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# Optimization of biotransformation from phytosterol to androstenedione by a mutant *Mycobacterium neoaurum* ZJUVN-08*

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**Abstract:** Biotransformation of phytosterol (PS) by a newly isolated mutant *Mycobacterium neoaurum* ZJUVN-08 to produce androstenedione has been investigated in this paper. The parameters of the biotransformation process were optimized using fractional factorial design and response surface methodology. Androstenedione was the sole product in the fermentation broth catalyzed by the mutant *M. neoaurum* ZJUVN-08 strain. Results showed that molar ratio of hydroxypropyl-β-cyclodextrin (HP-β-CD) to PS and substrate concentrations were the two most significant factors affecting androstenedione production. By analyzing the statistical model of three-dimensional surface plot, the optimal process conditions were observed at 0.1 g/L inducer, pH 7.0, molar ratio of HP-β-CD to PS 1.92:1, 8.98 g/L PS, and at 120 h of incubation time. Under these conditions, the maximum androstenedione yield was 5.96 g/L and nearly the same with the non-optimized (5.99 g/L), while the maximum PS conversion rate was 94.69% which increased by 10.66% compared with the non-optimized (84.03%). The predicted optimum conditions from the mathematical model were in agreement with the verification experimental results. It is considered that response surface methodology was a powerful and efficient method to optimize the parameters of PS biotransformation process.

**Key words:** Phytosterol, *Mycobacterium neoaurum* ZJUVN-08, Androstenedione, Hydroxypropyl-β-cyclodextrin
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## 1 Introduction

As two important pharmaceutical steroid precursors, androst-4-ene-3,17-dione (AD) and androsta-1,4-diene-3,17-dione (ADD), which belong to 17-ketosteroid family can be further used to produce a wide range of pharmaceutical steroid derivatives (Fernandes *et al*., 2003). Being an alternative to chemical methods, bioconversion of steroids has been studied extensively (Malaviya and Gomes, 2008). Selective side chain degradation of sterols to 17-ketosteroids is one of the most widely used biotransformation reactions of steroids. It is well-known that phytosterols (PSs) are suitable raw materials for microbial degradation to 17-ketosteroids because of low cost and easy availability (Fernandes and Cabral, 2007). In recent years, microbial selective cleavages of PS to AD and ADD have been reported by many researchers (Huang *et al*., 2006; Pérez *et al*., 2006; Sripalakit *et al*., 2006; Wang *et al*., 2006). All things considered, the major obstacle of microbial transformation of PS is the low aqueous solubility (1 μmol/L) of substrate (Malaviya and Gomes, 2008). Consequently, many efforts have been devoted to overcoming the low solubility of sterols and improving product yield. These include the application of various water-miscible organic solvents (Simon *et al*., 1998), water-immiscible organic solvents (Cruz *et al*., 2001), silicone oil (Kutney *et al*., 2000; Stefanov

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*et al*., 2006), polypropylene glycol (Kutney *et al*., 2000; Stefanov *et al*., 2006), cloud point system (Wang *et al*., 2004; 2006), and microemulsions (Stefan *et al*., 2002). However, organic solvents present many drawbacks such as toxicity to microbial cells and environmental hazards (Schimid *et al*., 1998; Kim *et al*., 2007). Thus, the main focus of steroid biotransformation is to increase the solubility of hydrophobic substrates as well as retain the activity and stability of microbial cells.

Cyclodextrins (CDs) are cyclic oligosaccharides with 6–8 glucopyranose units bonded by α-1,4-linkage and have a structure that conveys a hydrophobic central cavity in the molecule (Nalluri *et al*., 2005). Based on this molecular structure, CDs have been widely used to form host-guest complexes with hydrophobic compounds in their cavities. By inclusion with CDs, the hydrophobic compounds are encapsulated in this CD cavity. The hydrophilic outer surface of the inclusion complex shows advantageous solubility and bioavailability, favoring the solubility of hydrophobic compounds in aqueous media (Szejtli, 1998). Thus, the aqueous solubility of hydrophobic compounds is subsequently improved (Loftsson and Brewster, 1996). As a result, CDs have been extensively used in the microbial transformation of steroids (Hesselink *et al*., 1989; Roglič *et al*., 2005; 2007). Hydroxypropyl-β-cyclodextrin (HP-β-CD) is one kind of chemically modified β-cyclodextrin derivate, which has higher water solubility and complexing ability than CD, and has been widely added in medium to improve steroid biotransformation (Manosroi *et al*., 2008; Ma *et al*., 2009; Zhang *et al*., 2009).

This work used response surface methodology (RSM) to optimize the biotransformation process of PSs. RSM is an efficient mathematical and statistical technique applied to investigate the relationship between several independent variables and the generated responses (Senanayake and Shahidi, 2002). The application of RSM contributes to the rapid screening of experimental factors and factors interactions, thus obtaining the optimal process parameters, which significantly reduces the cost of experimentation (Ghosh and Hallenbeck, 2010). Moreover, this method has been extensively used to optimize the operational conditions of bioconversion process (Goswami *et al*., 2009; Liu *et al*., 2010).

In recent years, several studies have reported that *Mycobacterium neoaurum* strain is able to degrade PS and yield AD (Rodina *et al*., 2008; Wei *et al*., 2010). In the preliminary study, an *M. neoaurum* strain (designated as *M. neoaurum* ZJUVN), which could degrade PS, was successfully isolated from soil. The strain was capable of converting PS to AD and ADD. Further, this strain was bred using low-energy nitrogen ion ($N^+$) implantation method for accumulation of AD as the only product. A genetically stable mutant, which could substantially accumulate AD as the sole product, *M. neoaurum* ZJUVN-08 was screened and used in this work. The pathway of PS side chain degradation to AD is shown in Fig. 1. In the present study, the influence of inducer concentration, substrate concentration, molar ratio of HP-β-CD to substrate, pH, and incubation time on AD yield and PS conversion was studied using RSM. The optimized PS bioconversion conditions by *M. neoaurum* ZJUVN-08 were obtained.

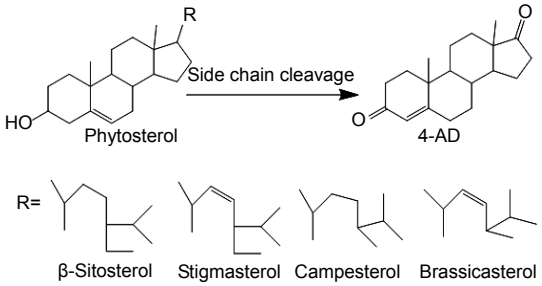


**Fig. 1 Side chain degradation of phytosterols by *M. neoaurum* ZJUVN-08**

## 2 Materials and methods

### 2.1 Materials

PS (95%) was obtained from Zhejiang DAVI Biochemistry Co., Ltd. (China). AD and ADD were obtained from Sigma (USA). HP-β-CD was obtained from Jiangsu Kunshan Chemical Industries Co., Ltd. (China). Methanol was of high performance liquid chromatography (HPCC) grade. All other chemicals and solvents in this work were of analytical grade.

### 2.2 Microorganisms

The soil samples were obtained from Taian suburb, Shandong, China. The strain *M. neoaurum* ZJUVN was isolated using gradient dilution of

soil samples. The genotypical and morphological identifications were performed by 16S ribosomal RNA (rRNA) sequence analysis and biochemical tests (Dogra and Qazi, 2001), respectively. The nucleotide sequence of this strain was submitted to GenBank and obtained the accession number JN935007. *M. neoaurum* ZJUVN-08 (CGMCC No. 5447) was evolved from *M. neoaurum* ZJUVN using a low-energy $N^+$ implantation method. The low-energy $N^+$ implantation was conducted with an ion beam bioengineering instrument at the Institute of Bioengineering, Zhejiang University of Technology, China. The energy of $N^+$ used in this work was 10 keV. The implantation doses ranged from $20\times2.6\times10^{13}$ to $180\times2.6\times10^{13}$ ions/cm$^2$, and were finally chosen at $100\times2.6\times10^{13}$ ions/cm$^2$. The mutation of the strain was carried out as described by Liu *et al*. (2007). After ion implantation treatment, samples were diluted by physiological sodium chloride solution, and then placed onto seed medium plates containing 1.0 g/L PS. Visible colonies were picked and further used to test PS degradation capabilities by thin layer chromatography (TLC) and HPLC. The selected mutant *M. neoaurum* ZJUVN-08 exhibited enhanced ability to produce AD and could accumulate AD as the sole product.

### 2.3 Culture medium

The stock culture was maintained on a medium which contained (g/L): yeast extract, 5.0; glycerol, 10.0; $MgSO_4\cdot7H_2O$, 0.5; $K_2HPO_4$, 0.5; $NH_4Cl$, 1.0; agar power, 20.0 (pH 7.0). The seed medium contained (g/L): glucose, 5.0; glycerol, 20.0; $NH_4NO_3$, 2.0; $MgSO_4\cdot7H_2O$, 0.5; citric acid, 2.0; $K_2HPO_4$, 0.5; ferric ammonium citrate, 0.05 (pH 7.0). Fermentation medium contained (g/L): glucose, 10.0; $K_2HPO_4$, 0.5; citrate acid, 2.0; $MgSO_4\cdot7H_2O$, 0.5; $(NH_4)_2HPO_4$, 3.5; ferric ammonium citrate, 0.05 (pH 7.0).

### 2.4 Biotransformation process

PS biotransformation by growing-cell culture was performed in two consecutive steps, namely, activation culture in seed medium and biotransformation culture in fermentation medium. The activation culture was grown in 250-ml shake flasks containing 50 ml seed medium at 30 °C, 200 r/min, and 48 h. PS was served as the inducer of bioconversion process, and was added into the seed medium with 0.1% Tween 80 after 24 h of incubation. After activation culture, 5 ml of seed culture liquid was extracted and transferred to 50 ml fermentation medium, then incubated in 250-ml shake flasks at 30 °C, 200 r/min, and 96–144 h. PS was added to the fermentation medium as an aqueous HP-β-CD solution, and sonicated for 15 min until complete homogenization before sterilization. As the optimization procedure was carried out, inducer concentration, substrate concentration, molar ratio of HP-β-CD/PS, pH value, and incubation time were varied to the designed conditions. Substrate controls and culture controls were grown under the same conditions. All experiments were carried out in triplicate.

### 2.5 Separation, purification, and structural elucidation of biotransformation products

At the end of fermentation period, 10 ml culture broth was taken from the flasks and extracted twice by vigorous shaking for 2 h with the same volume of ethyl acetate. Then, 5 μl samples were applied onto one TLC plate which was spread by silica gel GF254 (0.25 mm). The solvent system used for TLC was petroleum ether:ethyl acetate (6:4, v/v) and the compounds were visualized by iodine staining. Steroid products were observed as orange spots.

The products were isolated by preparative TLC (silica gel GF254, 1.0 mm). The spots obtained were scraped off and dissolved in methanol. After centrifugation, the products were further purified by preparative HPLC (Waters Sunfire $C_{18}$ column, 5 μm particles, and 250 mm×10 mm). The mobile phase was composed of methanol and water (80:20, v/v), flow rate was 5 ml/min, and detection wavelength was 254 nm.

The compounds obtained were identified by mass spectrum (MS), infrared spectrum (IR), and nuclear magnetic resonance (NMR) analyses. MS was performed using Waters Q-TOF micro MS (USA). IR spectra were obtained on Nicolet 6700 FT-TR spectrometer (USA). $^1H$ and $^{13}C$ NMR spectra were carried out on a Bruker AVIII500 MHz NMR spectrometer (Sweden) at 500 and 125 MHz, respectively. $CDCl_3$ was used as solvent.

### 2.6 Quantification of biotransformation product

Quantification of products was carried out using HPLC on Waters Symmetry $C_{18}$ column (5 μm particles, 250 mm×4.6 mm). The mobile phase was

composed of methanol and water (80:20, v/v), the flow rate was 1 ml/min and column temperature at 30 °C. The sample volumes injected were 10 μl and the detection wavelength was 254 nm.

Conversion rate of PS to AD was estimated using the following equation:

$$\mathrm{CR}=\frac{(m_{\mathrm{AD}}/\mathrm{MW}_{\mathrm{AD}})}{(m_{\mathrm{P}}/\mathrm{MW}_{\mathrm{P}})}\times 100\%, \quad (1)$$

where $m_{ad}$ and $m_p$ are the weights of AD and PS, respectively; $MW_{AD}$ and $MW_p$ are the molecular weights of AD and PS, respectively (Sripalakit *et al*., 2006).

### 2.7 Experimental design

In order to optimize the conditions of biotransformation for AD yield and PS conversion, a set of statistically designed experiments were conducted. First, fractional factorial design (FFD) was used to identify the significant factors affecting AD yield and PS conversion rate by *M. neoaurum* ZJUVN-08. Then central composite design (CCD) was utilized to optimize the most important factors for the maximum AD yield and PS conversion rate. A $2^{5-1}$ FFD which included 16 sets of trials with four replicates at center point was carried out in duplicate to screen the critical parameters influencing AD yield and PS conversion rate (Chen *et al*., 2002). The coded values of variables were determined by the following equation:

$$X_i=(x_i-x_0)/\Delta x_i, \quad (2)$$

where $X_i$ stands for the coded value, $x_i$ stands for the actual value, $x_0$ stands for the real value at the center point, and $\Delta x_i$ stands for the step change value (Chen *et al*., 2008).

The coded and actual values of variables are given in Table 1. The AD yield and PS conversion rate are considered as responses $Y_{AD}$ and $Y_{Conversion}$, respectively. After the analysis of FFD, a first-order model was gained. A five-level CCD was performed to fit the empirical quadratic polynomial model (Myers and Montgomery, 2002). The quadratic model equation was as follows:

$$y=b_0+\sum b_i x_i \sum b_{ii} x_i^2+\sum b_{ij} x_i x_j, \quad (3)$$

**Table 1 Coded and actual values of factors in FFD**

| $X$ | $x_1$ (g/L) | $x_2$ | $x_3$ | $x_4$ (g/L) | $x_5$ (h) |
|---|---|---|---|---|---|
| −1 | 0.05 | 6.5 | 1:1 | 5 | 96 |
| 0 | 0.10 | 7.0 | 2:1 | 10 | 120 |
| 1 | 0.15 | 7.5 | 3:1 | 15 | 144 |

$x_1$: inducer concentration; $x_2$: pH; $x_3$: molar ratio of HP-β-CD/PS; $x_4$: substrate concentration; $x_5$: incubation time; $X$: coded value

where $y$ is predicted response, $b_0$ is offset term, $b_i$ is linear offset, $b_{ii}$ is squared offset, $b_{ij}$ is interaction coefficient, $x_i$ and $x_j$ stand for independent variables (Chen *et al*., 2002).

The regression and graphical analysis of data were performed using Design Expert software (Version 6.0, Stat-Ease Inc., Minneapolis, USA).

### 2.8 Model verification and time-course experiment

Biotransformation process was performed by a procedure similar to normal transformation experiments using the optimized conditions under a 5-L fermenter (BIOF-2000, Shanghai Gaoji Bioengineering, China) containing 3 L fermentation medium. The pH was monitored by a pH probe (F-635-B120-DH, China) and maintained at 7.0 by addition of 5 mol/L NaOH. The fermenter was run at 30 °C, 350 r/min, and aeration rate of 1 vvm (air volume/culture volume/min).

After the seed was cultured for two days, PS was added into the transformation medium to start the conversion process. At a regular time interval, samples were taken to evaluate the yield of AD and PS conversion rate as described earlier, performed in triplicates.

## 3 Results

### 3.1 Bioconversion products of PS using *M. neoaurum* ZJUVN-08

As can be seen from Fig. 2, in bioconversion medium inoculated with parent *M. neoaurum* ZJUVN strain, PS was transformed into two products, the rate of flow ($R_f$) value were 0.31 and 0.48, respectively, which were in agreement with those of the authentic ADD and AD. There was only one product in the bioconversion medium inoculated with mutant *M. neoaurum* ZJUVN-08 strain. The $R_f$ value (0.48) and HPLC peak occurred at the retention time of 5.2 min,

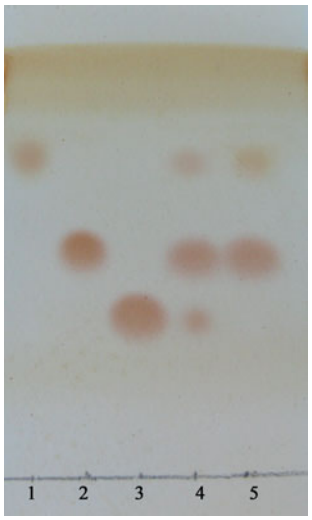


**Fig. 2 TLC of phytosterol biotransformation**
Lane 1: standard phytosterols; Lane 2: standard AD; Lane 3: standard ADD; Lane 4: bioconversion medium inoculated *M. neoaurum* ZJUVN strain; Lane 5: bioconversion medium inoculated mutant *M. neoaurum* ZJUVN-08 strain

which were identical with those of the authentic AD. It is proposed that the compound obtained after bioconversion was AD.

### 3.2 Structure characterization of purified product

The purified compound was white powder and was further identified using NMR, MS, and IR analyses. MS data of the purified compound exhibited a molecular ion $[M+H]^+$ at *m/z* 287, which indicated that the molecular mass was 286 and is in line with that of authentic AD. IR $V_{max}$ ($cm^{-1}$): 2920, 2 850, 1736, 1661, and 1615. IR spectra peaks at 1736 $cm^{-1}$ supported the structure of 17-C=O, 1661 $cm^{-1}$ (3-C=O), 1617 $cm^{-1}$ (4-CH=C), 2920 $cm^{-1}$ (-CH), and 2850 $cm^{-1}$ (-CH-$CH_3$), which are in full agreement with the structure of AD. The data obtained from $^1H$ NMR (500 MHz, $CDCl_3$) were as follows ($\delta$): 0.924, 1.223, and 5.747. Chemical shifts at $\delta$ 5.747 suggested the presence of 4-ene (de Brabandere *et al*., 1997). Chemical shifts at $\delta$ 0.924, and $\delta$ 1.223 demonstrated the $C_{18}$ and $C_{19}$ methyl groups, respectively. $^{13}C$ NMR (125 MHz, $CDCl_3$) spectra data from $C_1$ to $C_{19}$ were as follows ($\delta$): 35.70, 33.89, 199.31, 124.14, 170.32, 32.56, 30.75, 35.15, 53.82, 38.64, 20.31, 31.28, 47.49, 50.84, 21.74, 35.84, 220.12, 13.70, and 17.37. The chemical shifts of C-17 at $\delta$ 220.12 verified the structure of 17-C=O and were identical with the data of AD reported by the recent studies (Lin *et al*., 2009; Hegazy *et al*., 2012). From the above results, we concluded that the purified product was AD.

### 3.3 FFD analysis for process variables affecting AD yield and PS conversion

A screening design was performed to estimate the effects of five factors, namely inducer concentration ($X_1$), pH ($X_2$), molar ratio of HP-β-CD/PS ($X_3$), substrate concentration ($X_4$), and incubation time ($X_5$). The design and results of FFD are indicated in Table 2. Effects of the five variables were analyzed by multiple regression analysis method and are illustrated in Table 3. The results indicated that the molar ratio of HP-β-CD/PS ($X_3$) and substrate concentration ($X_4$) were highly significant factors ($P$<0.0001). However, inducer concentration ($X_1$), pH ($X_2$), and incubation time ($X_5$) were insignificant factors, which exhibited a probability level below 90%. As shown in Table 3, coefficient estimates of molar ratio of HP-β-CD/PS and substrate concentration were both negative, which indicated that the decreases of molar ratio of HP-β-CD/PS and substrate concentration would lead to the increases of AD yield and PS conversion rate. Consequently, molar ratio of HP-β-CD/PS and the

**Table 2 Design and the results of FFD**

| Run | $X_1$ | $X_2$ | $X_3$ | $X_4$ | $X_5$ | $Y_{AD}$ (g/L) | $Y_{Conversion}$ (%) |
|---|---|---|---|---|---|---|---|
| 1 | 1 | 1 | 1 | 1 | 1 | 1.01 | 9.51 |
| 2 | −1 | −1 | −1 | 1 | −1 | 2.11 | 19.93 |
| 3 | −1 | −1 | −1 | −1 | 1 | 2.67 | 75.83 |
| 4 | −1 | 1 | 1 | −1 | 1 | 2.11 | 59.88 |
| 5 | 1 | −1 | 1 | −1 | 1 | 2.10 | 59.83 |
| 6 | 0 | 0 | 0 | 0 | 0 | 6.00 | 85.26 |
| 7 | −1 | −1 | 1 | −1 | −1 | 2.06 | 58.47 |
| 8 | 1 | −1 | −1 | 1 | 1 | 2.38 | 22.56 |
| 9 | −1 | 1 | 1 | 1 | −1 | 0.96 | 9.07 |
| 10 | −1 | 1 | −1 | 1 | 1 | 1.81 | 17.09 |
| 11 | −1 | 1 | −1 | −1 | −1 | 2.63 | 74.75 |
| 12 | 1 | −1 | −1 | −1 | −1 | 2.61 | 74.18 |
| 13 | −1 | −1 | 1 | 1 | 1 | 0.95 | 8.99 |
| 14 | 1 | 1 | −1 | 1 | −1 | 2.07 | 19.58 |
| 15 | 1 | −1 | 1 | 1 | −1 | 0.90 | 8.55 |
| 16 | 0 | 0 | 0 | 0 | 0 | 5.95 | 84.49 |
| 17 | 0 | 0 | 0 | 0 | 0 | 6.06 | 86.04 |
| 18 | 0 | 0 | 0 | 0 | 0 | 5.99 | 85.12 |
| 19 | 1 | 1 | 1 | −1 | −1 | 2.08 | 59.14 |
| 20 | 1 | 1 | −1 | −1 | 1 | 2.68 | 76.05 |

substrate concentration were chosen to be investigated in the further optimization experiment.

The analysis of variance (ANOVA) of FFD was carried out and the results are shown in Table 4. *P* values for AD yield and PS conversion rate were 0.0005 and 0.0001, respectively. The higher values of determination coefficient ($R^2$=0.9990, 0.9999 for AD yield and PS conversion rate, respectively) further confirmed the effectiveness of the models. The variance between mean value of center points and factorial points in Table 2 was evaluated by *t*-test, which suggested that the difference was significant ($P$<0.01). Hence, a conclusion could be drawn that the optimum point was in the range of our design and the steepest decent experiment could be omitted.

### 3.4 Optimization of the significant factors by CCD and RSM

CCD and RSM were employed to optimize the significant factors for AD yield and PS conversion. Based on the above results, the value of inducer concentration ($X_1$), pH ($X_2$) and incubation time ($X_5$) were fixed at the center-point level. The final optimization experiment of biotransformation conditions was performed using a two-variable-five-level CCD with five repetitions at the center points, thus the total number of experiments needed was $n$=4+4+5=13. The central composite design and its results are summarized in Tables 5 and 6. The experimental data of CCD were fitted to a second-order polynomial model obtained by multiple regression analysis. ANOVA was used to test the adequacy of the model and the results are presented in Table 7. The *F*-value (33.34 and 71.23) and Probe >*F*-value ($P$<0.0001 and $P$<0.0001) of the models for AD yield and PS conversion rate implied that the models were very significant. The high values of determination coefficients ($R^2$) were found to be 0.9597, 0.9807 for AD yield and PS conversion rate, respectively, which indicates the experimental data and the predicted values are of good consistency. The quadratic polynomial equations in terms of coded factors were given below:

$$Y_{AD}=6.00-0.52X_3-0.33X_4-1.57X_3^2-0.75X_4^2-0.089X_3X_4, \quad (4)$$

$$Y_{Conversion}=85.23-7.36X_3-17.29X_4-22.20X_3^2-7.24X_4^2+0.39X_3X_4. \quad (5)$$

**Table 3 Regression analysis of FFD experimental results**

| Response | Variable | Coefficient estimate | *df* | *F*-value | Probe >*F* |
|---|---|---|---|---|---|
| AD yield | $X_1$ | 0.034 | 1 | 5.39 | 0.116 |
| | $X_2$ | −0.028 | 1 | 3.81 | 0.157 |
| | $X_3$ | −0.420 | 1 | 1431.12 | <0.0001* |
| | $X_4$ | −0.420 | 1 | 1419.71 | <0.0001* |
| | $X_5$ | 0.018 | 1 | 2.54 | 0.209 |
| PS conversion | $X_1$ | 0.340 | 1 | 4.48 | 0.125 |
| | $X_2$ | −0.200 | 1 | 1.65 | 0.289 |
| | $X_3$ | −6.660 | 1 | 1748.92 | <0.0001* |
| | $X_4$ | −26.430 | 1 | 27554.93 | <0.0001* |
| | $X_5$ | 0.380 | 1 | 5.58 | 0.106 |

* Significance at 95% confidence level. *df*: degree of freedom

**Table 4 ANOVA results of FFD for AD yield and PS conversion rate**

| Response | Source | *df* | Mean square | *F*-value | Probe >*F* |
|---|---|---|---|---|---|
| AD yield | Model | 15 | 0.42 | 207.05 | 0.0005 |
| | Curvature | 1 | 52.61 | 26172.13 | <0.0001 |
| | Pure error | 3 | 0.0020 | | |
| | Cor total | 19 | | | |
| PS conversion | Model | 15 | 795.32 | 1961.04 | <0.0001 |
| | Curvature | 1 | 6305.33 | 15547.29 | <0.0001 |
| | Pure error | 3 | 0.41 | | |
| | Cor total | 19 | | | |

*df*: degree of freedom

**Table 5 Coded and actual values of factors in CCD**

| $X$ | $x_3$ | $x_4$ (g/L) |
|---|---|---|
| −1.414 | 1.29 | 7.17 |
| −1 | 1.5 | 8 |
| 0 | 2 | 10 |
| 1 | 2.5 | 12 |
| 1.414 | 2.71 | 12.83 |

$x_3$: molar ratio of HP-β-CD/PS; $x_4$: substrate concentration; *$X_3=(x_3-2)/0.5$; $X_4=(x_4-10)/2$

**Table 6 Results of CCD for AD yield and PS conversion**

| Run | $X_3$ | $X_4$ | $Y_{AD}$ (g/L) | $Y_{Conversion}$ (%) |
|---|---|---|---|---|
| 1 | 0 | 0 | 6.03 | 85.69 |
| 2 | 0 | 0 | 5.93 | 84.26 |
| 3 | −1 | −1 | 4.43 | 78.47 |
| 4 | 0 | 0 | 6.01 | 85.38 |
| 5 | 1 | −1 | 3.44 | 61.02 |
| 6 | 0 | −1.414 | 4.97 | 98.34 |
| 7 | 0 | 0 | 5.97 | 84.76 |
| 8 | −1.414 | 0 | 3.78 | 53.64 |
| 9 | 0 | 1.414 | 4.59 | 50.37 |
| 10 | 1.414 | 0 | 2.50 | 35.56 |
| 11 | 0 | 0 | 6.06 | 86.05 |
| 12 | −1 | 1 | 3.57 | 42.21 |
| 13 | 1 | 1 | 2.22 | 26.33 |

The significance of linear terms and quadratic terms for $X_3$ and $X_4$ is summarized in Table 7, and the results implied that the molar ratio of HP-β-CD/PS and substrate concentration had a highly significant effect ($P<0.01$) on the yield of AD as well as the PS conversion. The interaction effect of $X_3$ and $X_4$ was not significant ($P>0.05$). As can be seen from the response surface plot (Fig. 3), a maximum value in this surface plot was observed. With the coded level of −0.16 ($X_3$) and −0.56 ($X_4$), AD yield and PS conversion rate reached the maximum, which further verifies that the maximum point existed in the fitted surface. The predicted maximum responses from the regression models for AD yield and PS conversion were 5.98 g/L and 93.26%, respectively. The residuals analysis of CCD data, presented in Fig. 4, further validates that the models simulated the actual process of PS biotransformation under the present system.

In order to confirm the validity of the models, experiments were carried out with the optimized parameters. The obtained values of AD yield and PS bioconversion rate were (5.93±0.075) g/L and ((94.74±1.19)%), respectively. This further indicates the validity of optimization design.

**Table 7 ANOVA results for AD yield and PS conversion obtained from CCD**

| Response | Source | Sum of squares | Mean square | $F$-value | Probe >$F$ |
|---|---|---|---|---|---|
| AD yield | Model | 22.16 | 4.43 | 33.34 | <0.0001 |
| | $X_3$ | 2.13 | 2.13 | 16.01 | 0.0052 |
| | $X_4$ | 0.85 | 0.85 | 6.42 | 0.0390 |
| | $X_3^2$ | 17.06 | 17.06 | 128.29 | <0.0001 |
| | $X_4^2$ | 3.89 | 3.89 | 29.26 | 0.0010 |
| | $X_3X_4$ | 0.032 | 0.032 | 0.24 | 0.6388 |
| | Residual | 0.93 | 0.13 | | |
| | Lack of fit | 0.92 | 0.31 | 119.73 | 0.0002 |
| | Pure error | 0.010 | 0.00256 | | |
| PS conversion | Model | 6387.45 | 1277.49 | 71.23 | <0.0001 |
| | $X_3$ | 433.64 | 433.64 | 24.18 | 0.0017 |
| | $X_4$ | 2390.19 | 2390.19 | 133.27 | <0.0001 |
| | $X_3^2$ | 3429.69 | 3429.69 | 191.23 | <0.0001 |
| | $X_4^2$ | 364.29 | 364.29 | 20.31 | 0.0028 |
| | $X_3X_4$ | 0.62 | 0.62 | 0.034 | 0.8582 |
| | Residual | 125.54 | 17.93 | | |
| | Lack of fit | 123.47 | 41.16 | 79.60 | 0.0005 |
| | Pure error | 2.07 | 0.52 | | |

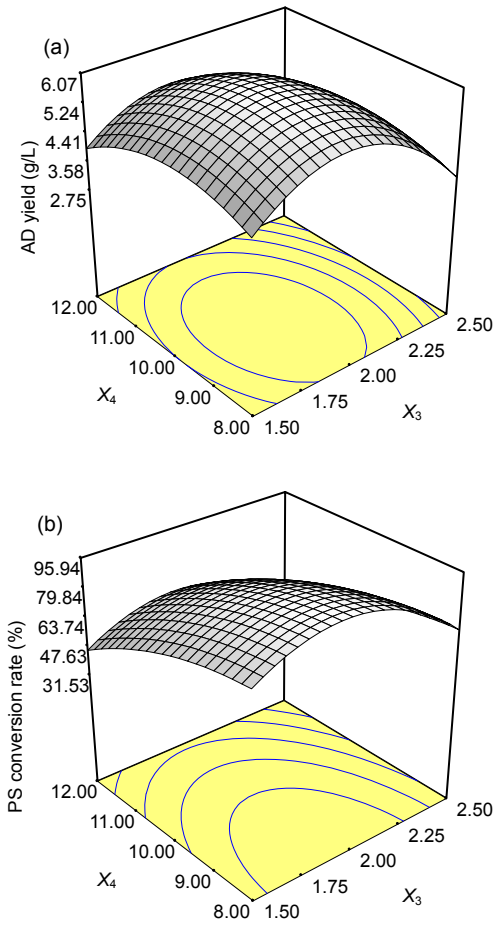


**Fig. 3 Three-dimensional response surface plots for AD yield (a) and PS conversion (b)**
$X_3$ is the molar ratio of HP-β-CD/PS, $X_4$ is the substrate concentration

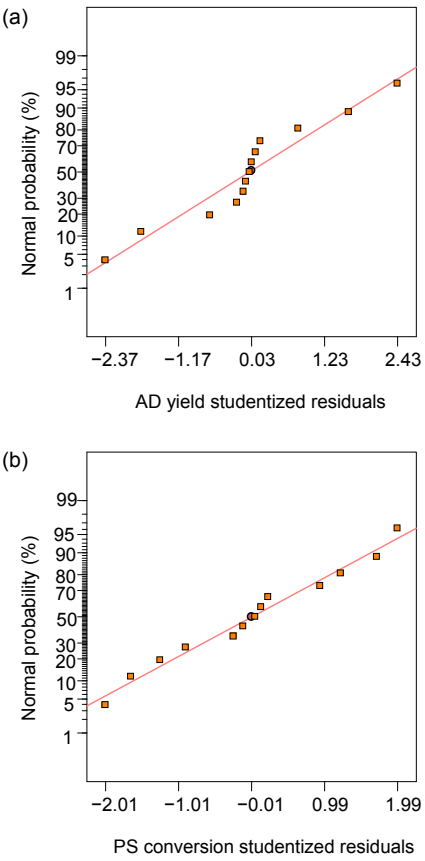


**Fig. 4 Residual analysis of CCD experiments for AD yield (a) and PS conversion rate (b)**

## 3.5 Biotransformation process examination under the optimum conditions in the 5-L fermenter

As the influencing factors had been optimized, biotransformation in a 5-L fermenter was examined and compared between the optimized and the non-optimized conditions. Fig. 5 shows the changes of AD yield and PS conversion rate versus time. After 24 h biotransformation, AD was accumulated and maximized at 120 h for both the optimized and non-optimized conditions. With the prolongation of biotransformation time, the concentration of final product in fermentation broth was decreased. This could be caused by the product degradation of mycobacteria cells (Malaviya and Gomes, 2008).

Under the optimized conditions, with lower substrate concentration (8.89 g/L) and lower molar ratio of HP-β-CD/PS (1.92:1), the maximum yield of

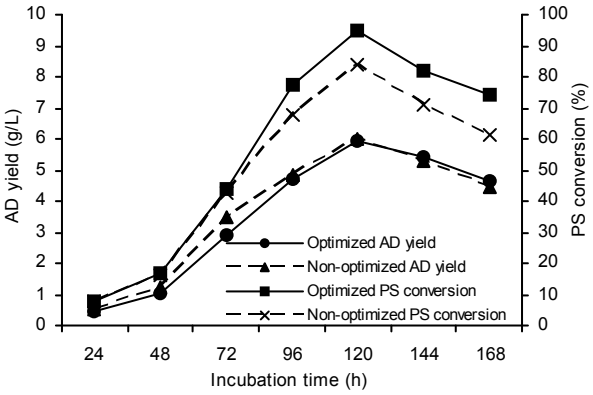


**Fig. 5 Time course of PS conversion and AD formation catalyzed by *M. neoaurum* ZJUVN-08**

AD was 5.96 g/L and was nearly the same with the non-optimized (5.99 g/L), while maximum PS conversion rate was 94.69% which increased by 10.66% compared with the non-optimized (84.03%). When the concentration of substrate and molar ratio of HP-β-CD/PS were decreased from 10 to 8.89 g/L and

2:1 to 1.92:1, the costs of PS and HP-β-CD are also decreased by 11% and 15%, respectively. This can result in significant cost savings when the biotransformation is implemented to large-scale production.

## 4 Discussion

HP-β-CD has been demonstrated to improve steroid biotransformation in aqueous media such as dehydrogenation of cortisone acetate (Ma *et al*., 2009) and the production of androstadienedione from progesterone (Manosroi *et al*., 2008). The results obtained in this paper indicated that the addition of HP-β-CD in transformation media was in favor of increasing substrate solubility. The yield of AD and PS conversion rate varied significantly according to the molar ratio of HP-β-CD/PS. As the present data were evaluated, the best molar ratio of HP-β-CD/PS was 1.92:1. This might be due to the formation of 1.92:1 HP-β-CD and PS complex. As shown in Table 2, when the molar ratio of HP-β-CD/PS were at 1:1 and 3:1, both the PS conversion rate and AD yield were very low. The reason might be that when low concentration of HP-β-CD was used, efficient inclusion complex cannot be formed between the PS molecule and HP-β-CD; and at high concentration of HP-β-CD, too much HP-β-CD could hinder the effective contact of enzyme and substrate. Several researchers have reported that when a higher concentration of substrate was used, HP-β-CD may serve as a reservoir of available substrate (Loftsson and Brewster, 1996; Zhang *et al*., 2009). Therefore, the presence of HP-β-CD makes it possible to maintain higher concentrations of PS in biotransformation media. Moreover, mycobacteria cells were proven to have good tolerance to HP-β-CD due to the unique composition of their cell wall (Shen *et al*., 2011), which facilitates the application of HP-β-CD in PS bioconversion.

The yield of the final product and the bioconversion rate are two critical elements to measure the economy of biotransformation procedure (Wang *et al*., 2004). It can be seen from Table 2 that as low substrate concentration was used, the PS conversion rate increased but the yield of product decreased. This made it more difficult for the downstream product separation, thus increasing the production cost from an economical viewpoint. With increased substrate concentration, the yield of AD was enhanced but the PS conversion rate decreased due to the low solubility of high concentration of substrate and could not effectively contact the microorganisms. Therefore, a threshold concentration of substrate must exist. Under this concentration, the highest product yield and conversion rate are both achieved. In the present work, the optimal substrate concentration was 8.89 g/L at 1.92:1 molar ratio of HP-β-CD/PS.

Many biotransformation approaches have been performed for improving of steroid bioconversion. As reported earlier, in organic media, the highest conversion rate from sitosterol to AD using immobilized *Mycobacterium* sp. NRRL B-3805 cells on Celite 545 was 89% when the initial concentration of substrate was 5.28 g/L (Cabral *et al*., 1997). In an organic-aqueous two phase media (dioctyl phthalate (DOP)-buffer system), also using *Mycobacterium* sp. NRRL B-3805 cells to catalyze the process, full conversion of 12 mmol/L sitosterol could be achieved at 24 h when biomass was 70 g/L (Staebler *et al*., 2004). In poly propylene glycol (PPG)/complex fermentation media, the conversion rate of PS to AD by *Mycobacterium* MB 3683 was found to be 90% when the initial concentration of substrate was 5 g/L at 168 h of incubation time (Kutney *et al*., 2000). Bioconversion of cholesterol to produce AD and ADD in cloud point system was studied using growing *Mycobacterium* sp. NRRL B-3683 cells, the obtained conversion rate was 93% at 168 h when the initial concentration of substrate was 14.5 g/L (Wang *et al*., 2004). Again, using resting mycobacterial cells, microbial conversion of PS to ADD in an optimized cloud point system was achieved. The yield of AD reached up to 12 g/L and incubation time was shortened to 96 h when the initial substrate concentration increased to 25 g/L (Wang *et al*., 2006). In a liquid polymer bioconversion system, silicone B oil was proven to be the best substrate carrier, and the conversion rate of sitosterol to AD at 120 h was 83%. It was also found that when the oil phase increased, the efficiency of conversion process decreased accordingly (Carvalho *et al*., 2009).

In this paper, the highest yield of AD (5.96 g/L) and PS conversion rate (94.69%) were obtained at 120 h of incubation time when the initial concentration of substrate was 8.98 g/L. Compared with

biotransformation processes catalyzed by industrial mycobacteria strains in different transformation systems, the conversion of PS catalyzed by mutant *M. neoaurum* ZJUVN-08 in HP-β-CD solution showed a certain advantage over the processes in organic media, PPG/complex fermentation media and liquid polymer of silicone B oil systems, which indicated it was a useful bioconversion process and further confirmed the improvement of the biotransformation process when HP-β-CD was added. Moreover, AD was the sole product in the fermentation broth, which made the mutant *M. neoaurum* ZJUVN-08 a useful strain for large-scale production of AD from PS.

## 5 Conclusions

In the present work, bioconversion of PS by *M. neoaurum* ZJUVN-08 was thoroughly investigated. AD was the sole product in the final fermentation broth catalyzed by the new mutant strain. FFD was conducted to screen the significant factors influencing the biotransformation process. Then CCD and RSM were employed for further optimization process. The optimized conditions were observed at inducer concentration of 0.01 g/L, pH 7.0, molar ratio of HP-β-CD/PS 1.92:1, substrate concentration of 8.89 g/L, and incubation time at 120 h. Under the optimum condition, more than 94% of PS was degraded and was obtained the maximum yield of AD 5.96 g/L, which is a cost-efficient process compared to the non-optimized condition. It can be seen that RSM is an effective method to optimize the PS bioconversion conditions. For in-depth study, further studies should be undertaken on the mode of adding HP-β-CD in fermentation medium.

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