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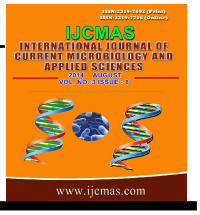

Original Research Article

Parameter optimization for progesterone to 17-ketosteroids bioconversion by co-culture of *Pseudomonas diminuta* and *Delftia acidovorans*

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Keywords

17-keto steroids; *Delftia acidovorans* MTCC 3362; progesterone bioconversion *Pseudomonas diminuta* MTCC 3361.

A B S T R A C T

Various physico-chemical parameters for the bioconversion of progesterone to 17-ketosteroid intermediates required for the production of male and female sex hormones by co-culture of *Pseudomonas diminuta* MTCC 3361 and *Delftia acidovorans* MTCC 3362 has been optimized. Nutrient broth inoculated with actively growing co-culture proved to be the ideal medium for bacterial growth and bioconversion. A temperature range of 30 - 35°C, incubation period of 72 h, dimethylformamide as a carrier solvent at 1.33% concentration and pH of the medium adjusted to 7.0 gave maximum 80 mol % bioconversion at 0.5 mg/ml progesterone concentration. Addition of 8-hydroxyquinoline improved the mol % conversion of progesterone to 17-ketosteroids to 88% by preventing further breakdown of 17-ketosteroids. Progesterone bioconversion in a five liter fermentor followed the trends observed in shake flask culture.

Introduction

Progesterone is an important precursor for the production of androst-4-ene-3, 17-dione (AD) by C-17 side chain cleavage and androsta-1, 4-diene-3, 17-dione (ADD) by side-chain cleavage and Δ^1-dehydrogenation reaction (Manosroi *et al*., 2008). Progesterone is available in large quantities through chemical degradation of steroidal alkaloids and sapogenins (Asolkar and Chadha, 1979).The production of male and female sex hormones form progesterone through chemical route requires its conversion to two key 17-ketosteroids, AD and ADD respectively (Martin, 1977).

Female hormones (Estrogen and analogous) are the major steroid hormones produced worldwide using AD and ADD. Estrogen replacement therapy is a major tool to combat postmenopausal osteoporosis in women (Neunzehn, J. et al., 2014), which employs estrogen from external sources. The side chain cleavage (Lin et al., 2009) and Δ^1-dehydrogenation activities are widely distributed in several microorganisms (Gharaei-Fathabad, and Chabra 2011; Manosroi et al., 2008). The bioconversion pathway of 16-DPA to ADD by a mixed culture of *Pseudomonas diminuta* MTCC

3361 and *Delftia acidovorans* MTCC 3362 has been reported earlier (Patil et al., 2002). The present work reports the optimization of parameters for maximum conversion of progesterone to 17-ketosteroids by co-culture of these bacteria.

Materials and Methods

Microorganisms

Pseudomonas diminuta MTCC 3361 and *Delftia acidovorans* MTCC 3362 were procured from Microbial Type Culture Collection and Gene Bank, Chandigarh, India.

Bioconversion

All bioconversion experiments were carried out in 150 ml Erlenmeyer flasks containing 30 ml culture medium. The medium was supplemented with 10 mg/l progesterone as an inducer, adjusted to pH 7.0 with 1N NaOH/ HCl and sterilized at 15 psi steam pressure for 15 min. The sterile medium was inoculated with 1 ml of actively growing co-culture of *Pseudomonas diminuta* MTCC 3361 and *Delftia acidovorans* MTCC 3362 raised in the medium of same composition. After 12 h growth period on a gyratory incubated shaker at 30 ± 2°C, 30 mg progesterone dissolved in 0.5 ml dimethylformamide (DMF) was added to the culture. The subsequent bioconversions were carried out adopting the parameters optimized during previous experiments.

Medium

The compositions (g/l) of various media used during the experiments were as follows : (A) : Peptone, 5.0; NH_4NO_3, 1.0; $MgSO_4.7H_2O$, 0.25; (B) : Yeast extract, 5.0; NH_4NO_3, 1.0; K_2HPO_4, 0.25; $MgSO_4.7H_2O$, 0.25; (C) : Glycerol, 10.0; Soy meal, 10.0; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.25; $FeCl_3.6H_2O$, 0.05; (D) : Sodium citrate, 3.0; 16-DPA, 1.0; Urea, 0.5; K_2HPO_4, 1.6; KH_2PO_4, 0.4; KNO_3, 1.0; $MgSO_4.7H_2O$, 0.1; $MnSO_4.7H_2O$, 0.0025; $ZnSO_4.7H_2O$, 0.0025; $FeSO_4.7H_2O$, 0.0025; (E) : Peptone, 5.0; Yeast extract, 2.0; Beef extract, 1.0; NaCl, 5.0.

Analysis

One ml sample of the incubation medium was aseptically drawn after regular intervals and extracted with ethyl acetate (2 + 2 ml). The organic phase separated after centrifugation (5000 rpm, 5 min.) was collected and dried over anhydrous sodium sulphate. The residue left after vacuum evaporation of ethyl acetate extract at room temperature was redissolved in 1 ml ethyl acetate. Aliquots of 0.1 ml each were used for qualitative analysis by TLC (Shah et al., 1980) and quantitative analysis of 17-ketosteroids following modified Zimmermann reaction (Pontius, 1962; Ahmad and Johri, 1991).

Bioconversion in fermentor

Three liter medium E was dispensed in five liter capacity fermentor vessel (BioFlo III, New Brunswick Scientific, NJ, USA) and sterilized at 15 psi steam pressure for 45 min. After cooling, the medium was inoculated with 100 ml co-culture of *P. diminuta* MTCC 3361 and *C. acidovorans* MTCC 3362 grown for 12 h. Aeration and agitation were set at 2.5 v/v/min and 180 rpm respectively. After 12 h growth period, 15 g progesterone dissolved in 30 ml DMF was aseptically added and the fermentation was continued at 30°C. Foaming was controlled by addition of sterile aqueous silicon oil emulsion through antifoam controller.

Results and Discussion

Prominent quantitative difference in the mol % conversion of progesterone to 17-ketosteroids was observed in various media used for bioconversion (Table 1). No qualitative difference in the product was detected in any of the medium. Maximum bioconversion was recorded in nutrient broth (Medium E) at 96 h incubation period. The 17-ketosteroid content declined on prolonged incubation in all the media.

A maximum of 53.6 mol % conversion of progesterone to 17-ketosteroids was recorded after 72 h incubation period when the initial pH of the medium was set at neutrality (Fig. 1). Adjusting media pH values away from 7.0 invariably resulted in lower bioconversion in the corresponding period of incubation.

The use of various substrate carrier solvents significantly affected the mol % conversion of progesterone to 17-ketosteroids.When ethyl acetate was used as a substrate carrier, a reduction in the overall conversion was recorded (Table 2). Whereas, when 1, 4-dioxan or acetone were used as carriers, slight increase in the conversion of progesterone to 17-ketosteroids was observed during initial period. DMF proved to be the most suitable carrier, which showed more than three-fold accumulation of 17-ketosteroids at 120 h incubation period as compared to ethyl acetate. DMF concentration up to 1.33% was well tolerated by the co-culture, attaining about 67 mol % conversion at 72 h incubation period. Higher concentration of DMF in the incubation medium hampered the bioconversion (Fig. 2).

In a temperature range of 25 – 45°C, the maximum bioconversion (80 mol %) was obtained at 72 h incubation period when temperature was maintained at 30°C (Table 3). Higher or lower temperatures adversely affected the bioconversion. Qualitative analysis by TLC revealed a rapid utilization of progesterone as well as 17-ketosteroids at higher temperatures.

Addition of 0.5 mg/ml progesterone as substrate resulted in its optimal conversion to 17-ketosteroids (80.8 mol %) at 72 h incubation. Higher substrate concentrations adversely affected the bioconversion. Increasing the substrate concentration to 1 and 2.5 mg/ml, the bioconversion of progesterone to 17-ketosteroids reduced by 2 and 4 folds respectively (Fig. 3). Substrate added at 12 h growth yielded a maximum of 79 mol % conversion (Fig. 4) at 72 h incubation period. Addition of substrate at 6 or 24 h resulted in low mol % conversion.

The effect of some 9α-hydroxylase inhibitors on the bioconversion of progesterone to 17-ketosteroids is depicted in Figure 5. The addition of 8-hydroxyquinoline resulted in marginal improvement in the bioconversion of progesterone to 17-ketosteroids, yielding 88 mol % conversion within 48 h of incubation. Moreover, the pertinent degradation of 17-ketosteroids was also checked to an appreciable extent. Other commonly used 9α-hydroxylase inhibitors including 2, 2'-dipyridyl and cobalt chloride not only failed to accumulate higher amount of 17-ketosteroids, but also reduced the mol % bioconversion significantly.

The pattern of accumulation of 17-ketosteroids remained same under shake flask conditions as well as in fermentor. However, the rate of progesterone utilization (as evident by TLC analysis) and 17-ketosteroids accumulation was faster under the conditions predominant in the fermentor (Fig.6).

Table.1 Effect of medium composition on the bioconversion of progesterone to 17-ketosteroids by co-culture of *P. diminuta* MTCC 3361 and *C. acidovorans* MTCC 3362.

	Mol % conversion					
	Time (hours)					
Medium	24	48	72	96	120	144
A	24.25 ± 2.20	39.53 ± 0.37	39.90 ±1.53	48.53 ±1.77	49.30 ± 0.48	48.10 ± 0.46
B	6.12 ± 0.70	14.20 ± 0.83	21.89 ±0.26	34.70 ±1.98	40.82 ±1.70	37.10 ± 0.68
C	6.60 ± 0.51	10.80 ± 0.60	13.69 ± 0.22	14.33 ± 0.83	13.41 ± 1.36	10.31 ± 1.17
D	ND	3.70 ± 0.30	6.10 ± 0.41	8.11 ± 0.39	9.02 ± 0.09	8.44 ± 0.18
E	23.54 ±1.72	41.44 ± 0.35	45.10 ±2.24	53.21 ± 0.37	50.00 ±1.50	47.20 ± 0.48

Data are mean of three replicates ± standard deviation ND: Not detected

Table.2 Effect of some carrier solvents on the bioconversion of progesterone to 17-ketosteroids by a co-culture of *P. diminuta* MTCC 3361 and *C. acidovorans* MTCC 3362.

	Mol% Bio-conversion					
	Incubation period (hours)					
Carrier solvent	24	48	72	96	120	144
DMF	30.19 ± 0.70	42.56 ± 0.37	48.82 ± 0.30	38.35 ± 0.73	37.38 ± 1.11	34.72 ± 0.34
Acetone	29.14 ± 1.07	40.62 ± 1.14	45.17 ± 0.58	29.50 ± 0.44	24.09 ± 0.78	21.91 ± 0.58
Ethyl alcohol	18.15 ± 1.85	29.52 ± 0.45	42.94 ± 1.42	38.04 ± 1.54	31.62 ± 0.74	29.12 ± 0.32
Ethyl acetate	6.00 ± 0.55	6.70 ± 0.46	10.25 ± 0.74	17.22±0.56	12.40 ± 1.63	11.15 ± 0.67
1,4-Dioxan	26.10 ± 1.33	34.12 ± 1.16	38.97 ± 0.89	32.22 ± 1.58	23.50 ± 0.55	21.67 ± 0.67

Data are mean of three replicates ± standard deviation

Table.3 Effect of temperature on the bioconversion of progesterone to 17-ketosteroids by a co-culture of *P. diminuta* MTCC 3361 and *C. acidovorans* MTCC 3362.

	Mol % conversion							
	Incubation period (hours)							
Temp (ºC)	12	24	36	48	60	72	96	120
25	19.72±0.31	22.61±0.25	25.82±0.42	28.90±0.36	30.75±0.15	32.45±0.41	32.10±0.24	29.63±0.29
30	33.67±0.61	38.50±0.14	49.92±0.51	64.33±0.49	70.88±1.53	79.78±1.39	70.13±0.49	54.23±0.22
35	36.30±0.28	45.80±1.09	42.36±0.76	39.56±0.24	38.40±0.03	37.08±0.22	36.20±0.19	33.86±0.59
40	38.91±0.83	46.95±0.28	47.70±0.14	51.00±0.45	53.55±0.28	52.60±0.29	51.43±0.27	47.11±0.34
45	37.90±0.27	39.35±0.57	40.30±0.24	42.58±0.40	35.65±1.48	34.50±0.24	27.01±0.44	25.10±0.23

Data are mean of three replicates ± standard deviation

Figure.1 Effect of pH of the medium on the bioconversion of progesterone to 17-ketosteroids by co-culture of *P. diminuta* MTCC 3361 and *C. acidovorans* MTCC 3361.

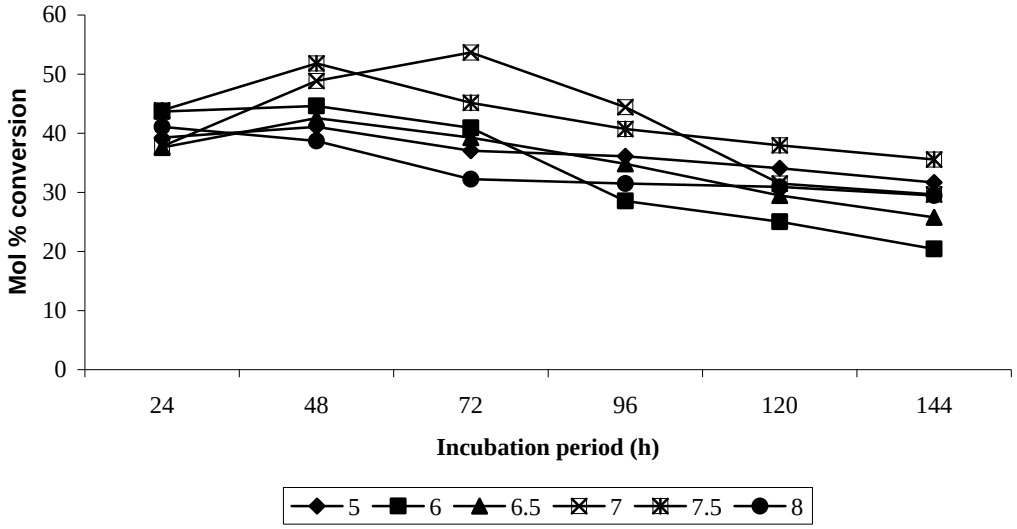


Figure.2 Effect of amount of DMF on the bioconversion of progesterone to 17-ketosteroids by co-culture of *P. dimiuta* MTCC 3361 and *C. acidovorans* MTCC 3361.

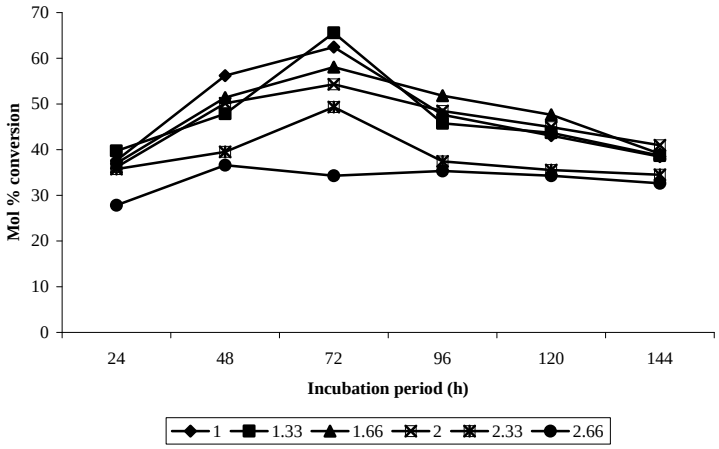


Figure.3 Effect of substrate concentration on the bioconversion of progesterone to 17-ketosteroids by co-culture of *P. diminuta* MTCC 3361 and *C. acidovorans* MTCC 3362.

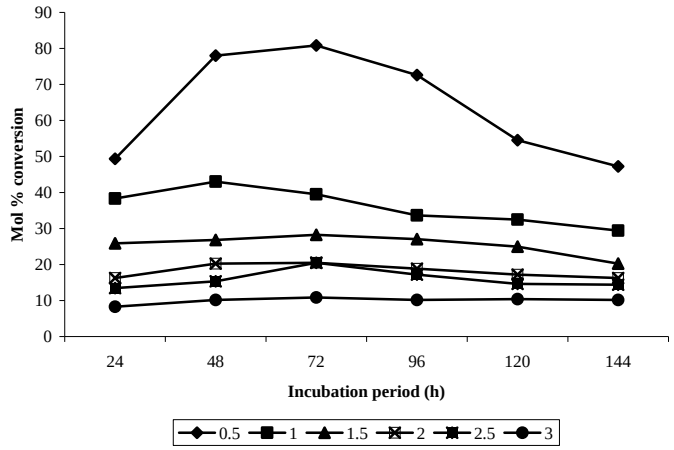


Figure.4 Effect of time of substrate addition on the bioconversion of progesterone to 17-ketosteroids by co-culture of *P. diminuta* MTCC 3361 and *C. acidovorans* MTCC 3362.

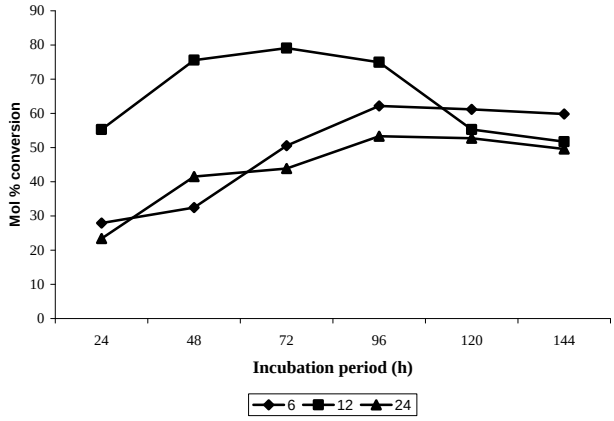


Figure.5 Effect of 9a-hydroxylase inhibitors on the bioconversion of progesterone to 17-ketosteroids by co-culture of *P. diminuta* MTCC 3361 and *C. acidovorans* MTCC 3362.

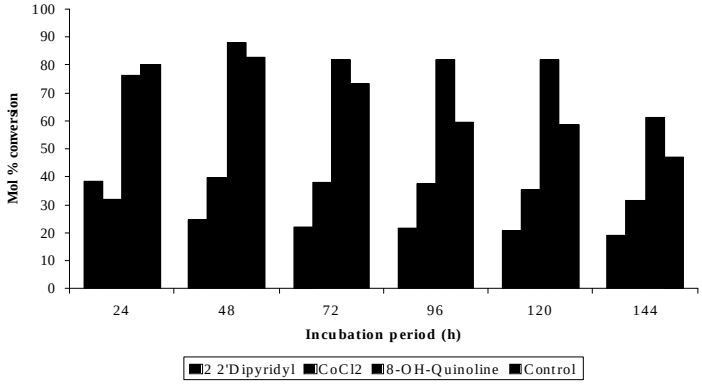
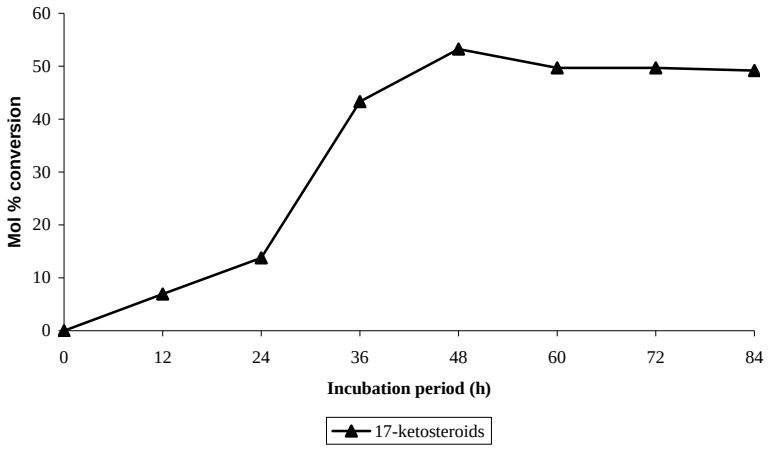


Figure.6 Changes in mol % conversion of progesterone to 17-ketosteroids by co-culture of *P. diminuta* MTCC 3361 and *C. acidovorans* MTCC 3362 in a fermentor at 0.5 mg/ml substrate concentration.



However, degradation of 17-ketosteroids was considerably slower in a fermentor. A maximum of 53.2 mol % conversion was recorded after 60 h incubation at 0.5 mg/ml substrate concentration in the fermentor.

Nutrient broth (Medium E) containing beef extract, yeast extract and peptone as the major nutrients appears to be the most suitable medium for the bioconversion of progesterone to 17-ketosteroids by the co-culture of *Pseudomonas diminuta* MTCC 3361 and *Delftia acidovorans* MTCC 3362. The accumulation of 17-ketosteroids in the culture medium represents a balance between their formation and degradation. The monocultures of *Arthrobacter simplex*, *Serratia marcescens*, *Brevibacterium lipolyticum* and *Protaminobacter alboflavus* have been shown to degrade 17-ketosteroids further to non-steroidal products (Shah et al., 1980). Addition of a variety of inhibitors for 9α-hydroxylation, a key step that initiates the further degradation, has been widely used to prevent the loss of 17-ketosteroids (Whitmarsh, 1964; Goswami et al., 1994). In the present case, only 8-hydroxyquinoline successfully blocked 9α-hydroxylase activity of the co-culture.

The alkaline or acidic pH of the medium adversely affected the mol % conversion, indicating the suitability of neutral pH for the bioconversion of progesterone. This observation supports the finding of Shiwei and Youhua, (1982) who observed higher conversion of 16-dehydro allo-pregnene-3β-acetoxy-3, 20-dione to ADD at neutral or slightly alkaline pH by a strain of *Arthrobacter simplex.*

Besides the chemical nature, the concentration of the carrier solvent has been shown to exert deleterious effect on microorganisms (Ceen et al., 1987; Pinheiro and Cabral, 1999). For the bioconversion of progesterone to 17-ketosteroids by the co-culture, the gradation in solvent toxicity observed is DMF < acetone < ethyl alcohol < 1, 4-dioxan < ethyl acetate. The adverse effect

of high concentration of steroid substrates on the bioconversion has been repeatedly shown (Sallam et al., 1977; Roy et al., 1991; Srivastava and Patil, 1992). Minimum concentration of progesterone tested gave maximum mol % conversion, indicating the toxicity of the substrate to the co-culture.

The phase of the bacterial culture at the time of substrate addition is critical, particularly when the substrate adversely affects the bioconversion. The suitability of 12 h grown co-culture, which corresponded to the onset of stationary phase, reconciles with the observation of El-Refai et al. (1975) of maximum 11β-hydroxylation of progesterone when added at stationary phase.

A wide gap observed between the mol % conversion obtained in shake flask cultures and fermentor emphasizes the necessity of optimization of every parameter in a fermentor during scaling up of progesterone to 17-ketosteroids bioconversion process. Moreover, slower degradation of 17-ketosteroids, offers a potential advantage for successful commercial exploitation of the present bioconversion in larger fermentors.

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