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Microbial Side-Chain Degradation of Progesterone I: Optimization of the Transformation Conditions

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The microbial side-chain degradation of progesterone for the production of C-19 androgens was investigated. Thirty seven locally isolated fungal cultures were screened for their ability to degrade the side-chain of progesterone. *Fusarium dimerum* showed the greatest bioconversion efficiency and was selected for further studies. 50% of the substrate was converted to androstenedione after 24 hrs. 72 hrs old culture was able to produce maximum yields of testosterone, androstenedione and androstadienedione. The maximum conversion activities (90%) of progesterone were recorded at pH 7. The capacity of the fungus to degrade the side-chain of progesterone was greatly diminished on using high concentration of progesterone. The bioconversion estimates sharply decreased by using glucose syrup, corn steep liquor and glucose-corn steep media.

Key words: Progesterone, Fusarium dimerum, Bioconversion, Androgens.

Introduction

One of the important microbial reactions on steroid substrates, is the microbial side-chain degradation of 4-dehydro-3,20-diketosteroids of pregnene series, leading to the production of C-19 androgens which may be further used as intermediates for the preparations of the C-18 estrogens.

This type of reaction was first reported by Turfitt (1948) when a C-8 side chain of C-27 steroid substance (cholestenone) was removed by the action of *Proactinomyces erythropolis* with the formation of a C-20 steroidal product identified as 3-oxo-eticholenic acid. Since that time many substrates were subjected to the action of different microorganisms with the formation of variant products (Peterson *et al* 1957; El-Tayeb 1966; Kondo and Mitsugi 1966; Helena and Igor 1968; Nagasawa *et al* 1970; Szentirmal 1990).

The microbial side-chain degradation of progesterone may lead to the formation of simple transformation products vis androstenedione and/or testosterone (Bodansky *et al* 1955; Peterson *et al* 1957). Androstenedione was formed from progesterone side-chain degradation by the action of *Gliocladium catenulatum*, *Aspergillus flavus* and *Penicillium lilacinum* (Peterson *et al* 1957), by *Cephalosporium subverticillatum* (Bodansky *et al* 1955) and by *Fusarium* spp. (Helena and Igor 1968). On the other hand, testosterone has been identified as a transformation product of progesterone side-chain degradation by the action of *Penicillium citrinum*, *P. notatum* and *P. decumbens* (Hanc *et al* 1957). Testolo-lactone was formed from progesterone by the action of *Aspergillus flavus* (Peterson 1953), *Cephalosporum subverticilatum* (Bodansky *et al* 1955), *Aspergillus oryzae* (Kondo 1960), *Pythium ullinum* and *Penicillum citrinum* (Shirasaka and Ozaki 1961), *Aspergillus tamarii* (Brannon *et al* 1965) and *Penicillum chrysogenum* (Schubert *et al* 1968).

In some cases another common reaction often coupled with the side-chain cleavage of steroids is the introduction of a double bond between C_1/C_2 of ring A. The product obtained in this case was androstadiendione (a valuable intermediate in the synthesis of estrone) and 1-dehydrotesto-sterone. Wix and Albrecht (1959; 1961) investigated the use of *Fusarium caucasicum* as a practical tool for the preparation of 1,4 dehydro-3-ketones.

The transformation processes are affected by the nature of the substrate, the microbial species and strains, as well as the constitution of the fermentation medium (Capek *et al* 1966; Abou El-Hawa *et al* 1993; Sallam *et al* 1977, 1994; Liu and Lo 1997).

The aim of the present work is to optimize the transformation conditions for progesterone side-chain degradation by *F. dimerum* since no studies with this organism have previously been reported.

Materials and Methods

Micro-organism and culture conditions. The fungal isolates used in this work were obtained from the Centre of Cultures of Microbial and Natural Chemistry Department;

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Plant Pathology Dept., NRC, Egypt as well as Botany Dept., Fac. of Assiut University.

Thirty seven different fungal cultures were tested for their ability to degrade the side-chain of progesterone within 48h, 16 different *Aspergillus* spp., 8 *Fusarium* spp. and 13 *Penicillum* spp.

Stock cultures were prepared by growing the pure organism on agar slopes of Kinawy's medium (Kinawy 1974) which has the following composition (g l^{-1}): glucose, 40; peptone, 1; yeast extract, 1; $MgSO_4.7H_2O$, 1; KH_2PO_4, 0.74; L-asparagine, 0.70; agar, 20.

Transformation process. The organisms were grown in 50 ml aliquots of a nutritive solution of the Kinawy's medium in 250 ml Erlenmeyer flasks. Flasks were autoclaved for 20 min and each flask was inoculated with 1 ml of a spore suspension prepared from a seven day-old slant of the test organism. The flasks were agitated on a reciprocal shaker at 200 rpm for 48 h at 30 ±2°C, supplemented with 5 mg of progesterone dissolved in 1 ml of 96% ethanol and fermentation was continued for another 48 h.

Transformation products assay. At the end of the transformation period, the contents of each flask (50ml) were extracted with 100 ml chloroform, The combined chloroform extracts were washed with an equal volume of 5% (w/v) $NaHCO_3$ solution and with distilled water, dried over anhydrous sodium sulphate, filtered, and then distilled to give a semi-solid residue (test material).

The test material was dissolved in a measured volume of chloroform:methanol (1:1, v/v) and analysed by using thin-layer chromatography (TLC) 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 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 distances 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 determination of 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.

Optimization Experiments. 1. Transformation conditions A. Bioconversion time course. Inoculated flasks containing Kinawy's medium (50ml each) were shaken at 30°C for 48 h, after which they were supplemented with 5 mg progesterone per flask, and samples were collected for analysis at 6, 12, 36, and 48 h intervals.

B. Culture age. Aliquots (50 ml each) of Kinawy's medium were inoculated with *F. dimerum* and after incubation for 24, 48, 72, 96 and 120 h under shaking condition at 30°C, different preparations (5 mg each) of progesterone were separately added to the culture at each period, the transformation period was continued for another 24 h, and the necessary analyses were carried out.

C. Substrate concentration. Inoculated flasks were shaken at 30°C for 72 h; after that, substrate (progesterone) was added to the medium with different concentrations ranging from (5-20 mg $flask^{-1}$). The transformation period was continued another 24 h, and the necessary analyses were carried out.

2. pH value. The effect of the pH value was studied by adjusting the fermentation medium initially before autoclaving with 1M NaOH or 1M HCl (pH 4-9). Flasks were inoculated and incubated under shaking condition for 72 h at 30°C and then progesterone (5 mg $flask^{-1}$) was added and transformation continued for another 24 h.

3. Concentration of the medium ingredients. The effect of varying the concentration of each of the constituents of the fermentation medium (glucose, peptone, yeast extract, L-asparagine, $MgSO_4$ and KH_2PO_4) on the bioconversion process was investigated. The concentration (g l^{-1}) of glucose ranged from 40 to 100, that of peptone from 0 to 4, yeast extract 0-4, KH_2PO_4 from 0-1.5 and that of $MgSO_4.7H_2O$ from 0-2. In all the treatments, the reaction of the transformation medium was initially adjusted to pH 7. Inoculated media were incubated for 72 h at 30°C, and then 5 mg of progesterone were added and the transformation period was continued for another 24 h.

Conversion of progesterone by F.dimerum under optimal condition. In the preceding experiments the nutritional requirements, pH value of the medium, age of the culture that influence the transformation of progesterone to the desired C_{19} steroids were investigated. Maximal bioconversion estimates were achieved when the fungus was grown under the following conditions:

Cultivation medium (g l^{-1}): glucose, 80; peptone, 2; yeast extract, 1; L-asparagine, 0.5; $MgSO_4.4H_2O$, 1; KH_2PO_4, 0.74; pH, 7.

The cultivation period extended for 72 h, progesterone was added at 5 mg $flask^{-1}$ and the transformation process was continued for another 24 h. Samples (5 ml) were taken out after 6, 12, 24, 36, 48, 72 and 120 h and necessary analyses were carried out.

The use of media with natural product. The following tested media were simply formed of available cheep carbon and nitrogen sources namely glucose syrup and corn steep liquor respectively.

Simplified media (gl^{-1}): Medium I: glucose, 80; corn steep liquor (solids); *Medium II:* glucose syrup, 80%; corn steep liquor (solids).

In the two media different concentrations of corn steep liquor were used. PH was initially adjusted to 7. The cultivation period should extend for 72 h; thereafter progesterone was added (5 mg $flask^{-1}$) and the transformation process was continued for another 24 h.

Results and Discussion

Screening for the most active organism. Thirty seven different fungal cultures were tested for their ability to degrade the side-chain of progesterone within 48 h. Table 1 presents the organisms that successfully performed this bioconversion.

The active progesterone side-chain degrading fungi namely *F. dimerum*; *F. oxysporum* No. 153; *F. oxysporum* No. 152; *F. moniliforme* were thereafter subjected to quantitative studies. The data presented in Table 2 shows that the tested organisms metabolized progesterone in different degrees. However, *F. dimerum* was the most active where only 18% of the added progesterone remained unchanged and highest yields of both testosterone and androstadienedione (16 and 6.1%, respectively) were recorded. On the other hand, androstenedione has not been detected in all cases.

The fact that *F. dimerum* was the most active organism with the formation of considerable yield of testosterone was not studied earlier led us to choose this organism for elucidating the most favourable conditions for it to perform the side-chain degradation of progesterone.

Bioconversion time course, culture age and substrate concentration. The results in Table 3 revealed that during the first 24 h, androstenedione was the major product (50.6%). However, a sharp decrease in its yield was recorded after 36 h. On the other hand the yield of testosterone increased gradually till it reached maximum (16.7%) after 48 h. In a similar view androstadienedione was detected after 6 h and its yield increased gradually along the fermentation time till it reached maximum yield after 48 h. Testololactone was not detected except after 36 h and reached maximum yield after 48 h. These data may suggest that the biodegradation of progesterone-side chain to yield the mentioned products by *F. dimerum* may be as indicated in Fig 1. These findings are

Table 1

Ability of different local fungi to transform progesterone

Organism	Residual P	T	AD	ADD
Aspergillus amstelodami	+	+	-	-
Aspergillus flavus	+	-	+	-
Aspergillus nidulans	+	-	+	+
Aspergillus niveus	+	-	-	+
Aspergillus versicolor	+	-	-	+
Aspergillus sydowi	+	-	+	+
Penicillium citrinum	+	-	-	+
Penicillium chysogenum	+	-	+	-
Penicillium nigricans	+	-	+	-
Penicillium piscarium	+	+	-	-
Penicillium waksmani	+	-	-	+
Penicillium corylophilum	+	-	-	+
Fusarium dimerum	Traces	+	-	+
Fusarium moniliforme	Traces	+	-	+
Fusarium oxysporum No. 152	+	+	-	-
No. 153	Traces	+	-	-
Fusarium solani No. 4	+	+	-	-
No. 5	+	+	-	-
Fusarium sp. 2	+	+	-	-
3	+	+	-	-

Activity index: (+), detected; (-), not detected; P, Progesterone; T, Testosterone; AD, Androstenedione; ADD, Androstandienedione.

Table 2

Amount of steroids detected by the fungi when acted on progesterone

Organism	Residual P%	T %	AD %	ADD %
Fusarium dimerum	18.5	16	0	6.1
Fusarium moniliforme	25.9	6.1	0	1.3
Fusarium oxysporum No 153	19.8	4.9	0	0
Fusarium oxysporum No 152	26.6	5.5	0	0

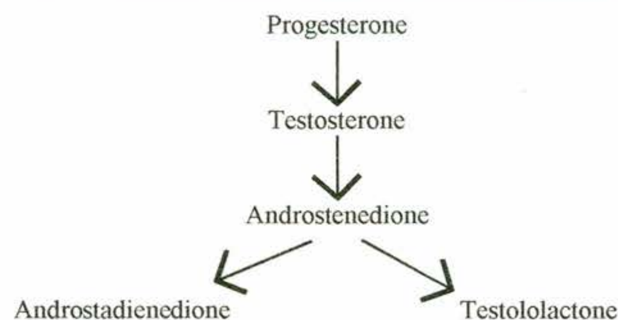

Fig 1. Scheme suggestion of progesterone side-chain degradation by *F. dimerum*.

Table 3
Transformation of progesterone by *F. dimerum* as influenced by transformation time, culture age and substrate level

Transformation	conditions	Residual P %	T %	AD %	ADD %	TL %
Transformation period (h)	6	30.3	5.2	22.5	1.7	-
	12	25.8	6.4	35.5	2.7	-
	24	22.5	12.3	50.6	5.8	-
	36	20.7	15.7	Traces	6.3	7.3
	48	19.2	16.7	Traces	6.8	13.1
Culture age (h)	24	36.5	10.3	40.8	5.1	-
	48	22.6	11.7	50.9	5.3	-
	72	18.1	13.2	52.8	5.9	-
	96	20.2	9.4	46.7	5.5	-
	120	45.0	4.4	35.6	1.8	-
Progesterone level (μg ml^{-1})	100	-	18.5	59.6	12.1	
	200	18.2	15.1	41.3	8.6	
	300	23.8	13.2	29.7	5.7	
	400	35.6	8.3	12.5	4.2	

in agreement with those recorded by Mahato *et al* (1988) using *Arthrobacter simplex* and Nishikawa *et al* (1955) using *Fusarium* sp. However, Sallam *et al* (1973) suggested that androstenedione was the first product formed during the side-chain degradation of progesterone by *A. fischeri*. The testosterone was formed from it during the fermentation periods starting from 12 h. Similar findings were repeated by Abdel-Fattah *et al* (1974) using *P. lilacinim* and progesterone as substrate. Moreover, the fermentation of testosterone from androstenedione by *Mycobacterium* sp. have been observed by Liu and Lo (1997). So 24 h was our choice of transformation period.

Culture age. The results (Table 3) showed that 72 h old culture has the greatest capacity for degrading the side-chain of progesterone. Highest consumption of progesterone as well as highest yields of transformation products were observed at that culture age.

Substrate concentration. The lowest progesterone concentration (100 μg ml^{-1}) was completely transformed (Table 3). 35% of progesterone remained unchanged when substrate concentration 400 μg ml^{-1} was used. Androstenedione was formed in 59% conversion yield at the lowest concentration of progesterone. As the amount of the added progesterone increased, a noticeable decrease in the percent conversion yield of androstenedione was observed. The lowest conversion yield percent (12%) was obtained at the highest level of progesterone (400 μg ml^{-1}). Similar pattern was obtained with testosterone, thus, highest conversion yield percent of testosterone (18.5) was obtained at (100 μg ml^{-1}) progesterone level while lowest percent of conversion yield (8.3%) was that at 400 μg ml^{-1} progesterone level.

pH value. The highest bioconversion rates of progesterone were obtained within initial pH range 6-7, where 80% of the charged progesterone was transformed (Table 4). At higher pH values (>pH 7) the bioconversion process was noticeably inhibited.

Concentration of the medium ingredients. The data (Table 5) showed that glucose 80 g l^{-1} was the best concentration as it yielded maximum bioconversion products by percentage. Addition of different concentrations of peptone to the fermentation medium led to an increase in the yield of different transformation products. Higher percent yields of all products were noticed at peptone levels ranging between 1-2 g l^{-1}. L-Asparagine (0.5 g l^{-1}), $MgSO_4.7H_2O$ (1 g l^{-1}) and KH_2PO_4 (0.7 g l^{-1}) were the most suitable for supporting the maximum bioconversion efficiency.

Table 4
Transformation of progesterone by *F. dimerum* as influenced by the pH value of the medium

Initial pH	Residual P %	T %	AD %	ADD %
4	27.6	10.5	27.7	5.1
5	19.3	10.9	42.1	5.5
6	18.2	12.1	50.3	5.5
7	18.7	12.6	52.2	6.0
8	36.9	9.6	33.2	4.3
9	39.2	8.5	22.3	4.1

Table 5
Bioconversion of progesterone by *F. dimerum* as influenced by the ingredients

Ingredient	g l^{-1}	Residual P%	T%	AD%	ADD%
Glucose	40	19.1	12.2	50.0	6.1
	60	15.3	13.5	56.9	7.6
	80	10.2	15.9	57.0	8.1
	100	10.3	15.4	57.5	8.2
Peptone	0.0	38.8	10.8	25.2	5.3
	0.5	12.3	12.7	39.4	7.6
	1.0	10.5	15.9	52.3	8.1
	2.0	10.0	15.3	54.0	8.2
	4.0	10.3	10.1	40.8	7.5
L-Asparagine	0.0	16.7	11.2	40.3	7.8
	0.5	12.1	14.4	45.1	8.9
	0.7	10.1	15.2	55.9	8.9
	1.0	10.2	15.2	55.4	8.9
	2.0	12.4	13.1	42.9	9.3
Yeast extract	0.0	45.1	12.1	20.6	3.8
	0.5	20.0	14.2	51.6	6.2
	1.0	9.3	15.3	56.7	9.3
	2.0	9.4	12.1	50.0	7.7
	4.0	19.4	9.5	44.9	3.3
KH_2PO_4	0.0	37.9	12.8	21.3	2.1
	0.5	15.6	12.5	53.2	8.4
	0.74	9.2	15.9	56.3	10.3
	1.0	17.1	15.9	56.2	10.8
	1.5	22.1	10.7	47.2	11.1
$MgSO_4.7H_2O$	0.0	39.8	12.9	29.7	8.6
	0.5	16.2	14.3	50.3	10.5
	1.0	8.0	16.2	57.5	10.2
	1.5	10.5	15.5	54.6	10.7
	2.0	12.1	14.4	50.6	11.7

Except for the ingredient under consideration, the medium consisted of (g l^{-1}): glucose, 40; peptone, 1; yeast extract, 1; $MgSO_4.7H_2O$, 1; KH_2PO_4, 0.74; L-asparagine, 0.70.

Conversion of progesterone by F. dimerum under optimal conditions. In this experiment, the organism was cultured in a medium containing the optimum concentration of each ingredient and had the following composition (g l^{-1}): glucose, 80; peptone, 2; yeast extract, 1; L-asparagine, 0.7; $MgSO_4.7H_2O$, 1; KH_2PO_4, 0.74. At pH 7, cultivation was made for 72 h, and the transformation process was continued for another 120 h.

The data given in Table 6 showed that the charged progesterone was nearly transformed at the end of the first 24 h. At the early stage of process (6-24 h) testosterone, androstenedione and androstadiendione were detected in the transformation mixture. The major product (androstenedione) reached maximum amount (58%) at the end of the first 24 h. Thereafter, the level of androstenedione decreased to about 53% after 36 h and almost completely disappeared during latter phases of the bioconversion process. After the first 36 h testololactone was formed as a new product and its concentration increased gradually till it reached maximum (20%) after 120 h transformation.

The use of media with natural products. Table 7 indicated that on using glucose-corn steep solid medium (medium I)

Table 6
Transformation of progesterone by *F. dimerum* under the optimum fermentation conditions as influenced by different transformation period

Transformation period (h)	Residual P %	T %	AD %	ADD %	TL %
6	33.8	4.5	28.8	3.5	0.0
12	18.3	10.6	35.6	4.5	0.0
24	8.4	16.3	58.5	11.1	0.0
36	6.3	17.4	52.6	12.4	6.2
48	5.4	18.1	00.0	11.3	10.1
72	Traces	18.3	00.0	11.6	17.5
120	Traces	19.2	00.0	8.8	20.4

Table 7
Bioconversion of progesterone by *F. dimerum* using natural products media

Corn steep liquor (g l^{-1})	2.5		5.0		7.5		10		15	
Medium No.	I	II	I	II	I	II	I	II	I	II
Residual										
P %	29.5	56.3	38.4	50.9	46.6	42.7	57.8	66.6	65.6	72.9
T %	4.9	4.4	4.3	5.4	4.3	5.2	3.6	4.8	36	4.9
AD %	32.1	-	30.5	-	25.6	-	17.2	-	11.7	-
ADD %	3.1	-	6.5	-	3.4	-	2.3	-	1.5	-

Medium I: glucose, 80; corn steep liquor (solids); Medium II: glucose syrup, 80%; corn steep liquor (solids).

progesterone was successfully transformed to the same products, testosterone, androstenedione and androstadienedione as usually obtained with synthetic medium; however, the transformation yields were comparatively of lower magnitudes (as compared with the conversion estimates obtained after 24 h using the optimal cultivation medium; Table 6). Best bioconversion estimates were recorded with the cultivation medium composed of glucose (80 g l^{-1}) and corn steep solids (2.5 g l^{-1}). Relatively lower yields of the transformation products were recorded with high levels of corn steep liquor. Conversely, the transformation activities were greatly restricted on replacing the pure glucose constituent by the commercial glucose syrups (medium II). Thus, the tested fungus failed to produce androstenedione or androstadienedione under these conditions and instead of that, only the testosterone was formed. Testosterone was optimally formed with corn steep solid equal to 7.5 g l^{-1} (Table 7).

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