SUBSTRATE CARRIERS FOR C-1(2)-DEHYDROGENATION OF 6-METHYLENE ANDROSTENEDIONE TO EXEMESTANE BY GROWING AND IMMOBILIZED *ARTHROBACTER SIMPLEX* NCIM 2449

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ABSTRACT

Objective: Permeability of hydrophobic steroid substrates across cell membrane is a critical factor during microbial bioconversion. To increase substrate intake, the feasibility of some organic solvents and emulsifiers as a substrate carrier on the bioconversion of 6-methylene androstenedione (6-MeAD) to exemestane was assessed.

Methods: AD, a commonly available steroid precursor, was chemically converted 6-MeAD. The time course of exemestane accumulation was estimated after addition of 6-MeAD dissolved in some organic solvents or dispersed with emulsifiers by growing and immobilized cells of *Arthrobacter simplex* NCIM 2449 in shake flask cultures.

Results: The use of substrate carriers for addition of 6-MeAD enhanced the bioconversion several folds. With growing bacterium in triplicate flasks, a peak mol % bioconversion recorded was- ethanol (67.25, 72 hrs); soybean oil + tween 80 (50.37, 48 hrs); acetone (38.84, 48 hrs); soybean oil (38.36, 48 hrs); lecithin (32.73, 48 hrs), methanol (32.71, 48 hrs) and tween 80 (10.37, 48 hrs). As compared to the growing cells, the bioconversion with Ca-alginate immobilized cells was delayed, and peak mol % bioconversion was recorded as ethanol (60.78, 120 hrs); soybean oil + tween 80 (42.98, 120 hrs); methanol (40.50, 72 hrs); soybean oil (38.36, 48 hrs); acetone (31.18, 72 hrs), and lecithin (33.67, 120 hrs); tween 80 (13.87, 120 hrs).

Conclusion: The use of substrate carriers for addition of 6-MeAD increased the permeability of substrate and may be used to increase the yield of exemestane and reduce incubation time.

Keywords: Steroid substrate carrier, Exemestane, *Arthrobacter simplex* NCIM 2449, Breast cancer, Steroid C-1(2)-dehydrogenation, 3-ketosteroid-delta-1-dehydrogenase.

INTRODUCTION

Exemestane (6-methylene androstadienedione [ADD]) is a suicidal aromatase inhibitor that lowers estrogen production in the body [1]. This drug is being used individually for the treatment of postmenopausal breast cancer patients [2] and under Phase III trial valuation in combination with bevacizumab [3]. Various schemes have been patented for chemical synthesis of exemestane depending on starting precursor such as androstenedione (AD) [4,5], boldenone [6], ADD [7], 6-methylene AD (6-MeAD) [8], and testosterone [9]. However, the involvement of multiple steps, use of hazardous chemicals like selenium oxide and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone required for the final step of C-1(2)-dehydrogenation and formation of extremely toxic hydrocyanic acid gas make these methods undesirable for the synthesis of exemestane. The sequence of reactions involved in the synthesis of exemestane from AD is presented in Fig. 1.

Alternative to the hazardous chemical reaction, biochemical reactions catalyzed by enzymes are preferred. Several microbial genera are known to secrete an enzyme 3-ketosteroid-Δ¹-dehydrogenase (ksdD, EC 1.3.99.4), which catalyzes C-1(2)-dehydrogenation of a variety of steroid substrates. *Arthrobacter simplex* has been frequently used for C-1(2)-dehydrogenation of steroids substrates such as hydrocortisone to prednisolone [10,11] and 16-methyl Reichstein's compound S-21 acetate [12]. Resting cells of *A. simplex* have also been used for the bioconversion of 6-MeAD to exemestane in the presence of water immiscible organic solvent and an exogenous electron carrier [13].

Microbial C-1(2)-dehydrogenation of 6-MeAD to exemestane by *Nocardioides simplex* VKM Ac-2033D has also been reported,and the process is patented [14,15].

Aggregation of steroidal substrate particles pose major mass transfer problem that decreases the bioavailability of substrate during the bioconversion, and a variety of methods have been adopted to obtain finely dispersed suspension of the substrate in the medium. Frequently, steroidal substrates have been added in the form of their solution in water miscible organic carrier solvents such as ethanol, dimethylformamide, acetone, dimethylsulphoxide, and methanol [16-19]. However, this method has limitations imposed by the toxic effect of the carrier solvents on the microorganisms at higher concentrations.

Water-immiscible organic solvents have also been used in biphasic systems and the toxicity exerted by various organic solvents and solvent mixtures on steroid-1(2)-dehydrogenase activity of *A. simplex* have been worked out [20]. The use of natural oils as carriers yielded higher conversion of soybean sterols to 17-ketosteroids by mycobacteria [21,22]. Using lecithin and tween 80 as substrate dispersing agents, higher yields of ADD have been reported [23,24]. This work was carried out to assess the feasibility of some organic solvents and emulsifiers as dispersing agents to increase mass transfer of substrate so that an efficient and eco-friendly alternative for C-1(2)-dehydrogenation of 6-MeAD to exemestane can be developed.
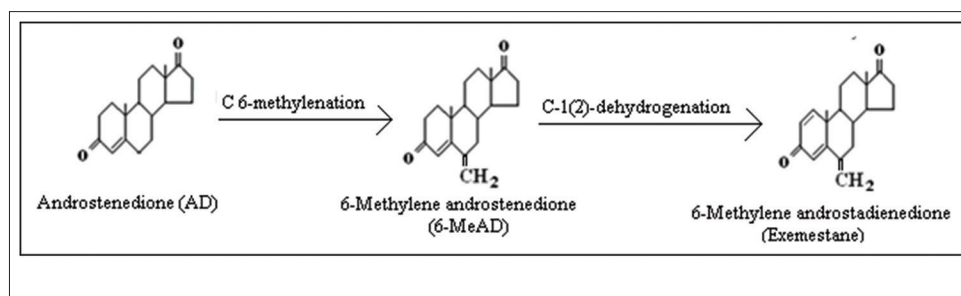
Fig. 1: Sequence of reactions involved in the synthesis of exemestane from androstenedione.

METHODS

Synthesis of 6-MeAD

AD procured from Jagsonpal Pharmaceuticals, New Delhi was converted to 6-MeAD following the method disclosed earlier [25] with suggested modification [26] as follows: 10 g of AD was dissolved in solvent mixture of 50 ml dioxane, 12 ml trimethylorthoformate and 10 ml absolute ethyl alcohol, heated to 45°C on a magnetic stirrer and 0.6 g *p*-toluenesulfonic acid was added. After 1 hr, 4 ml *N*-methyl aniline and 4.5 ml formaldehyde (37%) were added, and the reaction was continued for 3 hrs at 50°C. The reaction solution was cooled to 0°C, acidified by dropwise addition of 25 ml concentrated HCl and the reaction was continued further for 1 hr at 5°C. The reaction solution was diluted with 500 ml distilled water pre-cooled to 10°C, stirred for 1 hr and the precipitated solids were filtered, washed with pre-cooled 80 ml distilled water and vacuum dried. The dried precipitate was dissolved in 40 ml ethanol, decolorized with charcoal and crystallized from the filtrate. The crystalline product was dried under vacuum, weighed and subjected to thin layer chromatography (TLC), ultraviolet (UV), and infrared (IR) spectroscopic analyses.

Organism

Arthrobacter simplex NCIM 2449 was purchased from National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune.

Bioconversion of 6-MeAD by growing cells

About 30 ml nutrient broth (pH 7.2) was dispensed in 150 ml capacity Erlenmeyer flask, and 30 mg 6-MeAD was dispersed with glass beads on a gyratory shaker (control). Alternatively, the substrate was dissolved in 0.5 ml water soluble carrier (methanol/ethanol/acetone) and added to the medium with continuous stirring. When soybean oil was used as a carrier, 30 mg substrate was dissolved in 4.8 ml soybean oil or soybean oil containing 5 mg tween 80 and added to the medium. In case of lecithin (5 mg, hydrated with 1 ml distilled water) and tween 80 (5 mg), each of these ingredients was macerated with the 30 mg substrate in pestle and mortar and dispersed by addition of nutrient broth to make up the volume to 30 ml. After sterilization at 121°C for 15 minutes, the flasks were inoculated aseptically with 1 ml actively growing culture of *A. simplex* NCIM 2449 and incubated at 32±1°C on a gyratory incubator shaker (180 rpm, 2.5 mm eccentric throw).

Bioconversion of 6-MeAD by Ca-alginate immobilized cells

The method described by Kierstan and Bucke was adopted for immobilization of microbial biomass [27]. *A. simplex* NCIM 2449 was grown in four 500 ml capacity Erlenmeyer flasks, each containing 100 ml nutrient broth adjusted to pH 7.3. After sterilization, the flasks were inoculated with actively growing culture of *A. simplex* NCIM 2449 and incubated at 32±1°C on gyratory incubator shaker. After 72 hrs growth period, the biomass was aseptically harvested by centrifugation at 10,000 rpm for 10 minutes, washed twice with 0.85% saline and suspended in 200 ml sodium alginate solution. After thorough mixing on a cyclomixer, the alginate-biomass mixture was added dropwise through syringe into chilled 0.2 M calcium chloride solution. The beads were hardened overnight in 0.2 M calcium chloride solution at 4°C, washed with phosphate buffer (pH 7.0, 0.07 M) and stored in 0.02 M

calcium chloride solution at 4°C in refrigerator. For bioconversion, 10 g beads were taken in 150 ml flasks containing 30 ml sterile phosphate buffer (pH 7.0, 0.07 M). The substrate 6-MeAD was added to the reaction buffer as described above replacing buffer in place of nutrient and incubated on a gyratory incubator shaker at 32±1°C. Samples of reaction buffer were taken after regular intervals and processed for qualitative and quantitative analyses.

TLC

The samples of the incubation medium or reaction buffer were drawn periodically and subjected to qualitative and quantitative analysis of exemestane. To 1 ml sample of medium in an eppendroff tube, 0.5 ml chloroform was added and thoroughly mixed on a cyclomixer. After centrifugation at 5000 rpm for 5 minutes, lower chloroform phase was transferred to another tube and dried over anhydrous sodium sulfate. 10 μl extract was spotted on pre-coated TLC plates (silica gel 60 F254, Merck, Darmstadt) along with authentic sample of exemestane obtained from Cipla Ltd., Mumbai, India, and the plates were developed in benzene-ethyl acetate (5:2). The spots were visualized by spraying the plate with 50 % sulfuric acid followed by heating at 110°C for 5 minutes. In the case of oil as carrier solvent, sample of incubation medium was centrifuged at 10,000 rpm for 10 minutes, 0.1 ml oil phase was transferred to another eppendroff tube, mixed thoroughly with 0.5 ml methanol on a cyclomixer, centrifuged at 10,000 rpm for 10 minutes and upper methanolic phase was dried under vacuum. The residue was dissolved in 0.5 ml chloroform and subjected to TLC analysis as described above. Identification of exemestane spot on TLC plate was done by comparison of R_f value, color of the spot with authentic sample and spectroscopic methods.

Quantification of exemestane

Chloroform extract of the incubation medium (0.25 ml) was dried under vacuum, the residue was dissolved in 0.5 ml acetonitrile, filtered and 10 μl was subjected to high-performance liquid chromatography (HPLC) analysis as described earlier [28]. The specifications and the parameters of HPLC analysis were: Equipment JASCO Make, Model 1580; column: ODS HYPERSIL C 18 (250 mm × 4.6 mm); column temperature 45°C; Detection wavelength 245 nm; mobile phase acetonitrile: water (60:40); flow rate 0.45 ml/min; total run time 20 minutes, injection volume 10 μl.

Bioconversion, isolation, and purification of exemestane

Bioconversion of 6-MeAD was carried out in six 500 ml capacity Erlenmeyer flasks, each containing 100 ml nutrient broth. Substrate 100 mg dissolved in 2 ml ethanol was added in each flask with continuous stirring, sterilized and inoculated with 5 ml actively growing culture of *A. simplex* NCIM 2449. After 96 hrs incubation on gyratory shaker, the fermentation broth of all flasks was combined and extracted with 3 ml × 100 ml chloroform. The chloroform extract was dried over anhydrous sodium sulfate and chloroform was recovered in rotary flash evaporator. The obtained residue was dissolved in ethyl acetate, decolorized with charcoal; the filtrate was concentrated to 30 ml and cooled to 4°C in refrigerator for crystallization. Crystals separated after overnight cooling were collected by filtration, washed with chilled ethyl acetate and dried under vacuum.

RESULTS

Identification of product of chemical synthesis

10 g of AD taken for chemical synthesis yielded 7.136 g of crystalline product, which was identified as 6-MeAD on the basis of following characteristics:

- Color of spot on TLC plate: Bluish green; R_f - 0.46 (Fig. 2a)
- UV absorption spectrum: λ_{max} 261.4 nm (Fig. 2b)
- IR spectrum (KBr): Peaks, cm^{-1} at 3832.3, 3793.7 (6-CH$_2$), (17-C=O); 1454.2 and 1334.6 (Δ^4-3-C=O).
- The molar yield of 6-MeAD was 68.48 mol %.

Identification of bioconversion product

A total of 500 mg 6-MeAD was added in five bioconversion flasks and yielded 245 mg crystalline product. The product was identified as exemestane on the basis of following characteristics:

- Color of spot on co-TLC plate: Reddish pink; R_f - 0.40 (Fig. 2a)
- UV absorption spectrum: λ_{max} 245.5 nm (Fig. 2c)
- The molar yield of exemestane was 49.33 mol %.

HPLC analysis of crystalline bioconversion product indicated a single peak (Fig. 3) at average retention time (6 runs) at 11.67±0.28 minutes (mean±standard deviation). A fairly proportional relationship was observed between exemestane concentration (2.5, 5.0, 7.5 and 10.0 µg/ml) and peak area (r^2=0.9855) and was used for quantification of exemestane formed during the bioconversion.

Mole % bioconversion of 6-MeAD to exemestane by growing cells of *A. simplex* NCIM 2449 with different carriers is presented in Table 1. In the case of control and tween 80, no exemestane was detected in the medium at 24 h incubation period, whereas a rapid accumulation of exemestane was recorded with other carriers. As compared to control, the use of all other substrate carriers increased C-1(2)-dehydrogenation activity of *A. simplex* NCIM 2449. Peak bioconversion was recorded
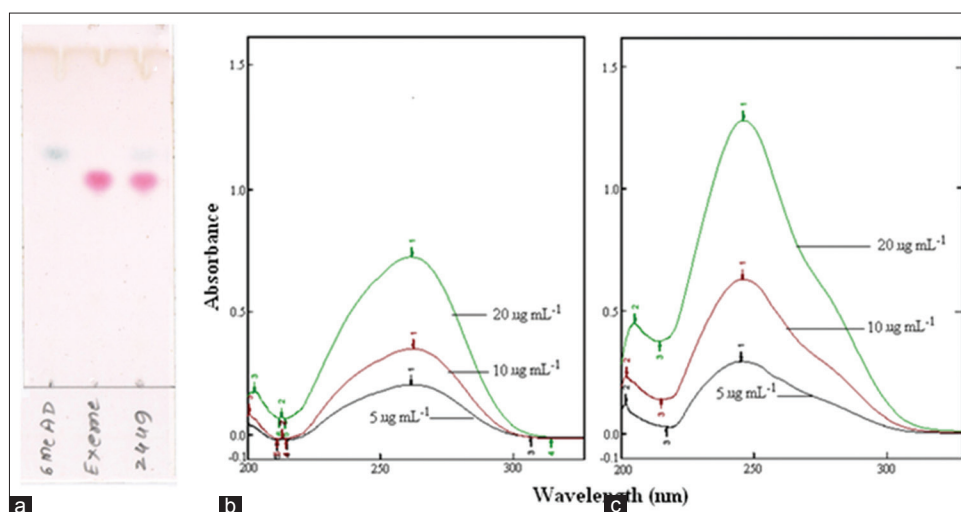

Fig. 2: Thin layer chromatography scan (a) and ultraviolet absorption spectra of 6-methylene androstenedione (b) and exemestane (c)

Table 1: Mol % bioconversion of 6-MeAD to exemestane by growing cells of *A. simplex* NCIM 2449

Substrate carrier	Mol % bioconversion±SD after incubation period (hrs)				
	24	48	72	96	120
Control	nd	nd	3.45±0.30	7.45±0.32	15.07±0.45
Methanol	31.53±1.81	32.71±2.05	20.53±0.93	18.31±1.13	16.10±0.09
Ethanol	29.45±2.30	55.6±2.05	67.25±3.04	63.19±2.96	60.68±0.36
Acetone	25.88±1.90	38.84±1.62	25.31±1.87	23.67±1.33	23.32±1.26
Soybean oil	34.52±1.63	38.36±1.92	24.22±1.75	20.14±1.52	18.73±0.98
Soybean oil+tween 80	38.97±2.02	50.37±2.21	32.62±2.30	28.44±1.56	28.02±1.44
Tween 80	nd	10.37±1.69	6.90±0.64	3.42±0.44	4.09±0.56
Lecithin	30.11±2.14	32.73±1.70	21.84±1.91	21.27±1.49	18.55±1.52

SD: Standard deviation (N=3), nd: Not detected, *A. simplex*: *Arthrobacter simplex,* 6-MeAD: 6-methylene androstenedione, NCIM: National Collection of Industrial Microorganisms

Table 2: Mol % conversion of 6-MeAD to exemestane by Ca-alginate immobilized cells of *A. simplex* NCIM 2449

Substrate carrier	Mol % bioconversion±SD after incubation period (hrs)				
	24	48	72	96	120
Control	nd	nd	nd	2.65±0.34	8.60±0.50
Methanol	18.46±0.91	38.78±1.84	40.50±1.79	36.85±1.97	35.88±1.50
Ethanol	19.53±1.34	36.55±3.06	56.39±3.14	57.29±3.40	60.78±2.97
Acetone	14.18±1.12	17.27±1.42	31.18±1.76	29.54±1.43	30.13±1.33
Soybean oil	34.52±1.63	38.36±1.92	24.22±1.75	20.14±1.52	18.74±0.97
Soybean oil+tween 80	12.05±1.04	25.40±1.97	40.11±3.78	40.32±3.17	42.98±2.02
Tween 80	nd	nd	8.36±0.96	12.54±0.87	13.87±1.05
Lecithin	15.32±0.73	21.61±1.13	25.89±1.83	28.12±2.11	33.67±2.88

SD: Standard deviation (N=3), nd: Not detected, *A. simplex*: *Arthrobacter simplex*, NCIM: National Collection of Industrial Microorganisms
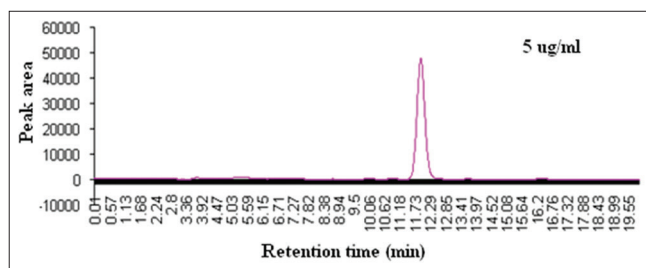
Fig. 3: High-performance liquid chromatography chromatogram of exemestane

at 48 hrs incubation period with exception of ethanol as a substrate carrier, which was observed at 72 hrs. A decline in the exemestane content of the medium was observed after reaching a peak, indicating the degradation of this compound by *A. simplex* NCIM 2449.

Table 2 shows the mol % conversion of 6-MeAD to exemestane by Ca-alginate immobilized cells of *A. simplex* NCIM 2449. Although the overall bioconversion indicated similar pattern as observed with growing cells, a delay in peak bioconversion was noted. Among various carriers used, the highest bioconversion was recorded again with ethanol as a substrate carrier till the end of incubation period at 120 hrs, indicating the suitability of ethanol for 1(2)- dehydrogenation activity of Ca- alginate immobilized cells of *A. simplex* NCIM 2449.

DISCUSSION

Permeability of hydrophobic steroid substrate across bacterial cell membrane plays a key role in bioconversion catalyzed by enzymes present inside the bacterial cell. The data obtained during this work clearly indicated an enhanced bioconversion when 6-MeAD was added in dissolved form in organic solvents or dispersed with emulsifiers. The enhancement in the bioconversion activity of *A. simplex* in the presence of organic solvents has been attributed to increased permeability of cell membrane to steroidal substrates [29]. The analysis of cell membrane of *A. simplex* grown in the presence of ethanol revealed an increment in *cis*-isomers of unsaturated fatty acids, thereby disorganization of native arrangement, resulting in increased permeability to steroid substrates [16]. In another study, reduction in the unsaturated and long chain fatty acids of *A. simplex* membrane in the presence of hydroxypropyl-β-cyclodextrin has been reported [30]. This study indicated that the organic solvents, soybean oil and lecithin increased the permeability of 6-MeAD, and resulted in early and higher bioconversion. Tween 80 alone proved deleterious to the bioconversion but favored the process when added along with soybean oil. It can be concluded that this anionic detergent lysed *A. simplex* cells at 5 mg/30 ml concentration when used alone. Similar observation was recorded during 9α-steroid hydroxylation by *Rhodococcus* sp. [31]. In comparison with the earlier reports of bioconversion of 6-MeAD to exemestane by *A. simplex* in xylene-water two-phase system [32] and β-cyclodextrin complexation technique [33], higher bioconversion was achieved in the present work.

The stability of C-1(2)-dehydrogenase activity of *A. simplex* cells immobilized in Ca-polygalacturonate [34], Ca- alginate [35], *k*-carrageenan beads, and polyurethane foam [36] has been shown against a variety of steroid substrates. The present work indicated the use of growing and Ca-alginate immobilized *A. simplex* cells as an environment-friendly alternative for 1(2)-dehydrogenation of 6-MeAD to exemestane.

Since exemestane is degraded simultaneously along with its formation both by growing as well as Ca-alginate immobilized cells of *A. simplex* NCIM 2449, the amount of exemestane accumulated in the fermentation broth is a result of rate of its formation and degradation. Therefore, precise termination of bioconversion reaction is suggested to harvest maximum product.

CONCLUSION

C-1(2)-dehydrogenase activity of growing and Ca-alginate immobilized *A. simplex* NCIM 2449 cells can be exploited for the bioconversion of 6-MeAD to exemestane. To overcome the steroid permeability barrier, 6-MeAD may be dispersed in the incubation medium using ethanol as substrate carrier. This organism and the described process may be scaled up to replace the hazardous chemical reaction by eco-friendly microbial bioconversion.

ACKNOWLEDGMENT

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