than that of PA. This difference mediated by HP-β-CD could be explained by the stability constant of the complex ($K_S$), which was determined by the phase-solubility technique [19] using Eq. (1). As shown in Table 1, a higher $K_S$ value was obtained by CA, which indicated the complex [CA-(HP-β-CD)] was more stable than the complex [PA-(HP-β-CD)]. In other words, HP-β-CD could form complexes with CA preferentially:

$$K_s = \frac{tg\alpha}{S0\,(1 - tg\alpha)} \quad (1)$$

where $S_0$ is the solubility of substrate in phosphate buffer without HP-β-CD, and $\alpha$ is an inclination angle of the phase solubility diagram for steroid in the HP-β-CD containing phosphate buffer.

### 3.4. Kinetics of CA 1-en-dehydrogenation

#### 3.4.1. Effect of substrate concentration on initial reaction rate

Substrate inhibition is commonly observed during steroids biotransformation process, which is generally caused by the effect of substrate on respiration chain not due to the interaction with $\Delta^1$-dehydrogenase by the hindering of electron transfer along the respiratory chain. Moreover, 6-methly steroids derivatives were demonstrated to have a more significant inhibition effect on respiratory activity than its precursors [20]. Compared with Fig. 4(a) and (b) shows a significant final conversion ratio improvement, especially when the $S_0$ was 30.0 and 45.0 mg mL$^{-1}$. Fig. 4 represents the kinetic curves of CA biotransformation by *ASP* whole cells. In this experiment, the initial CA concentrations ($S_0$) were ranged from 5.0 to 45.0 mg mL$^{-1}$ that is much higher than CA solubility either in HP-β-CD free or containing medium. By calculating the initial reaction rate from the slop of kinetic curves, Fig. 5 presents dependence between initial reaction rates and substrate concentrations in both reaction systems. The features of this dependence suggested the kinetics of substrate inhibition did not exist at first sight. However, we should note that at high concentrations of CA, it was difficult to determine the actual concentration of free CA in transformation medium. We assumed that the kinetics of substrate inhibition might be manifested more clearly with the correspondence of introduced and actual CA concentrations in the transformation medium. Moreover, it was certain that HP-β-CD enhanced CA solubility, which significantly accelerated the initial reaction rate and shorten the time for reaching the highest CA conversion ratio especially under the high concentration of substrate. This was similar to the results presented in Manosroi et al.'s research [18].

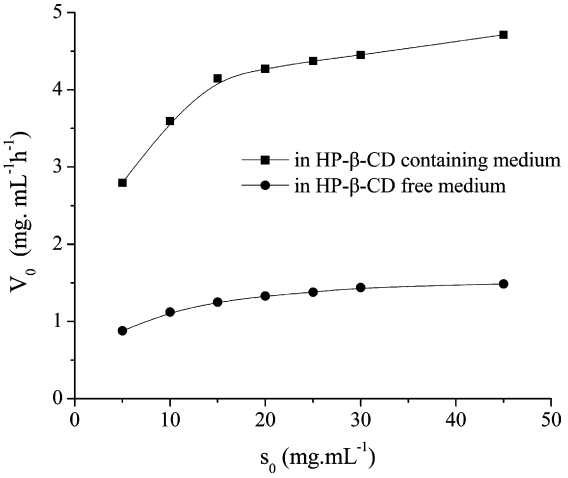


**Fig. 5.** Relation between initial substrate concentration and initial reaction rate in HP-β-CD free or containing medium ($C_{HP\text{-}\beta\text{-}CD}$ = 45 mg mL$^{-1}$).

The relationship between initial reaction rate and CA concentration was linear when graphed on a Linweaver–Burk plot. $K_m$ and $V_{max}$ values were listed in Table 2. The $K_m$ values in HP-β-CD containing or free medium were 4.19 and 4.24 mg mL$^{-1}$, respectively. It was approximate to that provided by Constantides [21]. Since the *ASP* $\Delta^1$-dehydrogenase was a kind of intracellular enzyme located on the cell membrane [22], HP-β-CD could not access it. The slight variation of $K_m$ could be ascribed to the pH or ionic strength changes of buffer properties that resulted from HP-β-CD introduction, as mentioned by Yeates and Krieg [23], but not due to the complex with enzyme or surface activity. Table 2 also suggested a notable variation of $V_{max}$. It was not consistent with Sergey et al.'s research [24] that the kinetic parameters for degradation of the free steroid in the presence and absence of methylated β-cyclodextrin did not differ. Nevertheless, compared to the high initial substrate concentrations in this experiment, it must take account of the extremely low concentrations of free steroid in Sergey et al.'s experiment. Thus, it indicated that HP-β-CD could be used to overcome constraints of substrate dissolution by acting as reservoirs for accessible substrate through its complexation. The exchange rate of the substrate between its free and complexed forms was fast and might not conspicuously retard the digestion of the substrate by the enzyme. Meanwhile, it was clear to see that the d$x$/d$t$ decreased when the CA conversion ratio was about 50% (Fig. 4(a)), but in presence of HP-β-CD (Fig. 4(b)) it did not decrease until the CA conversion ratio reached 75% or higher, especially at high substrate concentrations (30.0 or 45.0 mg mL$^{-1}$). It appeared reasonable to assume that HP-β-CD could alleviate the product inhibition in some extent.

#### 3.4.2. Effect of product inhibition

In order to reveal whether the product inhibition exists in this dehydrogenation process, initial product concentration ($P_0$) ranging from 0% to 40% of the substrate was added accompanying with the equivalent substrate. Fig. 6 represents the specific value of $V'$ (initial reaction rate in addition of PA) and $V_0$ (initial reac-

**Table 2**
Values of $K_m$ and $V_{max}$ in HP-β-CD free or containing medium.

| | $K_m$ (mg mL$^{-1}$) | $V_{max}$ (mg mL$^{-1}$ h$^{-1}$) | $R^2$ |
|---|---|---|---|
| 0.05 mol L$^{-1}$ phosphate buffer | 4.24 | 1.62 | 0.998 |
| 45 mg mL$^{-1}$ HP-β-CD medium | 4.19 | 5.14 | 0.994 |

32 °C in 0.05 mol L$^{-1}$ $KH_2PO_4$–NaOH phosphate buffer, pH 7.2, $E$ = 2.0 mg mL$^{-1}$.

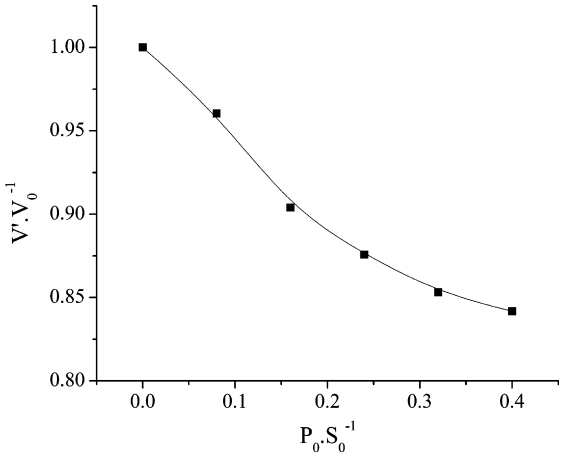


**Fig. 6.** Effect of initial product concentrations on initial reaction rates in HP-β-CD free medium ($S_0$ = 5.0 mg mL$^{-1}$).

tion rate without additional PA) was decrease with the increase of PA content, and Fig. 7 also shows an obvious decrease in d$x$/d$t$ when the CA conversion ratio was higher than 40% in HP-β-CD free medium. Both of them could be interpreted by the product inhibition. However, a Michaelis–Menten-like curve was exhibited in HP-β-CD medium in Fig. 7, and the d$x$/d$t$ did not decrease until the CA conversion ratio was higher than 75–80%. This illustrated that the introduction of HP-β-CD effectively alleviated the production inhibition.

Interestingly, when the experimental data in Fig. 4(b) were substituted into integral formula of the Michaelis–Menten Eq. (2), every group basically corresponded with the typical Michaelis–Menten kinetics. The calculated data and the experimental data were listed in Table 3. Taking $S_0$ = 30 mg mL$^{-1}$ group as an example, all error values between measured times ($T_m$) and calculated times ($T_c$) were no more than 4.00% even when CA conversion ratio reached about 80%. Therefore, it was reasonable to conclude that HP-β-CD could alleviate the product inhibition and the process in HP-β-CD containing medium was determined by its intrinsic kinetics. This could be ascribed to HP-β-CD preferentially forming inclusion complexes with CA. Because CA molecular was more hydrophobic and had a higher $K_s$ value, it could combine with most of HP-β-CD molecular by occupying the hydrophobic cavity; while most of production PA amassed in the transformation process was crystallized and precipitated. For this reason, the dissolved CA concentration always kept much higher than that of PA until the total CA content was far below

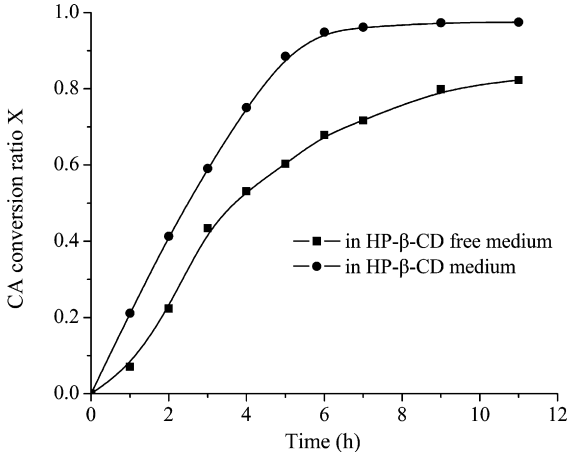


**Fig. 7.** Effect of HP-β-CD on the product inhibition in conversion process ($S_0$ = 5.0 mg mL$^{-1}$, $P_0$ = 2.0 mg mL$^{-1}$, $C_{HP\text{-}\beta\text{-}CD}$ = 45 mg mL$^{-1}$).

**Table 3**
Comparison of calculated data with experimental data.

| CA conversion ratio $X$ | $T_m$ (h) | $T_c$ (h) |
|---|---|---|
| 0.076 | 0.50 | 0.51 |
| 0.277 | 1.50 | 1.50 |
| 0.308 | 2.00 | 2.08 |
| 0.447 | 3.00 | 3.09 |
| 0.539 | 4.00 | 3.78 |
| 0.685 | 5.00 | 4.94 |
| 0.791 | 6.00 | 5.89 |
| 0.920 | 9.00 | 7.43 |

$S_0$ = 30 mg mL$^{-1}$, $P_0$ = 0 mg mL$^{-1}$, $E$ = 2.0 mg mL$^{-1}$.

the amassed PA. As a result, HP-β-CD could effectively alleviate the product inhibition. We also assumed there was a quantitative relation between the additional HP-β-CD content and the extent in the alleviation of product inhibition. It would be investigated in further research:

$$V_{max}t = S_0X_S + K_m \ln\frac{1}{1-X_S} \quad X_S = \frac{S_0 - S_T}{S_0} \tag{2}$$

where $V_{max}$ = 5.14 mg mL$^{-1}$, $K_m$ = 4.19 mg mL$^{-1}$ h$^{-1}$, $S_0$ means the initial CA concentration, $S_T$ means the CA concentration in bioconversion process.

## 4. Conclusion

As is shown above, HP-β-CD enhances the 1-en-dehydrogenation of CA catalyzed by *A. simplex* TCCC 11037. It not only increases the reaction rate but also improves the final substrate conversion ratio. HP-β-CD has no affect on the biocatalytic stability, contrarily, it exhibits a good biocompatibility with microorganisms. It is also an effective solublizer or reservoirs for accessible substrates. Solubility studies demonstrate that the solubilities of CA and PA increased 37.6 and 21.9 times, respectively, by complexation with HP-β-CD at 32 °C. By comparing the kinetics characteristics in presence and absence of HP-β-CD, the reaction rate is determined by the dissolution process of the insoluble CA in the HP-β-CD free medium, while in HP-β-CD containing medium, it is determined by the intrinsic kinetics. Furthermore, product inhibition significantly influences the reaction of CA 1-en-dehydrogenation especially at high substrate concentrations. It is lucky that HP-β-CD could alleviate the product inhibition. Hence, HP-β-CD provides a potential resolution to the key problem in hydrophobic compounds biotransformation. Considering the inertness of HP-β-CD to the microorganisms, it could be cyclically utilized. This may be especially useful for the continuous process. In summary, this work not only provides a systematic study on the influences of HP-β-CD on the CA 1-en-dehydrogenation reaction, but also suggests an applicable and effective method for enhancing enzyme-catalyzed steroids biotransformation.

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## References

[1] P. Fernandes, A. Cruz, B. Angelova, H.M. Pinheiro, J.S.M. Cabral, Enzyme Microb. Technol. 32 (2003) 688–705.
[2] D.L. Alexander, J.F. Fisher, Steroids 60 (1995) 290–294.
[3] C.H. Wong, Science 244 (1989) 1145–1152.
[4] M. Smith, J. Zahnley, D. Pfeifer, D. Goff, Appl. Environ. Microbol. 59 (1993) 1425–1429.
[5] S.T. Bie, F.P. Lu, L.X. Du, Q. Qiu, Y. Zhang, J. Mol. Catal. B: Enzym. 55 (2008) 1–5.

[6] A. Cruz, P. Fernandes, J.M.S. Cabral, H.M. Pinheiro, J. Mol. Catal. B: Enzym. 19 (2002) 371–375.
[7] A. Cruz, P. Fernandes, J.M.S. Cabral, H.M. Pinheiro, J. Mol. Catal. B: Enzym. 11 (2001) 579–585.
[8] M.E. Brewster, T. Loftsson, Adv. Drug Deliv. Rev. 59 (2007) 645–666.
[9] V.V. Fokina, A.V. Karpov, I.A. Sidorov, V.A. Andrjushina, A.Y. Arinbasarova, Appl. Microbiol. Biotechnol. 47 (1997) 645–649.
[10] Y. Singer, H. Shity, R. Bar, Appl. Microbiol. Biotechnol. 35 (1991) 731–737.
[11] J. Jadoun, R. Bar, Appl. Microbiol. Biotechnol. 40 (1993) 230–240.
[12] J. Jadoun, R. Bar, Appl. Microbial. Biotechnol. 40 (1993) 477–482.
[13] U. Roglič, I. Plazl, P. Plazl, Biocatal. Biotransform. 25 (2007) 16–23.
[14] J.B. Harper, C.J. Eastona, S.F. Lincolnb, Curr. Org. Chem. 4 (2000) 429–454.
[15] M.V. Donova, V.M. Nikolayeva, D.V. Dovbnya, S.A. Gulevskaya, N.E. Suzina, Microbiology 153 (2007) 1981–1992.
[16] F. Mantzouridou, T. Roukas, B. Achatz, Enzyme Microb. Technol. 37 (2005) 687–694.
[17] U. Roglič, P. Plazl, I. Plazl, Biocatal. Biotransform. 23 (2005) 299–305.
[18] A. Manosroi, S. Saowakhon, J. Manosroi, J. Steroid Biochem. Mol. Biol. 108 (2008) 132–136.
[19] J. Szejtli, Cyclodextrin Technology, Kluwer, Dordrecht, 1988, pp. 188–190.
[20] A.Y. Arinbasarova, A.V. Karpov, V.V. Fokina, A.G. Medentsev, K.A. Koshcheyenko, Enzyme Microb. Technol. 19 (1996) 501–506.
[21] A. Constantinides, Biotechnol. Bioeng. 22 (1980) 119–136.
[22] I. Molnar, K.P. Choi, M. Yamashita, Mol. Microbiol. 15 (1995) 895–905.
[23] C.A. Yeates, H.M. Krieg, Enzyme Microb. Technol. 40 (2007) 228–235.
[24] M. Sergey, M.S. Khomutov, G.V. Sukhodolskaya, M.V. Donova, Biocatal. Biotransform. 25 (2007) 386–392.