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MICROBIAL Δ¹-DEHYDROGENATION OF ANDROST-4-ENE-3, 17-DIONE (AD) BY *DELFTIA ACIDOVORANCE* MTCC 3364 IN PRESENCE OF ORGANIC SOLVENT AS SUBSTRATE CARRIERS

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Key words : *Androsta-1, 4-diene 3, 17-dione, Androst-4-ene 3, 17-dione,* Delftia acidovorans*, 1,4 Dioxane, Steroid biotransformation*

Abstract– Microbial Δ¹-dehydrogenation of androst-4-ene-3,17-dione (androstenedione, AD) was carried out by highly efficient strain *Delftia acidovorans* MTCC 3364. This is the first report on use of *D. acidovorans* MTCC 3364 for bioconversion of AD. The work emphasizes on the screening of suitable organic solvent for biotransformation of AD to yield androsta-1,4-diene,3,17-dione (androstadienedione, ADD).Various alcohols and organic solvents were included in the study as carriers for the addition of AD in the incubation medium. As compared to control, the use of methylene chloride and 1,4 dioxane enhanced the current biotransformation by 130 % and 135 % respectively. Chloroform, ethanol and propanol neither affected biomass accumulation nor the ADD accumulation. *n*-Butanol drastically affected the process, showing reduced biomass accumulation as well as biotransformation. However, at higher concentration of organic solvents used, decreased yield of bioconversion has been observed as compared to yield obtained at optimum concentration.

INTRODUCTION

Androst-4-ene-3, 17-dione (AD) and androsta-1, 4-diene-3, 17-dione (ADD) have been identified as key intermediates necessary for the production of estrogens, androgens and anabolic drugs extensively used in allopathic system of medicine (Martin, 1984; Ahmad *et al.,* 1991). The synthesis of whole range of steroid drugs and their derivatives used as medicine is based on the availability of these steroid precursors. Androsta-1, 4-diene-3, 17-dione (ADD) has been established as the key industrial intermediate for the synthesis of female sex hormones and to a major extent as the starting material for the production of contraceptive drugs. AD is converted to androsta-1, 4-diene-3, 17-dione (ADD) by microbial Δ¹-dehydrogenation reaction by inserting double bond between carbon number 1 and 2 in ring A of steroid nucleus. Microbial Δ¹-dehydrogenation is usually performed with whole cells; which are a co-factor-dependent reaction and the active cell machinery continuously regenerate the necessary co-factors (Cruz *et al.,* 2002). The ability to dehydrogenate C-C bonds within the steroid nucleus is distinctive feature of actinobacteria and thus used for the manufacture of prednisolone and its derivatives.

In last few years, various microorganisms have been used to convert AD into ADD including *Mycobacterium sp.* (Wang *et al.,* 2008), *Gordonia neofelifaecis* (Liu *et al.,* 2010), *Arthrobacter spp.* (Lee *et al.,* 1992; Arinbasarova *et al.,* 1996; Fokina, 2003) and *Nocardioides simplex* (Fokina, 2003). Besides high efficacy of various organisms to convert the steroidal substrates, hydrophobicity of a substrate remains a formidable barrier in steroid fermentations. To overcome this obstacle, precursor steroids have been frequently added in dissolved form using organic solvents (Ceen *et al.,* 1987b; Phase and Patil, 1994; Wang *et al.,* 2008; El Refai *et al.,* 2010; Pendharkar *et al.,* 2014a,b; Banerjee *et al.,* 2014; Pendharkar *et al.,* 2022).

In the present study, highly efficient biotransformation of AD to ADD is reported using some organic solvents as steroid substrate carrier. This is also the first report on use of *Delftia acidovorans* MTCC 3364 for Δ^1-dehydrogenation of AD.

MATERIALS AND METHODS

Strain

Delftia acidovorans MTCC 3364 was procured from Microbial Type Culture Collection and Gene Bank, Chandigarh, India. The organism was revived and maintained on nutrient agar at 4 °C.

Materials

Chemicals and solvents were purchased from different suppliers. AD and ADD were purchased from Sigma Chemicals, USA. Acetone, benzene, methanol, 1, 2-propanol, chloroform, tetrahydrofuron (THF), toluene, methylene chloride, ethyl acetate and sodium sulphate were procured from Merck, India. Dimethyl formamide (DMF) and 1,4 dioxane were procured from S D Fine Chemicals, India. Dimethyl sulfoxide (DMSO) (Loba chem., India); ethanol (Bengal chemical, India); butyl alcohol (Glaxo Lab, India); diethyl ether (Ranbaxy, India) and ammonium ceric sulphate was procured from HiMedia Laboratory, India.

Substrate addition

For control experiments, micronized suspension of AD was prepared by adding AD in incubation medium along with 30 g glass beads in Erlenmeyer flask and incubated it on gyratory incubator shaker. After 30 minutes of incubation, medium was dispensed in three flasks and sterilized in an autoclave. Alternatively, AD dissolved in organic solvent was added to the sterile flask containing incubation medium.

Biotransformation of AD

The inoculum was prepared in 150 ml nutrient broth (HiMedia Laboratory, India) and pH adjusted to 7.0 in 500 ml capacity flasks. After sterilization at 121°C for 15 min. in an autoclave, the medium was inoculated with 1 ml actively growing *Delftia acidovorans* MTCC 3364 and incubated on a gyratory incubator shaker (200 rpm, 32±2 °C) for 24 hours. Bioconversion of AD was initiated by addition of 1ml inoculum in 29 ml sterile nutrient medium containing substrate in 150 ml capacity flask. The flasks were incubated on a gyratory incubator shaker (200 rpm, 32±2 °C). Samples of the incubation medium were aseptically drawn after regular intervals of 12 hours and analyzed for product(s) formation.

Cell growth studies

To study the effect of solvents on cell growth, sterile incubation media containing solvents (without substrate) was inoculated. After regular interval of 24 hours the sample was drawn and absorbance was taken at 660 nm.

Analytical method

The bioconversion of AD to ADD was monitored by Quantitative Thin Layer Chromatography (QTLC) as described by Gulla *et al*. (2010) and Pendharkar *et al.* (2014a, b). Briefly; 1 ml fermentation broth was extracted with equal volume of chloroform, the separated organic phase after centrifugation was dried over sodium sulphate and 10ìl of the chloroform extract of bioconversion medium was spotted on pre-coated silica gel plates (Merck-1.05554.007, Darmstadt, Germany). On the same TLC plate, calibration was prepared by spotting three different concentrations of authentic ADD. The TLC plates were developed in solvent system containing ethyl acetate: benzene in ratio of 4:5. Spots were visualized by spraying the plates with 2% ammonium ceric sulphate dissolved in 60 % sulfuric acid followed by heating at 110°C for 5 min. The plates were scanned on Samsung SCX-4100 scanner and spots were quantified by Image Quant Software (G.E. Life Sciences) Ver. 7.0, based on image densitometric analysis.

RESULTS

Effect of the water miscible organic solvents on biotransformation

The work reported in this paper attempts to find a suitable solvent for bioconversion of AD to ADD. Various water miscible solvents such as methanol, ethanol, 1,4-dioxane, propanol, acetone, dimethyl formamide (DMF), dimethyl sulfoxide (DMSO) and tetrahydrofuran (THF) were investigated (Fig.1). Each solvent was added to the basal medium at a concentration of 1%, 2% and 3% (v/v). Along with the bioconversion, solvents effect on growth was also studied.

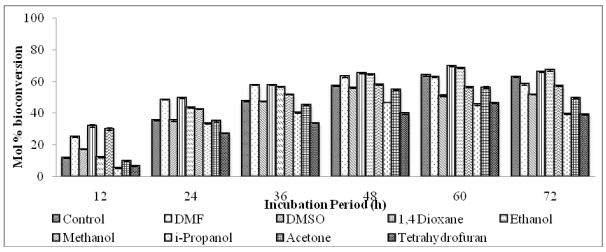

Fig. 1. Graph showing effect of **1%** water miscible organic solvents on the bioconversion of AD to ADD.

Among the solvents studied, the optimum conversion was obtained with 1,4-dioxane followed by DMF at 2%. At 2% concentration 1, 4 dioxane showed nearly 90 mol % bioconversion in 48 hours of incubation (Fig. 2). Simultaneously, control, ethanol, DMSO and DMF showed 67, 80, 81 and 86 mol % conversion respectively. After 50 hours of incubation, product formation got arrested without any further degradation of ADD. However, the bioconversion was negatively affected in presence of 3% organic solvents as compared to 2 % solvent concentration, showing only 62 Mol % conversion in 1,4 dioxane (Fig. 3).

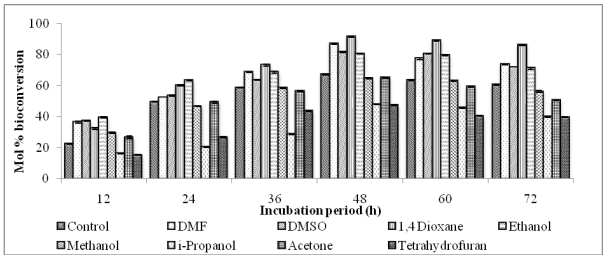

Fig. 2. Graph showing effect of **2%** water miscible organic solvents on bioconversion of AD to ADD.

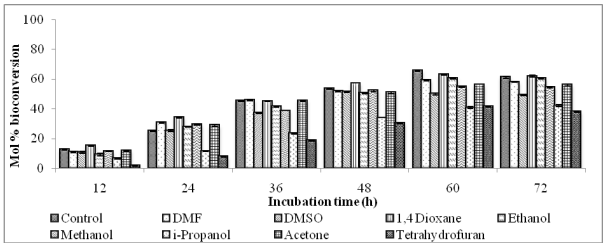

Fig. 3. Graph showing effect of **3%** water miscible organic solvents on bioconversion of AD to ADD.

Effect of the immiscible organic solvents

Six water immiscible organic solvents were investigated for bioconversion of and rostendione. Each solvent was added to the basal medium at a concentration level of 1%, 2% and 3% (v/v) (Table 1). At 2%, Methylene chloride showed nearly 86 Mol % conversions in 60 hours of incubation followed by Diethyl ether which showed 80 Mol % conversions.

As compared to control, both the solvents show approximately 18% and 12% increase in bioconversion respectively (Table 2). As like miscible solvents, Table 3 showed the conversion was negatively affected in presence of 3% organic solvents.

Effect of organic solvents on cell growth

Biomass accumulation in different concentrations of water miscible organic solvents yielded similar results. No significant difference was observed in presence of water miscible solvents as compared to control experiments (Fig. 4 to Fig. 6).

However, 2% concentration of *n*-butanol showed toxic effect on cell growth, which affected the cell growth up to 48 hours of incubation. Some water immiscible solvents *viz*, *n*-butanol, diethyl ether and toluene proved toxic at concentration of 3%. They showed deleterious effect on the cell growth compared to control (Fig. 7 to Fig. 9).

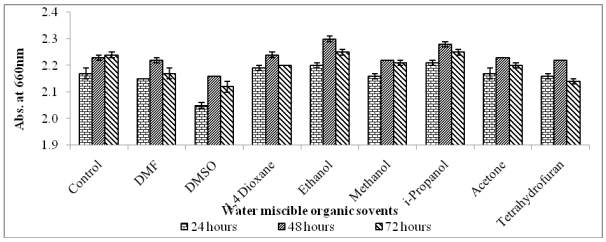

Fig. 4. Effect of **1%** water miscible organic solvents on cell growth of *D. acidovorans* MTCC 3364

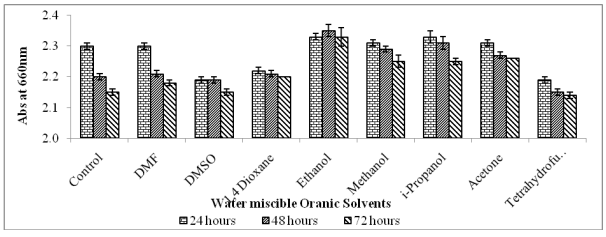

Fig. 5. Effect of **2%** water miscible organic solvent on cell growth of *D. acidovorans* MTCC 3364.

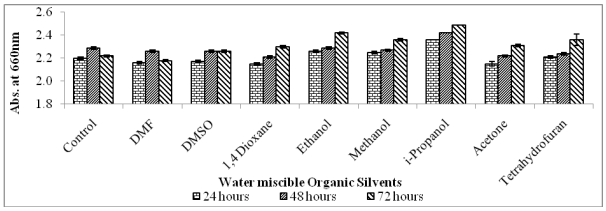

Fig. 6. Effect of **3%** water miscible organic solvent on cell growth of *D. acidovorans* MTCC 3364.

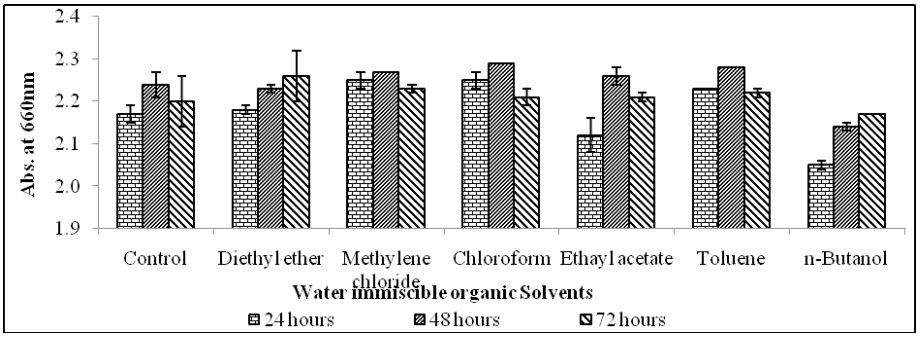

Fig. 7. Effect of **1%** water immiscible organic solvent on cell growth of *D. acidovorans* MTCC 3364.

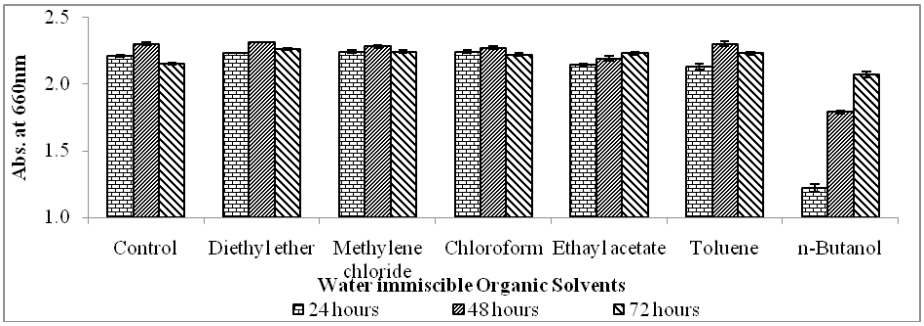

Fig. 8. Effect of **2%** water immiscible organic solvent on cell growth of *D. acidovorans* MTCC 3364

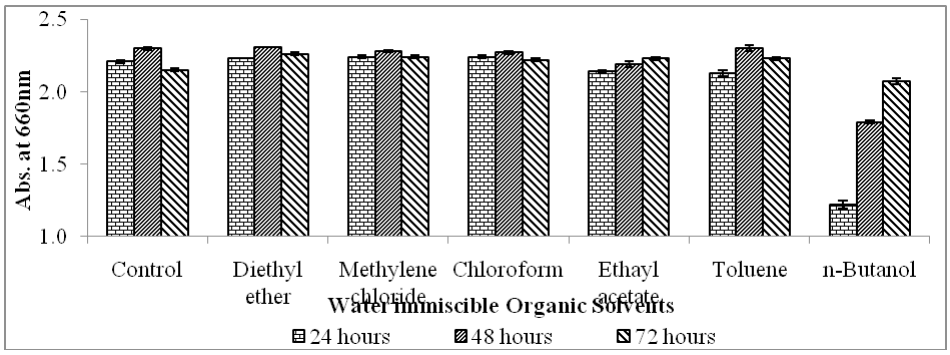

Fig. 9. Effect of **3%** water immiscible organic solvent on cell growth of *D. acidovorans* MTCC 3364.

Table 1. Effect of 1% water immiscible carrier solvents on bioconversion of AD to ADD by *Delftia acidovorans* MTCC 3364.

Carrier solvents	Mol % conversion ± S.D. after incubation period (h)					
	12	24	36	48	60	72
Control	12.9 ± 0.52	33.3 ± 0.5	46.8 ± 0.52	55.6 ± 0.4	63.9 ± 0.35	60.0 ± 0.53
Diethyl ether	13.9 ± 0.15	33.1 ± 0.48	47.1 ± 0.27	53.3 ± 0.9	60.1 ± 0.57	55.0 ± 0.36
Methylene chloride	12.3 ± 0.38	38.1 ± 0.4	50.7 ± 0.62	65.3 ± 0.2	72.8 ± 0.3	66.1 ± 0.41
Chloroform	06.1 ± 0.25	09.5 ± 0.58	13.5 ± 0.36	41.1 ± 0.3	33.2 ± 0.52	26.7 ± 0.65
Ethyl acetate	13.9 ± 0.35	35.6 ± 0.35	46.8 ± 0.56	51.4 ± 0.5	67.3 ± 0.23	63.3 ± 0.15
Toluene	nd	07.8 ± 0.27	19.6 ± 0.41	35.6 ± 0.3	42.9 ± 0.41	38.9 ± 0.12
n-Butanol	8.4 ± 0.12	15.7 ± 0.35	20.8 ± 0.44	20.7 ± 0.4	22.7 ± 0.5	22.7 ± 0.27

Data are the mean of triplicates ± standard deviation; nd: not detected

Table 2. Effect of 2% water immiscible carrier solvents on bioconversion of AD to ADD by *Delftia acidovorans* MTCC 3364.

Carrier solvents	Mol % conversion ± S.D. after incubation period (h)					
	12	24	36	48	60	72
Control	29.2 ± 0.15	39.7 ± 0.42	50.4 ± 0.21	67.6 ± 0.76	58.9 ± 0.45	55.5 ± 0.3
Diethyl ether	32.7 ± 0.62	47.7 ± 0.15	54.9 ± 0.35	72.1 ± 0.41	80.8 ± 0.36	78.9 ± 0.56
Methylene chloride	32.9 ± 0.59	45.3 ± 0.15	50.2 ± 0.4	70.5 ± 0.15	43.5 ± 0.48	83.8 ± 0.27
Chloroform	1.24 ± 0.35	20.5 ± 0.76	31.6 ± 0.48	41.5 ± 0.65	86.2 ± 0.35	49.1 ± 0.5
Ethyl acetate	19.5 ± 1.02	29.6 ± 0.76	41.4 ± 0.36	64.7 ± 1.01	53.0 ± 1.01	45.2 ± 0.45
Toluene	06.3 ± 0.36	15.4 ± 0.56	41.3 ± 0.81	42.2 ± 0.56	17.9 ± 0.25	46.7 ± 0.36
n-Butanol	05.6 ± 0.27	08.0 ± 1.17	13.5 ± 0.15	18.6 ± 0.25	55.5 ± 0.46	15.1 ± 0.2

Data are the mean of triplicates ± standard deviation; nd: not detected

Table 3. Effect of 3% water immiscible carrier solvents on bioconversion of AD to ADD by *Delftia acidovorans* MTCC 3364.

Carrier solvents	Mol % conversion ± S.D. after incubation period (h)					
	12	24	36	48	60	72
Control	25.6 ± 0.46	28.7 ± 0.45	33.6 ± 0.51	43.5 ± 0.7	59.9 ± 0.46	53.2 ± 0.61
Diethyl ether	17.3 ± 0.55	23.9 ± 0.46	31.9 ± 0.51	56.8 ± 0.52	52.7 ± 0.35	44.7 ± 0.66
Methylene chloride	15.3 ± 1.07	23.8 ± 0.71	35.0 ± 1.31	46.8 ± 0.56	55.0 ± 0.72	49.5 ± 0.68
Chloroform	nd	10.8 ± 0.48	33.6 ± 0.88	44.6 ± 1.53	40.6 ± 0.62	36.4 ± 0.52
Ethyl acetate	nd	nd	nd	nd	nd	nd
Toluene	nd	nd	06.6 ± 0.41	11.20 ± 0.32	28.9 ± 0.56	42.2 ± 0.56
n-Butanol	nd	nd	nd	nd	nd	nd

Data are the mean of triplicates ± standard deviation; nd: not detected

DISCUSSION

The bioconversion of steroidal precursors suffers a serious drawback of low substrate availability owing to the hydrophobic nature of the starting material. Co-addition of solvents (carriers) has shown tremendous improvement in the bioconversion processes like C-17 side chain cleavage and nucleus modification reactions (Wang *et al.,* 2008; El Refai *et al.,* 2010; Banerjee *et al.,* 2014; Pendharkar *et al.,* 2022). Thus, has potential of increasing the industrial productivity of the bioconversion reaction leading to improved performances and reduced costs.

Biotransformation of AD to ADD was enhanced when 2% of 1,4 dioxane and methylene chloride was usedas carriers. At lower concentration of carrier solvents, the product accumulated in fermentation broth is more as compared to control in both the cases (water miscible as well as immiscible organic solvents). The amount of product formed with 2% of organic solvents was increased to nearly 90 mol percent. Above 2% solvent concentration, the bioconversion of AD was reduced. The present work revealed the effect of organic solvents, at lower concentration of solvents the bioconversion is enhanced but as the concentration of solvent increased it inhibits the bioconversion. This result supported the conclusion of Miller (1985) that most of the organic solvents in a concentration range of 2-10% (v/v) damaged the functional integrity of enzyme-coenzyme system required for bioconversion of steroids. However the 1,4-dioxane, DMF, DMSO, ethanol, methylene chloride and diethyl ether exhibited low toxicity at lower concentration on bioconversion of AD to ADD by *D. acidovorans* MTCC 3364.

In case of biomass accumulation, water miscible solvents did not exert toxic effects on cell growth when compared with control. However, the water immiscible solvents showed toxic effects on cell growth with increase in concentration of solvents. In presence of chloroform, ethanol and propanol better cell growth was observed but cell unable to accumulate bioconversion product ADD. This demonstrated that the bioconversion is independent of cell biomass. After 60 hours of incubation period, the rate of bioconversion of AD was arrested, possibly due to the accumulation of product in fermentation broth. As observed, the mol % bioconversion of AD was stopped at a particular value in all experiments done considering controls. This could be attributed to the toxicity exerted by the product ADD. The concentration of ADD above

0.8 to 1.0 mg/ml of has been shown to exert toxic effect on the bacterial cells (Srivastava, 1985; Gottschaldt *et al.*, 1993). Another plausible explanation could be that the substrate concentration falls below the critical level required for the uptake or entry of the substrate in the bioconversion system.

Conflict of Interest: Authors declare no conflict of interest.

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