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ORIGINAL ARTICLE

Biotransformation of cholesterol to 1,4-androstadiene-3,17-dione (ADD) by *Nocardia* species

Preeti Sharma · Parvez S. Slathia · Priti Somal · Pardeep Mehta

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Abstract *Nocardia* sp. was isolated from an exotic soil of the northwestern Himalayas and was capable of selectively cleaving the side chain of sterols (cholesterol and phytosterol) yielding androstane steroids, in the presence of a hydrophobic metal chelating agent, after an incubation period of 24 h. *Nocardia* sp. was identified on the basis of morphological and biochemical characteristics accomplished with 16S rDNA sequencing. An extracellular production of 1,4-androstadiene-3,17-dione (ADD) was observed in the fermentation medium. The conversion studies were carried out with a cholesterol concentration ranging from 0.3 to 3 g/l, but the fermentation conditions in biotransformation experiments gave the maximum yields (theoretical yield was 90 %) at 0.5 g/l cholesterol concentration with pH 7.2 in the presence of Tween 80 concentration 2 g/l; in addition, th effects of the media were also studied.

Keywords *Nocardia* sp. · 1,4-androstadiene-3,17-dione (ADD) · Cholesterol

P. Sharma (✉) · P. S. Slathia
School of Biotechnology, Shri Mata Vaishno Devi University,
Kakrial,
Katra, Jammu & Kashmir, India 182320
e-mail: preeti.res@gmail.com

P. Somal
Indian Institute of Integrative Medicine (CSIR),
Near Irrigation Office,
Jammu, Jammu & Kashmir, India 180001

P. Mehta
Microbial Institute of Technology, University of Saugar,
Madhya Pradesh, India 470003

Introduction

Production of [1,4-androstadiene-3,17-dione (ADD) and 4-androstene-3,17-dione (AD)] androstane steroids from sterols involves the use of microbes (Kieslich 1985). It is one of the most successful microbial technologies due to the high regio- and stereo-selectivity associated with these biological reactions. The use of whole cells is prefered in these microbial conversions due to the necessity of cofactor regeneration and the possibility of multistep conversions with a single biocatalyst (Fernandes et al. 2003).

Microbial selective side chain cleavage of the sterols (β-sitosterol, stigmasterol, cholesterol) is the only biological method available for the production of ADD and AD (Ahmed and Johri 1991). The demand of ADD and AD compounds exceeds 1,000 tons per year (Biojournal 2000). AD and ADD, belonging to the family of 17 ketosteroids, have served as potential substrates for the production of sex hormones, anticoncipients, and antiphlogistics as well as blood pressure regulating agents (Ahmed et al. 1992, 1993; Mahato and Garai 1997; Fernandes et al. 2003). AD has been the starting material for the preparation of androgens and anabolic drugs and more recently for the production of spironolactone (Fernandes et al. 2003; Malaviya and Gomes 2008). ADD has served as a precursor for estrogens and other contraceptive agents. To meet this increasing demand, more than 60% of the raw materials for the steroid drugs are produced by the selective microbial side chain cleavage of sterols (Szentirmai 1990), which are abundantly present in the form of phytosterols (a plant origin) and cholesterol (an animal origin).

The chemical synthesis of these steroids having pharmacological effects associated with them has attracted the attention of various researchers. The microbial bioconversions have

been well established, and are still preferred as they are considered to be environment friendly (Fernandes et al. 2003). An effort to an increase in the efficiency of the existing processes as well as isolation of microorganisms capable of performing the desired transformations can help us in giving large-scale industrial processes. Production of AD and ADD as the side chain cleavage product of sterols (β-sitosterol, stigmasterol, cholesterol, ergosterol, and campesterol) has been reported from various bacteria and fungi genera *Arthrobacter, Bacillus, Rhodococcus, Pseudomonas, Mycobacterium, Corynebacterium, Nocardia, Clostridium, Micrococcus, Brevibacterium, Streptomyces, Serratia, Protoaminobacter* and *Fusarium* (Ahmed and Johri 1991; Wilson et al. 1999). The conversion of cholesterol to AD and ADD from soil isolates, *Rhodococcus* sp. (Ahmed and Johri 1991) and *Micrococcus roseus* RJ6 (Dogra and Qazi 2001) has highlighted the preferential use of cholesterol as a substrate. Lee et al. (1993) reported ADD yield from cholesterol, using two-step microbial transformation. Cholesterol was initially converted to cholestenone by *Arthrobacter simplex* U-S-A-18. Cholestenone was prepared directly from the fermentation broth of *A. simplex* and converted to ADD by *Mycobacterium* sp. NRRL B-3683. Conversion of AD to ADD has been reported in mixed culture of *Mycobacterium–Nocardioides* by Perez et al. (2003a). ADD yield by *Mycobacteria* sp. was enhanced by the addition of fresh cultures of *Nocardioides.* The 1,2-dehydrogenase activity from *Nocardioides simplex* NCIMB 8929 was used for the conversion of AD to ADD. Fermentation of cholesterol and its conversion to AD was carried out using the mycobacterial strain, *Mycobacterium* sp. MB3683.

Research of potentially useful steroids has highlighted the need for the isolation of microorganisms capable of performing the required transformations. Many reports on the isolation of microorganisms capable of producing AD and ADD by utilizing cholesterol as a substrate have been reported in the literature (Ahmed and Johri 1991). The enrichment of a bacterial population as a selective mechanism for isolating strains capable of utilizing cholesterol as sole source of carbon and energy is in agreement with the earlier observations (Ahmed et al. 1992). Compared to earlier studies (Lee et al. 1993; Perez et al. 2003b), present studies report the isolation of a novel isolate which was capable of producing ADD as a single isolate when transformation was terminated at 96 h, and the yields of ADD and AD by *Nocardia* sp. were higher using cholesterol as a substrate rather than β-sitosterol. The low conversion yield of stigmasterol as a substrate has been explained due to presence of the C-22 double bond having a depressing effect on degradation activity of microbial strains (Fernandes et al. 2003). Cholesterol as a substrate has good properties for microbial side chain oxidation (Dias et al. 2002; Donova et al. 2005), and the steps involved for the conversion of sterols to AD and ADD are: oxidation of the 3β-hydroxy group to the keto group; oxidative cleavage of the aliphatic side chain of sterol; isomerisation; and dehydrogenation.

Uptill now there has not been a single report where ADD has been found as a single isolate, after 120 h of fermentation, using single step microbial transformation. An improvement in the yield of ADD has been reported after nitrosoguanidine (NTG) treatment, and the mutants of mycobacterial strains were capable of tolerating 1 mg/ml dose of ADD, which the parent strain could not (Perez et al. 2003b). The fermentation studies for the production of androstane steroids from sterols revolves around the following parameters: to overcome the constraints like low solubility of the substrate; product feedback inhibition; long incubation periods; and facilitating culture–hydrophobic substrate interactions in order to obtain an increase in the rate of biotransformation (Malaviya and Gomes 2008).

In our present work, the potential strain was identified by 16S rDNA as *Nocardia* sp. which has the ability to degrade the cholesterol completely. Fermentation studies were carried out by *Nocardia* sp. in the presence of hydrophobic metal chelating agents for the conversion of sterols to an androstane steroid (Fig. 1).

Materials and methods

Chemicals

Substrates used were cholesterol and stigmasterol, purchased from Sisco Laboratories (Mumbai, India), while sitosterol (purity 98%) was purchased from Acros Organics (USA). Deodorized sterol mixture was procured from an unknown source and used as a substrate. Media ingredients used were obtained from Hi Media (India). All the chemical reagents used were of analytical grade and were from Ranbaxy and Hi-media Laboratories (India). Alugram Sil G/UV254 fluorescent TLC plates were obtained from Macherey-Nagel (Germany). Standards of AD and ADD used for identification of the biotransformed products were procured from Sigma-Aldrich (Banglore, India). All the reagents used for sequencing were from Applied Biosystems (USA). The DNA sequence thus obtained was aligned with the sequences in the GenBank by the BlastN program (Altschul et al. 1997).

Isolation of sterol degrading microorganisms and their maintenance

Screening studies were carried out after isolation of microbial strains by a culture enrichment technique employing a basal medium. The basal medium was incubated with a soil sample (1 g) obtained from the areas adjacent to hot springs (located in Manikaran region, Himachal Pradesh, India), and

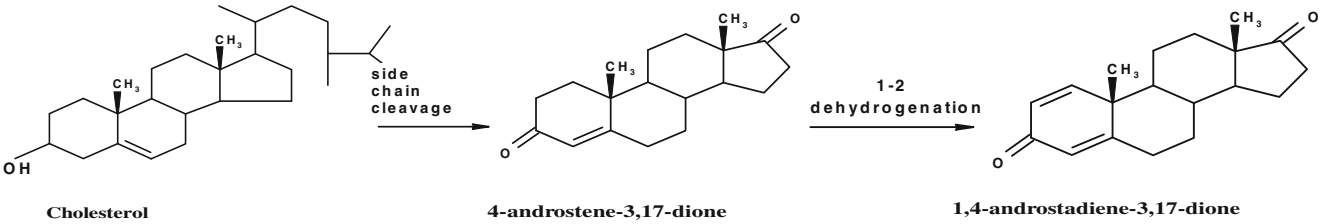

Fig. 1 Biotransformation of cholesterol to 1,4-androstadiene-3,17-dione by *Nocardia* sp

the potential strain was obtained which was capable of selectively cleaving the side chain of sterols (cholesterol, sitosterol, and stigmasterol), and producing androstane steroids (ADD and AD). The basal medium contained (w/v): NH_4NO_3 0.1%, KH_2PO_4 0.025%, $MgSO_4{\cdot}7H_2O$ 0.025%, NaCl 0.0005%, $FeSO_4$ 0.0005%, and cholesterol 0.05%, as a sole source of carbon (pH 7.2). Four fresh transfers of 1 ml mixed bacterial strains were made after an incubation of the basal medium with the soil sample (1 g). Enrichment (mixed) cultures were incubated at 30°C under shaking condition (220 rpm) for 1 week. The enriched (mixed) cultures were then transferred into the fresh basal medium for four consecutive times, and pure cultures (isolates) were obtained from the plating of an enrichment culture on the YET agar medium containing (w/v): yeast extract 0.5%, tryptone 1%, NaCl 5%, Tween 80 0.1% and agar 2% at pH 7.2. The colonies were picked up and purified by the streaking method. The cultures were grown on slants at 30° C and stored at 4°C. Identification was carried out by biochemical tests (data not shown).

Fermentation conditions

Thirteen colonies (isolates) were inoculated in the nutrient broth (25 ml) containing cholesterol (0.05% w/v, dissolved in 0.5 ml chloroform, and Tween 80 0.1% w/v, was also added), in 250-ml Erlenmeyer flasks. Incubation was carried out at 30°C under shaking conditions (220 rpm) for 96 h. The fermented broth extract was analyzed for the biotransformed products, ADD and AD, after regular intervals. One of the isolates that degraded the cholesterol completely and gave a positive result for the production of ADD in the presence of 2,2-dipyridyl was selected for further investigation. A 5- to 6-day-old culture was transferred from the slant to Tween 80 water (0.1% w/v) and shaken on vibro-mixer. The culture suspension was inoculated in the fermentation medium containing (w/v): yeast extract 0.5%, tryptone 1%, NaCl 0.5% and Tween 80 0.1% at pH 7.2. The seed culture was grown for 24 h at 30°C on a shaker at 220 rpm in an Erlenmeyer flask (250 ml) containing 25 ml of YET medium supplemented with cholesterol (0.05%, w/v dissolved in 0.5 ml chloroform). Samples (1 ml) were taken and suspended in the fermentation medium with cholesterol to an OD_{600} of 0.8. For the biotransformation experiments, 2 ml of inoculum was added to 25 ml of above stated medium, and incubated on rotary shaker (220 rpm) at 30°. After 16 h of growth or as specified in the experiments, metabolic inhibitors (2,2-dipyridyl or 8-hydroxyquinoline 1.5 mM) were added to prevent sterol ring degradation. Samples were taken for the qualitative and quantitative analysis, after regular intervals. All the experiments were performed with controls without sterol and culture.

Extraction and isolation of transformed metabolites

The fermented broth was transferred to the separating funnels after an incubation period of 120 h or as stated in the further experiments. Extractions were carried out by adding twice the volume of ethyl acetate. The process was repeated thrice and the ethyl acetate extract was combined. Anhydrous sodium sulphate was added to the extract to remove the excess of moisture. Sodium sulphate was removed by filtration and the ethyl acetate extract was concentrated on a rotary evaporator (240 mbar pressure) at 40°C. The controls were processed similarly.

Steroid analysis and identification

The concentrated ethyl acetate extract (10 μl) was applied (2 μl aliquots) onto Alugram Sil G/UV254 plates (Macherey-Nagel) for thin layer chromatography (TLC). These plates were developed in benzene/ethyl acetate (4:1 v/v) and visualized by spraying with 1% ceric ammonium sulphate in sulphuric acid followed by heating at 110°C. TLC analysis of the ethyl acetate extracted incubation mixtures revealed two colored spots and a dull spot of untransformed sterol. The R_f values of the two colored spots running immediately below the sterol/s on the TLC plate matched with the authentic standards of AD and ADD. All the metabolites produced a spectrum of different colors with ceric ammonium sulphate reagent. The identification of the metabolites was further confirmed by HPLC, H^1NMR, IR and mass spectral data.

Using preparative TLC, cholesterol metabolites were isolated and their structures were determined by ^{1}H nuclear magnetic resonance at 500 MHz (DRX 500 NMR system; Bruker, Switzerland). The red colored spot on the TLC plate had downfield signals for 1-H (δ 7.04) and 4-H (δ 6.24)

showing dehydrogenation in ring A. The structures were further confirmed using Fourier transform infrared (FT-IR) spectroscopic analyses which were carried out with an FT-IR impact 410 spectrometer (Nicolet, USA). The IR peaks were also identical to that of authentic ADD (Table 1). AD was produced as a minor metabolite and its structural confirmation was depicted by change in the downfield shift of the 4-H (δ 5.76) signal. The IR bands (1,736 cm^{-1}, 1,661 cm^{-1}) confirmed the structure.

AD and ADD were not detected in control samples, which confirmed the role of the organism in cholesterol/sterol side chain cleavage.

High performance liquid chromatography (HPLC) was performed on Agilent 1100 series with Si 60 5 μm, 125× 4 mm Merck column using 3:2 isocratic mixture of hexane: isopropyl alcohol, at a flow rate of 0.5 ml/min.The UV chromatograms were recorded at 210 and 240 nm. The samples were analyzed at 30°C in order to achieve an efficient separation.

For mass spectrometry (MS), a Bruker Esquire 3000 MS (Germany) was used to identify steroid metabolites by direct injection of a sample into an atmospheric pressure chemical ionization (APCI) interface of the LC-MS system. Full scan mass spectra were acquired in positive ionization mode. Mass range was from m/z 50 to 500. The protonated ions $[M + H]^+$ at m/z 285 for ADD and 287 for AD were identified and considered for qualitative as well as quantitative LC/MS analysis by single ion monitoring detection.

The HPLC retention time (14.2) and molecular ion $[M + H]^+$ peak of this compound (m/z 284) as depicted by LC-MS coincided with the standard of ADD. For AD, the authenticity of the compound $[M + H]^+$ m/z 286, was identical to that of the standard AD.

For quantification of cholesterol, $[M- H_2O + H]^+$ ions were monitored at m/z 369. The calibration curves resulting from the reference compounds (cholesterol, AD and ADD) in the concentration range of 100–5,000 pg on column exhibited good linear correlation ($r^2 \geq 0.996$). The method was validated by analyzing six replicates of broth samples fortified with three compounds, viz. cholesterol, AD, and ADD at 0.050 and 0.5 μg/g levels. The mean recoveries for these fortifications ranged from 90 to 98% with RSD in the range of 3.36 to 9.78%. The method was developed to study the qualitative as well as quantitative conversion of cholesterol to AD and ADD by *Nocardia* sp. (Khajuria et al. 2007)

Table 1 Physical characteristics of the biotransformed products by *Nocardia* sp. isolated on preparative silica gel G-coated TLC plates

AD	ADD	
Melting point	141–142°C	171–172°C
R_f values	0.46±0.03	0.48±0.55
IR (cm^{-1})	1,738 cm^{-1} (17-C = O)	1,736 cm^{-1} (17-C =O)
	1,663 cm^{-1} (3-C = O)	1,661 cm^{-1} (3-C = O)
^{1}HNMR (δ ppm)	0.94 (C_{18}-H; 3) s	0.93 (C_{18}-H; 3) s
	1.25 (C_{19}-H;3)s	1.22 (C_{19}-H;3)
	6.24 (C_4-H; 1) d,j	5.76 (C_2-H;1) d,j
	7.04 (C_1-H; 1) d,j	

s singlet, *d* doublet, *j*coupling constant. Chemical shifts (δ) are expressed in ppm from TMS and coupling constants (*j*) in Hz

16S Ribosomal DNA sequencing

The 16S ribosomal gene analysis was followed by set of primers (forward primer, 5' CAGCAGCCGCGGTAATAC 3', and reverse primer, 5'ACGAGCTGACGACAGCCATG 3') used to amplify a ca. 500- to 700-bp fragment of this gene. The PCR conditions used for amplification were: initial denaturation at 94°C for 3 min followed by 30 cycles of 94°C for 15 s; 60°C for 15 s; 72°C for 30 s; and a final extension of 72°C for 5 min. The 50 μl reaction mixture contained 1×PCR buffer, 200 μM each dNTP, 1.5 mM of $MgCl_2$, 10 p mol of each primer, 1–10 ng of DNA and 2.5 U of Taq DNA polymerase. Then, 5 μl of the amplified product was checked on 1% (w/v) agarose gel. The amplified products were purified by Microcon-100 columns (Millipore, USA), and 40 ng were used in a 20-μl sequencing reaction using the Big Dye Terminator sequencing kit (v. 3.0) and loaded on ABI Prism 310 Genetic Analyzer (Perkin-Elmer) for sequencing.

Optimization of fermentation parameters for production of ADD

The effects of media constituents on the conversion of cholesterol at 0.5 g/l concentration were used to yield 1,4-androstadiene-3,17-dione as a single end product. Other than the YET medium, three other media (M5, M8, and M9) with different constituents were tested. Media composition: M5 (w/v) (NaCl 0.5%, beef extract 0.5%, peptone 1%, pH 7.2): M8 (w/v) (NaCl 0.5%, yeast extract 0.5%, tryptone 1%, glucose 0.5%, pH 7.2): M9 (w/v) (NaCl 0.5%, yeast extract 0.5%, tryptone 1%, Tween 80 0.01%, pH 7.2) were used. In order to study the effect of the nitrogen/carbon source, phosphate buffer (pH 7.2) with cholesterol as carbon source was used for fermentation studies. pH of the medium was initially adjusted in the range of 4.0–12.0 (before autoclaving using 0.5 M NaOH/HCL) for studying its effect on the process of biotransformation. In order to determine the effect of the substrate concentration on production of ADD, five concentrations of cholesterol (0.3–3.0 g/l) were dealt with. Mode of addition of substrate: sterol dissolved in 1 ml chloroform was added in the biotransformation (fermentation) medium (containing 0.l% w/v Tween 80) prior to autoclaving which led to the formation of an emulsion.

The effects of the surfactants (Tween 80, Tween 20) at different concentrations in the YET media were studied to determine the growth of the microorganism and its ability to utilize cholesterol as a substrate for the biotransformation process. Simultaneous qualitative and quantitative routine analysis of the cholesterol, ADD, and AD in the fermentation broth was carried out by TLC and HPLC-MS. The sensitivity of the LC-APCI-MS method for the simultaneous detection made it the most suitable method for analyzing large numbers of samples with the varying concentrations.

Conversion yields of ADD or AD

Conversion of the substrates into ADD or AD was estimated according to Perez et al. (2003a):

$$\text{Conversion}(\%) = \frac{\text{Weight of androstenones/MW of androstenone} \times 100}{\text{Weight of sterol/MW of sterol}}$$

where MW is molecular weight. The substrate consumption was calculated on the basis of mass of the substrate unconsumed to produce AD and ADD compared to the mass of initial substrate provided and were expressed as percentages of the residual substrate.

Results and discussion

The isolated strain showed tolerance towards a substrate concentration of 3.0 g/l, and maximum ADD yield with the wild strain was 8 mg/l as compared to the earlier reports where the substrate used was cholesterol and the ADD produced was 5.28 mg/l (Perez et al. 2003b).

Isolation and identification of strain

An actinomycete was isolated that had the ability to consume cholesterol completely and to produce ADD as a single end product. It was identified as a slow growing Gram-positive filamentous bacterium, capable of reducing nitrite, characterized as: white-colored colonies appearing after 24 h which gradually changed to red-pink color, with entire edges developed in PDA medium after 5 days of incubation at 30°C. The culture was aerobic, mesophilic and urease as well as catalase positive. Negative results for oxidase and starch hydrolysis suggested that the organism was consistent with the description of *Nocardia* sp. as given in the 9th edition of Bergey's manual (Holt et al. 1994).

The alignment of the 16S ribosomal gene sequence with the sequences present in GenBank showed 97% identity with the genus *Nocardia* and was assigned as *Nocardia* sp. This strain is now a part of the culture collection of Department of Biotechnology, Regional Research Laboratory, presently known as the Indian Institute of Integrative Medicine, Jammu, India (Accession No: RRL 450).

Biotransformation of sterols

Nocardia sp. converts cholesterol preferentially as compared to phytosterols, and conversion rates were studied for β-sitosterol and stigmasterol, respectively (Table 2). Our results were in accordance with the earlier observations (Marsheck et al. 1972; Ahmed and Johri 1991). The formation of AD and ADD from sterols indicates the presence of sterol side chain oxidation, while the formation of ADD also shows the presence of delta-1-dehydrogenase activity. The modification of 3-beta-5-ol to 3-keto-4-ene-moiety was the result of 3-beta-ol oxidation and delta-5 to delta-4 isomerisation.

Bioconversion of cholesterol to ADD in shake flasks

The time-course studies of the cholesterol conversion by *Nocardia* sp. are presented in Table 3. ADD accumulated as a major end product and reached a maximum conversion yield of 25% in 120 h, while the yield of AD amounted to 0.6% (calculations were formulated by the equation stated above). Time-course studies have shown that with an increase in the incubation time AD is converted to ADD indicating the presence of 1, 2 dehydrogenase activity. Similar results have been reported in our previous paper while studying the side chain cleavage of progesterone by *Bacillus sphaericus* (Venugopal et al. 2008). AD and ADD production was observed in fermentation medium after an incubation of 24, 48, and 72 h, respectively. A 1:2 ratio of AD: ADD was seen, and later on, at 96 h, conversion of AD to ADD took place. ADD was obtained as an end product. The liquid chromatograph of fermented broth showed peaks, which were different from the products, and these disappeared in the late phase of cultivation. However, the mass

Table 2 Bioconversion of phytosterols to ADD and AD by *Nocardia* sp.; conversion (×%) = [weight of AD(D)/weight of substrate] × (MW substrate) / [MW AD(D)]×100 (*MW* molecular weight)

Substrate	Conversion %	
Sterols	ADD (1,4-androstadiene-3,17-dione)	AD (4-androstene-3,17-dione)
Stigmasterol	3.1	1.2
β-Sitosterol	4.3	1.1
Sterol mixtures (deodorized)	3.0	1.8
Cholesterol	16.30	0.6

Biotransformation of substrate (0.5 gl^{-1}) after an incubation of 96 h 2,2 –dipyridyl (1.5 mM) was added in fermentation broth (25 ml) after 16 h approximately. It was dissolved in 0.5 ml ethanol prior to addition

Table 3 Biotransformation of cholesterol at different concentrations to yield ADD and AD by *Nocardia* sp

	Initial cholesterol concentration	Incubation (h)			
		48	72	96	120
Androst-1,4-diene-3,17-dione	0.3 g/l	4.30	4.20	4.60	4.90
4-Androstene-3,17-dione		0.70	0.40	0.30	0.10
Residual cholesterol		0.20	0.10	0.20	0.30
Androst-1,4-diene-3,17-dione	0.5 g/l	17.30	16.30	21.70	25.0
4-Androstene-3,17-dione		6.50	8.00	0.60	0.50
Residual cholesterol		1.36	0.60	0.40	0.50
Androst-1,4-diene-3,17-dione	1.0 g/l	2.99	7.60	4.85	7.30
4-Androstene-3,17-dione		0.30	1.80	0.13	1.38
Residual cholesterol		7.00	12.27	7.20	0.13
Androst-1,4-diene-3,17-dione	2.0 g/l	1.15	4.77	2.85	4.10
4-Androstene-3,17-dione		0.11	1.40	0.05	0.11
Residual cholesterol		6.30	7.00	6.10	0.05
Androst-1,4-diene-3,17-dione	3.0 g/l	1.50	3.35	2.60	1.65
4-Androstene-3,17-dione		0.14	5.85	0.06	0.07
Residual cholesterol		7.70	10.15	0.55	2.93

Concentration of Tween 80 in YET medium was 2 mg/ ml; product yields were expressed as conversion % and inhibitor used was 2,2-dipyridyl. Units used for ADD and AD are Molar % but cholesterol is expressed as residual percentage

Mean of triplicate inhibitor added 1.5 mM after 24 h approx.

spectral analysis of these metabolites exhibited the formation of 24-bisnorchol-4-ene-3-one (HCBC), 22-dihydroxy-23,24-bisnorchol-4-en-3-one (HBC) and one unidentified compound (data not shown).

ADD and AD were not produced in controls, showing that the production of metabolites was organism and sterol dependent.

Effect of hydrophobic metal chelating agents on conversion of cholesterol to ADD

The key enzyme in the sterol ring fission is 9α-hydroxylase, therefore hydrophobic metal chelating agents which are inhibitors for the above stated enzyme were used for preventing the fission of the sterol ring (Goswami et al. 1984; Ahmed and Johri 1991). In order to determine the effect of an enzyme inhibitor on selective side chain cleavage of cholesterol, enzyme inhibitors 2,2-dipyridyl and 8-hydroxyquinoline were added to the culture grown for 16 h (Fig. 2). Simultaneous addition of the substrate and inhibitor resulted in the blockage of enzymes concerned with the bioconversion, leaving the substrate unutilized. Utilization of cholesterol started after an incubation of 12 h and it was used up by 96 h. In the absence of an enzyme inhibitor, 17-ketosteroids were not formed in the fermentation broth, so an important condition for the production of ADD and AD from cholesterol by *Nocardia* sp. was the need to add an enzyme inhibitor to prevent cleavage of sterol ring.

Inhibitor (2,2-dipyridyl or 8-hydroxyquinoline) concentration of 0.5 mM when added to the fermentation broth resulted in the absence of a biotransformed product, while use of 2.0 mM inhibitor concentration inhibited the growth of the culture. Usually, enzyme inhibitors are added to the stationary phase of a bacterial culture (Goswami et al. 1984). However, in our studies, addition of 2,2 dipyridyl as an enzyme inhibitor affected the product yields to a greater extent, and an optimized concentration was 1.5 mM in the culture grown for 24 h (data not shown). In earlier studies, a similar concentration of an enzyme inhibitor has been used for *Arthrobacter* sp. (Mathur et al. 1992). An increase in the yield of AD and ADD was observed on conversion of the cholesterol in the presence of 8-hydroxyquinoline, when the culture used was *Fusarium solani* (Sallam et al. 2005). However, the effect of different time intervals between the addition of substrate and inhibitor, i.e. 2,2-dipyridyl, on the conversion of cholesterol to ADD by *Nocardia* sp. was studied as shown in Fig. 2. Addition of inhibitor after 48 h resulted in a 20% decline of the desired product which may be attributed to the degradation of the sterol ring (data not shown), although an increase in androstane (AD + ADD) yield was observed at 72 h (Fig. 2). At 96 h incubation time, there was a decline in AD yield (0.6%) as shown in Table 4 with a gradual increase in ADD (22%) showing 1, 2-dehydrogenase ability of the culture.

Effect of pH

The effect of initial pH on the production of steroids was studied in the range of pH 4.0–12.0. There was a remarkable influence of initial pH values on the production of ADD. An increase in ADD yield was observed with an increase in pH

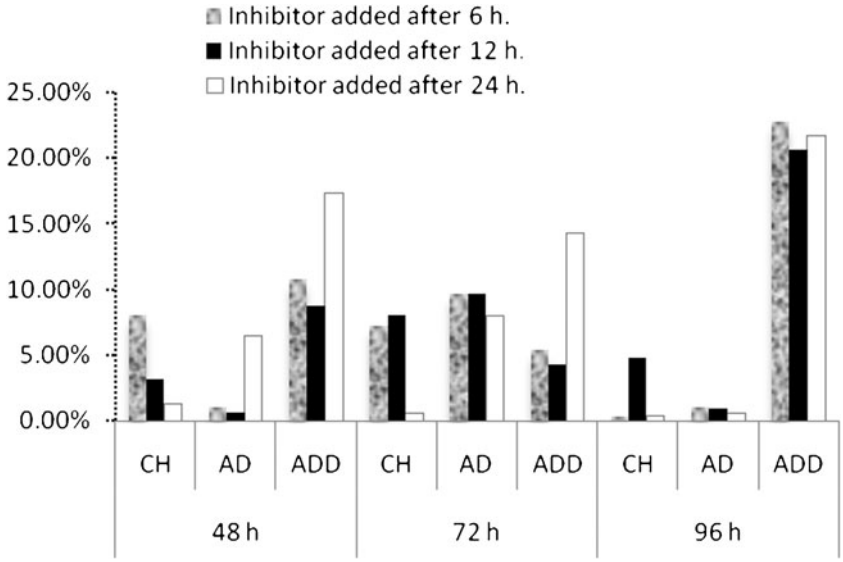

Fig. 2 Biotransformed products were calculated as: conversion (%) = weight of androstenones / MW of androstenone × 100/weight of sterol / MW of sterol. (*MW* molecular weight). Concentration of substrate was 0.5 g/l and the fermentation medium was YET with pH 7.0

above 5.0; maximum yield was 25% at pH 7.0, but a further increase in pH led to a decline in ADD production (Fig. 3). Llanes et al. (1995) had reported an increase in reduction reactions at pH 7.0 when sugars were added. But in our studies, the presence of glucose gave 14% yields of ADD. No production was found at pH 4.0 and 12.0, which was attributed to poor growth of the organism. Inhibition of biotransformation at pH 10 was possibly due to the inhibition of the C1 dehydrogenation reaction. The optimum pH for the bioconversion of cholesterol to ADD was 7.0.

Effect of media composition

ADD production and the cholesterol consumption was maximum when culture was grown in the YET media. In M5 media, beef extract and peptone were used as a nitrogen source and most of the substrate was not transformed because of the poor growth of the culture. On the other hand, yeast extract and tryptone served as a better nitrogen source (M9) for transformation of cholesterol. Angelova et al. (1996) and Venugopal et al. (2008) stated that nitrogen sources such as yeast extract produce high amounts of cells at the stationary phase with low biotransformation ability. In our experiments, cells in the stationary phase were harvested and biotransformation was carried out in phosphate buffer (pH 7.0) containing 0.5 g/l cholesterol as a substrate; the conversion of cholesterol to ADD was 19.0% (not shown in Fig. 4). However, in M8 media, in the presence of glucose (5 g/l) with yeast extract and tryptone as a nitrogen source, the yield of ADD was 14% (Fig. 4). The concentration of glucose in the biotransformation of cholesterol to ADD is an important parameter, whereas use of nitrogen sources such as yeast extract provided biomass with the ability to perform a biotransformation reaction. Carbon sources induce different metabolic activities or lead to different cell envelope characteristics (Borrego et al. 2000), thus interfering with substrate mass transfer, so production of ADD was hampered in the presence of glucose. Production of the biotransformed product was enhanced in the presence of Tween 80 as a surface active agent.

Table 4 Effect of types of enzyme inhibitor on the biotransformation of cholesterol to ADD and AD by *Nocardia* sp

Time duration (h)	Enzyme inhibitors			
	2,2-dipyridyl		8-hydroxyquinoline	
	ADD	AD	ADD	AD
24	13.00 (12,574.567 ng)	6.0 (5,920.0 ng)	2.00	0.90
48	17.00 (16,854.49 ng)	6.5 (6,271.0 ng)	4.00	0.2
72	16.30 (15,541.77 ng)	8.0 (742.16 ng)	2.00	0.8
96	21.7 (20,175.01 ng)	0.6 (649.824 ng)	ND	

Enzyme inhibitor was added at concentration of 1.5 mM after 24 h approximately and substrate used was cholesterol (0.5 mg/ ml).Medium used was YET. Product yields are expressed as Conversion % (Perez et al. 2003b)

ND not detected

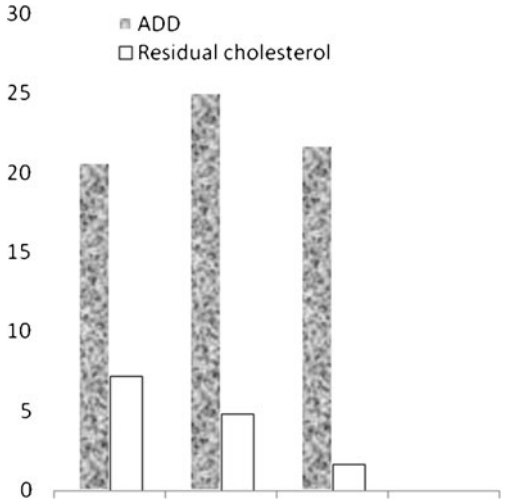

Fig. 3 Effect of pH (5.0, 7.0, and 8.0, respectively) on transformation of cholesterol (0.5 g/l) by *Nocardia* sp. in the presence of 2,2-dipyridyl (1.5 mM). Extractions were done at 120 h. AD values ranged from 0.3 to 0.6%

Effect of initial substrate concentration

Five different substrate concentrations in the range of 0.3 to 3 g/l were studied up to 120 h of incubation time (Table 3). At 0.5 g/l cholesterol concentration, maximum yields of ADD were achieved, after an incubation period of 96 h. With an increase in the incubation time, the formation of AD increased up to 72 h and then declined. In case of initial concentrations of cholesterol at 1, 2, and 3 g/l, production of ADD reached its maximum after incubation of 72 h, and at 96 h it declined. ADD conversion decreases from 48 to 72 h which shows that the culture is capable of utilizing ADD; however, the concentration of AD + ADD is higher at 72 h and an increase in ADD yield with a decline in AD yield at 96 h shows the 1,2-dehydrogenase ability of the culture. At 120 h, ADD was produced as a single isolate with AD concentrations dropping to 0.6%. The culture was showing

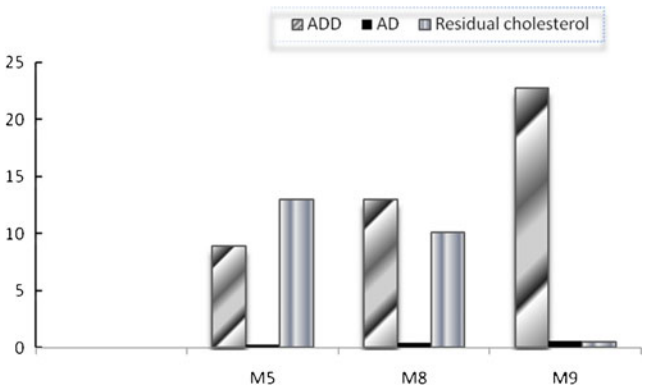

Fig. 4 Effect of media constituents (in presence of *Nocardia sp.*) on the biotransformation of cholesterol (0.5 g/l) to ADD and AD (conversion yield % on *y*-axis), respectively. Inhibitor added was 2,2-dipyridyl (1.5 mM) in 24 h culture, and extractions were done at 96 h

the ability of transforming the cholesterol completely at 0.5 g/l of initial concentration, while similar readings were observed at 0.3 g/l cholesterol concentration. The low productivity of ADD at a substrate concentration above 0.5 g/l may be considered as an ADD inhibitory effect. The culture had the ability to utilize the substrate even at concentrations up to 3.0 g/l. In order to enhance the production of the biotransformed product, the effect of Tween 80 was studied.

Effect of surfactant concentration

Tween 80 as a surfactant was most acceptable, as Tween 20 inhibited the production of ADD. The studies were in accordance with Smith et al. (1993). Three different concentrations (1, 2, and 3 g/l) of Tween 80 were studied using cholesterol as a substrate. Ethyl acetate extractions of (96 h) fermented broths were collected and analyzed for end product. ADD yields were 25% when the concentration of Tween 80 was 2 g/l (Table 5). Tween 80 was added as a surfactant, which altered the components of the cell wall causing changes in mycolic and fatty acid composition (Smith et al. 1993; Sripalakit et al. 2006). ADD yields were obtained with a significant increase in the growth of the culture, but an increase above 3 g/l in Tween 80 concentration was found to be toxic to the culture (data not shown).

Various biotransformation systems have been set up in the earlier studies, e.g., an organic–aqueous phase system, where Cruz et al. (2001, 2002) concluded that the biotransformation takes place at the organic–aqueous interface. The biotransformation of β-sitosterol to AD using *Mycobacterium* sp. NRRLB-3805 in the case of biphasic medium depended on the solvent molecular structure and its specific interactions with the cell envelope, which determined the catalytic behavior of the cells, in our case, chloroform was used as an organic solvent. Studies have shown that the choice of solvent also affects the product concentration; in addition, the water vesicles containing the biocatalyst when homogenously dispersed in the surfactant-rich medium (single phase) reduce the resulting toxic substrate inhibitory effect as well as the organic phase effect (Malaviya and Gomes 2008).

Table 5 Effect of Tween 80 surfactant on biotransformation of cholesterol to ADD and AD by *Nocardia* sp

Tween 80 concentration (g/l)	Conversion (%)		Residual cholesterol (%)
	ADD	AD	
1	14.1	0.4	28.2
2	21.7	0.4	0.6
3	12.8	0.5	20.6

Concentration of cholesterol was 0.5 g/l and extractions were done at 96 h, pH 7.0, and the medium used was YET. Enzyme inhibitor was added at concentration of 1.5 mM after 24 h approx.

Conclusion

Bioconversion yields of cholesterol to ADD were affected when different media constituents were used. An optimized medium concentration when subjected to varied concentrations of the inhibitor had an enormous effect on the conversion efficacy; however, optimized inhibitor concentration when added at different time intervals had a tremendous effect on the yields. The substrate concentrations used in the optimized medium varied from 0.3 to 3 g/l and the effect of initial pH was studied (4.0–12.0); maximum yields were observed at pH 7.0 with a substrate concentration of 0.5 g/l. The effect of the concentration of Tween 80 was also studied. In our studies, 2 g/l concentration of Tween 80 was considered as an optimum for utilization of cholesterol (0.5 g/l) within 96 h, and the yields were higher in the parent strain than the earlier reports by Perez et al. (2003a). The pH effect also showed an increase in the yield of ADD at pH 7.0 in the absence of glucose as a carbon source.

Our studies showed that, in the presence of nitrogen sources such as yeast extract (5 g/l) and tryptone (10 g/l), the culture was showing 25% transformation ability. The addition of 2,2-dipyridyl, inhibitor concentration 1.5 mM, in the culture grown for 24 h was mandatory for obtaining the maximum yield of ADD in 25 ml of fermented broth containing a cholesterol concentration of 0.5 g/l. Chloroform was used as a solvent for dissolving the sterols and added to the fermentation medium when the inoculums used were 2 ml.

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