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MICROBIAL SIDE - CHAIN DEGRADATION OF PROGESTERONE II. APPLICATION OF DIFFERENT TECHNIQUES FOR PROGESTERONE CONVERSION BY *FUSARIUM DIMERIUM*

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A local isolate of *Fusarium dimerium,* firstly reported to be able to degrade progesterone side chain to give certain C-19 androgenes derivatives, proved to perform this important conversion more efficiently adopting the cell immobilization technique. Thus, relatively more amounts of Androst - 4 - ene - 3, 17 - dione (AD), Testololactone (TL) (major products) as well as, Testosterone (T), Androsta - 1,4 - diene 3, 17 - dione (ADD) (minor products) were recorded after 72 h using 2% Ca - alginate immobilized fungal cells. Relatively lower bioconversion rates were achieved when the bioconversion process was carried out in top - laboratory fermentor using free cells of the promising fungus.

Key words: *Fusarium dimerium,* Progesterone, Bioconversion.

Introduction

Microbial side - chain degradation of steroids has become an important transformation process for the production of 17 - ketosteroid intermediates, such as androsta - 1, 4 - diene - 3, 17 -dione (ADD) and androst - 4 - ene - 3, 17 dione (AD) (Imada *et al* 1981). Much attention has been focussed upon the transformation of steroids by immobilized microbial cell (Atrat 1982; Kolot 1982; Koshcheyenko *et al* 1983; Rehm and Omar 1993; Gemeiner *et al* 1994; Manosroi *et al* 1999).

Immobilization of whole microbial cells has gained considerable interest, mainly due to the possible industrial applications.

The utilization of immobilized cells as industrial catalysts could also be advantageous when compared to traditional fermentation procedures for several reasons:

1) Immobilized microorganisms permit easy separation of the products.
2) They are reusable and suitable for continuous or repeated batch operation allowing better process control.
3) Immobilized cells and in particular, entrapped ones are less susceptible to microbial attack.
4) Pollution effects e.g. the problem of disposal of spent cell mass, are less pronounced with immobilized cells.

Due to these facts the present investigation was mainly designed to evaluate the efficiency of applying the immobilization techniques in the bioconversion of progesterone into C - 19 derivatives by *F. dimerium.* Moreover, the utilization of the top - lab fermentor in the bioconversion process was also carried out.

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Materials and Methods

Microorganism and culture conditions. Fusarium dimerium used as an experimental organism in this study was obtained from the centre of Plant Pathology Department, National Research Centre (NRC), Cairo, Egypt. The fungus was maintained on a medium composed of (g / l) glucose, 40; peptone, 1; yeast extract, 1 $MgSO_4.7H_2O$, 1; KH_2PO_4, 0.7; L - asparagine, 0.70; agar, 20 (Kinawy 1974). The same composition (except agar) was used as a liquid fermentation medium.

Immobilization technique. F. dimerium cells were entrapped in 2% Ca alginate as described by El - Diwany *et al* (1992), then standard weight of 2% Ca - alginate pellets was suspended in 50 ml 0.05 M tris HCI buffer (pH7) containing 0.7% NaCI and 0.02% Tween 80, in a 250 ml Erlenmeyer flasks. Progesterone (5 mg / 50 ml buffer) was then added as ethanolic solution and the flasks were agitated on a reciprocal shaker (150 rpm) at 30°C for certain time.

Utilization of free fungal cells. The bench top chemostat C_{32} "Bioflo" standard 2 litres vessel was used. One litre of the fermentation medium (Adham *et al* 2002) was sterilized, then inoculated with 20 ml of the standard fungal suspension. The fermentation was conducted at 30°C, agitation speed 200 r/min, aeration rate 1 / v / v / mm, initial pH 7, and exhaust gas escaping was applied under gauge pressure 17 cm water column for 72 h, progesterone was then added as solution in 96% ethanol

at a concentration of 100 µg / ml. The fermentation was continued for 24 h unless otherwise stated.

Transformation product assay. At the end of the transformation period, the culture filtrate was extracted with chloroform (2 volumes), washed with distilled water, then dried over anhydrous sodium sulphate. The combined extracts were evaporated under vacuum to give a semi-solid residue (test material). The different transformation products in the test material were separated and determined by two methods:

A - THIN - LAYER CHROMATOGRAPHY (TLC). It was performed on silica gel G plates (Sallam *et al* 1969), for identification and resolution of the test material, the following solvent systems proved to be suitable:

I - Cyclohexan: acetone: chloroform (15:5:2 v / v / v).
II - Cyclohexane: chloroform: isopropanol (10:5:2 v / v / v).
III - Benzene: ethyl acetate: acetone (4:1:1 v / v / v).

Two different colour reagents were used for the identification of the different steroids present. (i) Libermann - Burchard reagent. (ii) Chlorosulphonic acid: acetic acid (3:1 v / v).

The experimental results revealed the presence of more than one steroid compounds in the transformation medium. The distance between these were sufficient to enable them to be separated and eluted with chloroform. The extract was filtered and evaporated to dryness in a test tube in a water bath. For the determination of the different steroid products, 8 ml aliquot of chromogen reagent (45 ml conc. H_2SO_4 + 55 ml absolute ethanol) were added to the test material, heated in boiling water bath for 15 min, and cooled, the absorbance was measured photometrically at specific λ_{max} for each product in the tested material.

B - ISOLATION BY COLUMN CHROMATOGRAPHY: The collected extract (test material) was dissolved in the minimum volume of n - hexane and then fractionated on silica gel S column. The following sequence of solvents were used: n - hexane, n - hexane: benzene (1 : 1, v / v), benzene containing different concentrations of chloroform, chloroform containing different concentrations of methanol and finally methanol.

Results and Discussion

Bioconversion of progesterone by immobilized cells of F. dimerium. In a previous communication (Adham *et al* 2002) thirty seven different fungal cultures were tested for their ability to degrade the side - chain of progesterone. The data shows that some tested organisms metabolized progesterone in different degrees. Also *F. dimerium; F. oxysporum* No. 153; *F. oxysporum* No. 152; *F. moniliforme* were the most active progesterone side - chain degrading fungi and were, thereafter, subjected to quantitative studies. The obtained data shows that the tested organisms metabolized progesterone, however, *F. dimerium* was the most active and highest yields of both testosterone and androstadienedione were obtained by this species.

To our knowledge, side - chain degradation with *F. dimerium* have not yet been reported. In literature also it is the most active with the formation of considerable yield of testosterone (Adham *et al* 2002). Due to the above reasons, we choose this organism for elucidating the most favourable conditions for it to perform the side - chain degradation of progesterone. The physiological aspects of the bioconversion process were also evaluated (Adham *et al* 2002). In the preceding work, the bioconversion processes were routing carried out by the shaked free fungal cultures. Therefore, in the present study the biotransformation of progesterone with the immobilized cells of the tested fungus was investigated. The transformation capacity of the immobilized cells of *F. dimerium* seems to be relatively low at the early stage of the transformation process (about 40% of the steroid substrate remained unchanged), thereafter, the bioconversion rates were markedly enhanced as indicated by the consumption of the total amount of the charged progesterone (Fig 1). Androstenedione (AD) was traced as the major product during the different phases of the transformation period.Δ^1 - dehydroderivative; namely androstadiendione (ADD) was only detected in minor amounts indicating a weak dehydrogenase activity. On the other hand, testolactone seems to be the second major product which was produced with increasing amounts particularly during the early phase of the transformation process. Similarly, testosterone was produced in good yields after 96 h. This may reflect the remarkable lactonization activity catalyzing the formation of testololactone.

Bioconversion of progesterone by free fungus cells in a stirred tank fermentor. Transformation in a stirred tank fermentor using different aeration rate was carried out. The results (Table 1) showed that the same pattern of trans-

Table 1
Effect of aeration on transformation of progesterone by *F. dimerium* using Bioflo fermentor

Aeration Vvm	Residual P%	T %	AD %	ADD %	TL %	Missing steroids %
0.5	Traces	23.9	25.6	8.2	20.5	21.8
1.0	Traces	25.8	32.3	7.5	20.7	13.7
0.5	Traces	22.6	32.2	7.0	20.0	18.2

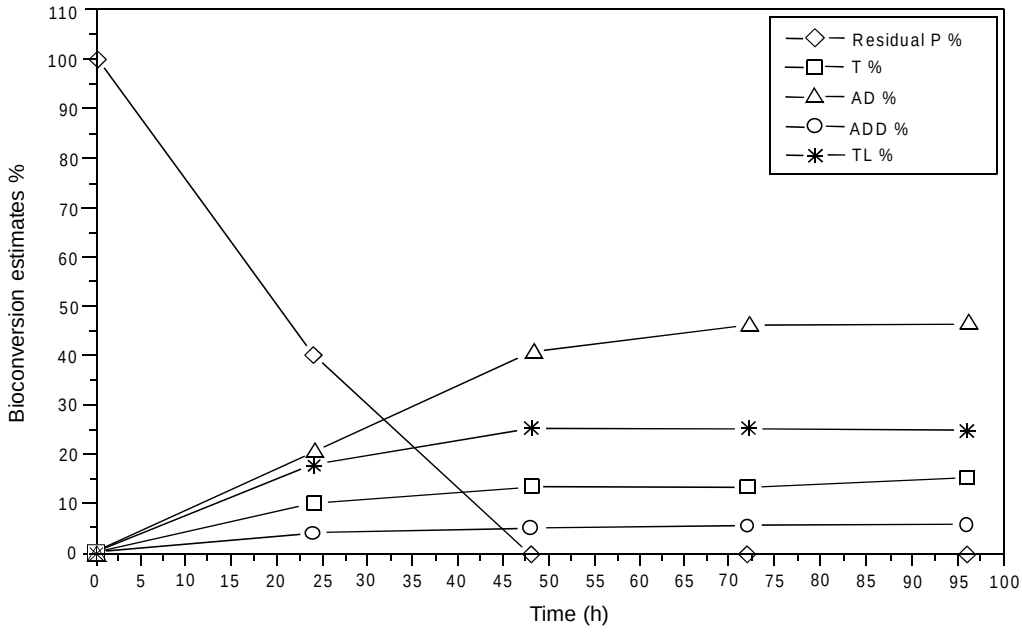

Fig 1. Bioconversion of progesterone by shaked immobilized cells of *F. dimerium* entrapped in 2% calcium alginate.

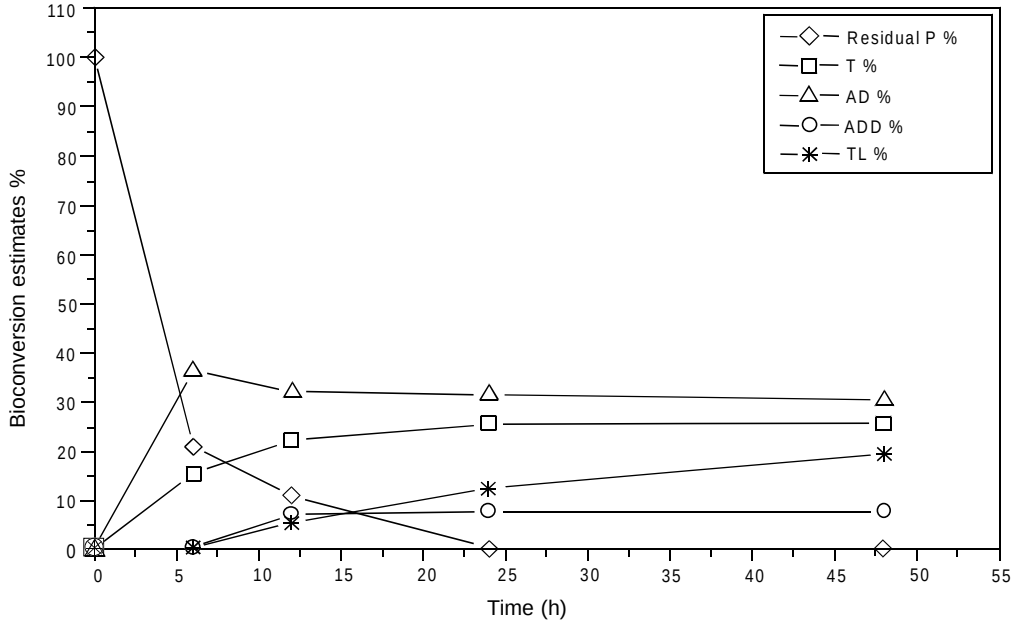

Fig 2. Bioconversion of progesterone by *F. dimerium* using Bioflo fermentor.

formation was observed regardless of the aeration rate applied. Thus, the charged progesterone concentration was almost converted mainly to AD and TL as major products and ADD in relatively minor levels. However, the adjustment of the aeration rate at 1 v / v afforded relatively higher bioconversion yields. In subsquent experiment, the bioconversion activities were traced at different time intervals while the aeration rate was adjusted at 1 v / v level. The results revealed that 80% of the progesterone substrate was successfully converted mainly into AD and after the first 6 h (Fig 2). The following phase of the bioconversion course (mainly 6 - 12 h) exhibited further progesterone conversion in favour of AD and T production with the concomitant production of ADD and TL in relatively lower yields. As the bioconversion process prolonged to 24 and 48 h, the charged progesterone was completely converted with the formation of AD and T as major products and the detection of TL in elevating yields.

Table 2
Properties of the transformation products of progesterone bioconversion by *F. dimerium*

Isolated compounds	Eluted solvents	Solvent of crystallization	Crystal properties
ADD	Benzene : Chloroform 70 : 30	Chloroform: Methanol	m.p. 140 - 142 ˚C UV λ_{max} 244 nm
AD	Benzene : Chloroform 1 : 1	Chloroform : Methanol	m.p. 174 - 176 ˚C UV λ_{max} 239 nm
T	Chloroform	Chloroform : Methanol	m.p. 153 - 158 ˚C UV λ_{max} 240 nm
TL	Chloroform : Methanol 1 : 1	Methanol	m.p. 207 - 209 ˚C UV λ_{max} 242 nm

Assessment of the progesterone side-chain derivatives produced by F. dimerium. It seems appropriate to verify the identity of the different products formed as a result of progesterone bioconversion with the tested fungus, since the identity so far has been based solely on the TLC separation of each compound. Therefore, the column chromatographic resolution of the transformation mixtures was performed. The fractions containing the same product (as judged by TLC analysis) were collected, then evaporated through vacuum and crystallization, from suitable solvent. Identification of each compound was made by determination of m.p. and UV absorption spectra (Table 2).

The aforementioned investigations clearly proved that the tested fungus *(F. dimerium)* can transform progesterone (C_{21}-steroid) into the same product, namely AD, T as major products in addition to TL and ADD as minor derivatives. This transformation pattern existed upon using the immobilized or the free cell conversion techniques. However, relatively higher transformation outputs were recorded with the immobilized fungal cells, whereby, the progesterone substrate was completely converted after 72 - 96 h into AD, TL, T and ADD at 48, 26, 16.5 and 7 %, respectively.

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