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Optimization of Progesterone 11α-Hydroxylation in the Presence of β-Cyclodextrin

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Aspergillus ochraceus NRRL 405 was used to hydroxylate progesterone to 11α-hydroxyprogesterone (11α-HP). This study described the effect of some fermentation parameters and the intermittent addition of β-cyclodextrin on the bioconversion process. The Kinaway's medium with pH 6 produced the best result of the used culture media. The transformation period was 48 h for the maximum hydroxylation. The maximum production of 11α-HP (93.10%) was obtained by the addition of 4g/I β-cyclodextrin at 12 h after inoculation compared to the control culture (56.8%). The results also showed the ability of the mould culture to carry out the transformation reaction at high substrate levels without by products formation in the presence of β-cyclodextrin.

Key words**:** 11α-Hydroxyprogesterone (11α-HP), Residual progesterone (RP), β-cyclodexrin.

Introduction

The discovery that some microorganisms could be used in modifying the steroid nucleus gave some lights about the field of steroid transformation (Petresson and Murry 1952). The most important reactions in the field of steroid transformtion are the hydroxylation reactions. A large number of fungi have the ability to hydroxylate steroids in various positions. The major industrial interest has been focused on those capable of hydroxylating steroid at the 11 position, because it is the key step in cortisone and predinsone production.

The biological hydroxylation of steroids at this strategic position is by far the most important, where the presence of an oxygen function at C_{11} is necessary for the anti-inflammatory activity of these compounds. This type of biological oxidation of steroids was not only confined to the enzymatic systems of microorganisms but also found in the mammalian tissue enzymes (Hanch *et al* 1949; Morfin 2000; Soffer *et al* 1961).

The discovery that steroid hormones or the intermediates used for their preparation could be made economically by the use of microorganisms, created a new trend in the biochemistry and also a new microbiological industry for exploiting steroid metabolism and its enzymatic basis (Mahato and Banerijee 1985).

In the transformation reaction other minor products (by products) may be produced. For example *Cephalosporium aphidcola* had been shown to hydroxylate progesterone to 11α and 6β positions (Farooq *et al* 1994) in addition to minor metaolities include testeosterone acetate and 12β, 17α dihydroxy progesterone.

Special problems of steroid transformation originate in most cases from nearly total insolubility of steroid substrates in water. There are several possibilities for increasing the solubility of such substances and to facilitate contact between the steroids and the biocatalysts. By using both free and immobilized cells (Schmauder *et al* 1991) one possibility is the use of water miscible organic solvents such as methanol, dimethyformamide or dimethyl sulphoxide. However, due to the toxic effects of such compounds, their use is commonly restricted to only low concentrations (Flagare 1998). Another approach is to carry out conversions in the presence of cathrating agents such as cyclodextrins.

Cyclodextrins are doughnut-shaped cyclic oligosaccharides of six to eight glucose units linked by a (1, 4) glycloside bonds. They are dissolved easily in water and have a hydrophobic cavity to include hydrophobic agent molecules by Van-der-Waals interactions and hydrogen bond formation. Cyclodextrins are presently used as stabilizers of several steroid drug such as hydrocortisone, cortisone acetate and testosterone.

The previous studies were focused on β-cyclodextrin effects on the side chain degradation of sterols. The aim of the present study was to investigate the effect of β-cyclodextrin on the 11α-hydroxylation reaction specifically of progesterone. β-Cyclodextrin is applied to improve the poor solubility of

steroid substrates and products in aqueous systems results in the formation of so called inclusion complexes. The solubility may be estimated according to the modified solubility equation given by (Higuchi and Connors 1965; Schmauder *et al* 1991). The solubility constant K is given by:

$$K = \{P_n - C_m\} / (P)^n . (C)^m$$

Where;

P_n = Concentration of uncomplexed progesterone

gC_m = Concentration of uncomplexed β-cylodextrin

$[P_n - C_m]$ = conc. of inclusion complexes of (progesterone and β-cyclodextrin) at the equilibrium state

n, m = represents the stochiometric number

The solubility of progesterone in the absence of β-cyclodextrin S_0 obtained by the following equation

$K = Slope/S_0 (1 - slope)$

$S_0 = Slope/k (1 - slope)$

Materials and Methods

Chemicals. The steroids progesterone and 11α-hydroxyprogesterone were provided by Sigma company, USA and Ciba, Giegy company, Switzerland. All other chemicals used in the current work were laboratory reagents grades purchased from Merck.

Microorganism. The fungal isolate *Aspergillus ochraceus* NRRL 405 was obtained from the Natural and Microbial Products Chemistry Department, National Research Center (NRC), Dokki, Cairo, Egypt.

Maintenance of the microorganism. The organism was maintained on agar slant of the modified Dox' S medium g/1(glucose 20.0, $NaNO_3$ 1.0, KC1 0.5, KH_2PO_4 1.0, $MgSO_4$. $7H_2O$ 0.5, $FeSO_4$. $7H_2O$ 0.005, agar 20). The inoculated slants, were incubated at 30 ± 1°C for 5 days and then stored at 4°C and subcultured at monthly intervals.

Transformation process. Erlenmeyer flask (250 ml) containing 50 ml sterile Kinaway's medium (Kinawy 1974) g/1 glucose 40.0, peptone 1.0, yeast extract 1.0, $MgSO_4$. $7H_2O$ 1.0, KH_2PO_4 0.75 and asparagines 0.7 (pH 6) were inoculated by transferring 2 ml of 48 h old culture and continued to grow for 48 h at 200 rpm, 30 ± 1°C. 1 Milligram progesterone was added to each flask and incubated for 24 h (induction period), then 5 mg progesterone was added and the transformation process was continued for another 48 h. Extraction of the transformation products and their qualitative and quantitative analysis were carried out (Sallam *et al* 1969).

Qualitative analysis. The transformation products presented in the fermented broth were identified using thin layer chromatographic technique (Sallam *et al* 1969) comparing colors and Rf value with those of authentic samples using the solvent system benzene: acetone: ethyl acetate (4:1:1v/v) and iodine reagent as a color reagent.

Quantitative analysis. Progesterone and 11α-hydroxyprogesterone were determined by HPLC (waters Co. Alc/GPC 204) under the following conditions: C_{18} Radial - PaK A coloum, d = 10 µm, mobile phase water: methanol: acetic acid (10:90:0.02) by volume, flow rate 2 ml/min, pressure 70 $kgcm^2$ under these conditions the respective retention times of 11α-HP and RP were 2.30 and 3.85 min.

Results and Discussion

Suitability of the fermentation medium. The present investigation was conducted to determine the influence of chemical composition of the fermentation medium on the activities of the experimental organism. Five different nutritive media differing in the nature and the concentration of some constituents were used.

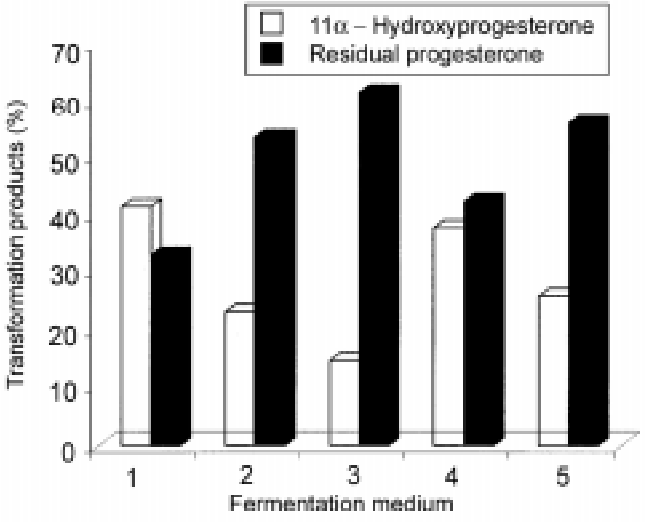

Fig 1. Comparison of different fermentation media for the bioconversion of progesterone to 11α-hydroxyprogesterone.

Medium 1(g/1): Glucose 40.0, Peptone 1.0, Yeast extract 1.0, $MgSO_4$. $7H_2O$ 1.0, KH_2PO_4. 0.075 and Asparagine 0.70. Medium 2(g/1): Malt extract 40.0, yeast extract 39.0. Medium 3(g/l): Malt extract 30.0, peptone 20.0, Soybean meal 10.0, KH_2PO_4 5.0 and $MgSO_4.7H_2O$ 0.50. Medium 4 (g/1): Glucose 20.0, KCL 0.5, $NaNO_3$ 2.0, $MgSO_4.7H_2O$ 0.5, KH_2PO_4 1.0, $FeSO_4$. $7H_2O$ 0.005. Medium 5(g/1): Sucrose 50.0, $MgSO_4$. $7H_2O$ 0.5, $NaNO_3$ 7.5, $FeSO_4$. $7H_2O$ 0.01, KH_2PO_4 1.0. The transformation period 48 h, pH 6, substrate concentration 5 mg/50 ml, inoculum size 2 ml/50 ml.

The results presented in (Fig 1) indicated that the constituents of Kinawy's medium (Kinawy 1974) proved to be the most suitable and conducive medium for the bioconversion of pro-gesterone to 11α-hydroxy progesterone, since it gave the maximum yield 41.50% of 11α-HP.

Time course. It was intended to follow up the rate of the transformation of progesterone to 11α-HP to select the proper time at which the maximal activities were achieved (Fig 2). This was approached by assaying the transformation products at different time intervals (24, 48, 72, 96 and 120 h). The results showed that the maximum production of 11α-HP (49.58%) was obtained in 72 h after incubation. This finding is according with the work reported by Ohlson *et al* 1980; Sallam *et al* 1989; Smith 1998, but differ from that reported by Hamdi *et al* 1999. However, the best yield of 11α-HP was obtained in 48 h by *Mucor racemoses* NRRL 3639.

pH Dependence. The effect of different initial pH values (4-9) of the selected fermentation medium on the bioconversion process was investigated (Fig 3). The result revealed that the best yield of 11α-HP (49.54%) was obtained at pH 6. Several investigators have reported that the optimum pH value for the maximal hydroxylation was at pH 6 (Ohlson *et al* 1980; Somal and Chopra 1985; Yusef 1991; Schlosser *et al* 1993; Smith *et al* 1994; Hamdi *et al* 1999).

Substrate level. Effect of different progesterone concentration (5 - 50 mg/50 ml medium) on the bioconversion process was investigated (Fig 4).The results showed that the maximum yield of 11α-HP (56.87%) was obtained by the

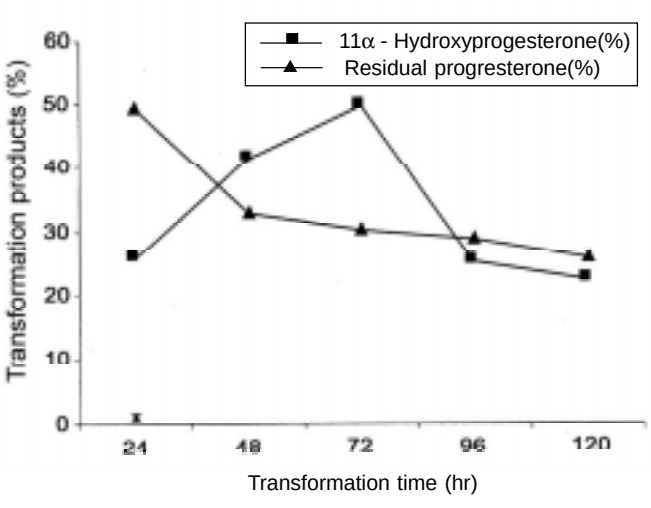

Fig 2. Time course fermentation of progesterone to 11α-hydroxyprogesterone by *A. ochraceus* NRRL 405.

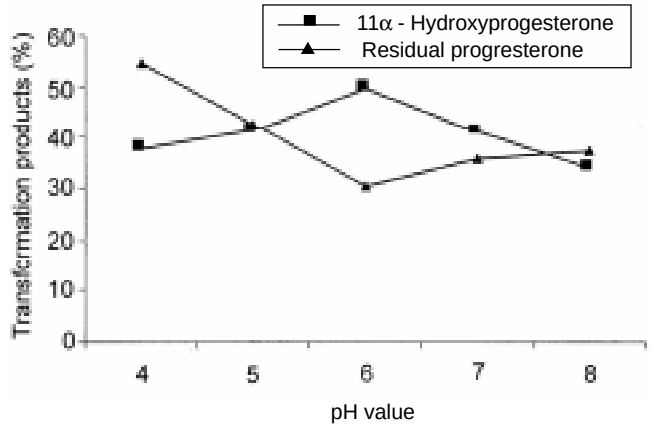

Fig 3. Effect of different initial pH of the fermentation medium on the transformation of progesterone using *A. ochraceus* NRRL 405.

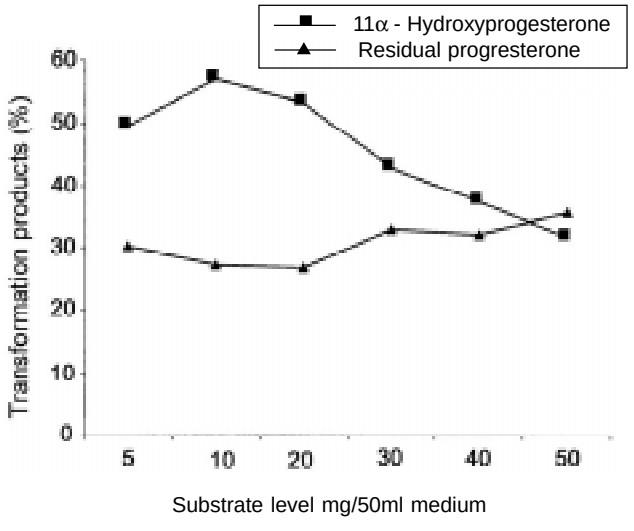

Fig 4. Effect of different substrate levels on the bio-conversion of progesterone to 11α-hydroxyprogesterone by *A. ochraceus* NRRL 405.

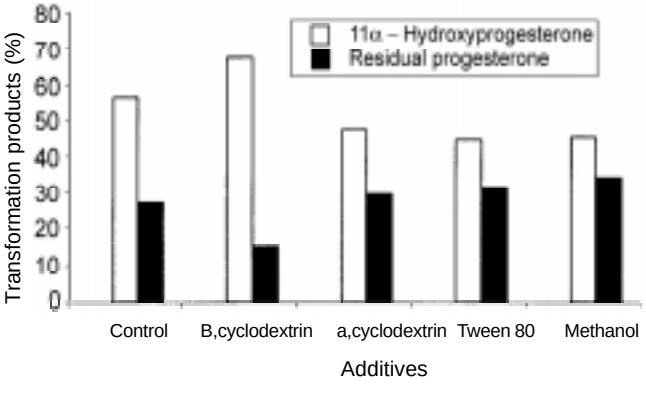

Fig 5. Effect of additives on the bioconversion of progesterone to 11α-hydroxyprogesterone.

addition of 10 mg/50 ml medium whereas further increase in its concentration reduced the yield. In accordance to these results (Somal and Chopra 1985; Schlosser *et al* 1993) who stated that the high substrate concentration induced by products formation.

Additives effects. The effect of some additives (β-cyclodextrin, Tween 80, α-cyclodextrin or methanol) on the transformation of progesterone to 11α-HP were tested (Fig 5). The results showed the enhancement effect of β-cyclodextrin on the bioconversion process, where it gave the best yield of 11α-HP (67.27%). The enhancement effect of β-cyclodextrin on the sterol degradation was stated by (Paul *et al* 1989) who reported that by the addition of β-cyclodextrin the yields of AD and ADD were increased by 35-40%.

Effect of the intermittent addition of β-cyclodextrin. The present study was carried out to evaluate the effect of β-cyclodextrin on the bioconversion process. It was added at different time intervals (0, 12, 24 and 48 h) of incubation and assaying the transformation yield at 72 h (Fig 6). The results showed that the addition of β-cyclodextrin at 12 h increases the yield of 11α-HP by 33.02% as compared to the control culture. The improvement of products formation may be due to both better solubility and bioavailability of progesterone as well as denovo synthesis of steroid hydroxylating enzyme during fungal growth (Irrgang *et al* 1992; Abdel-Salam 2003).

β-*Cyclodextrin concentration*. The effect of different β-cyclodextrin concentration (1.0-8.0g/1) on the transfor-

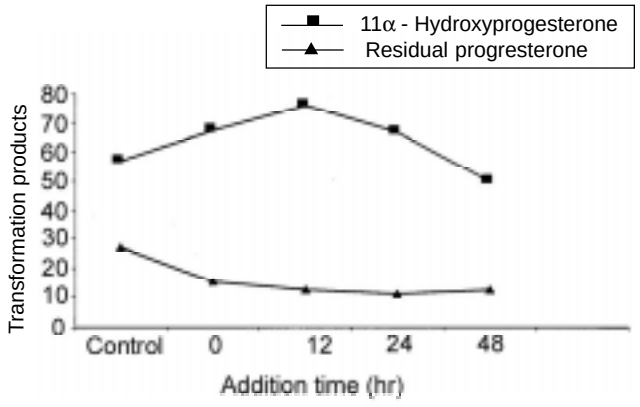

Fig 6. Effect of intermittent addition of β-cyclodextrin on the bioconversion of progesterone to 11α-hydroxyprogesterone.

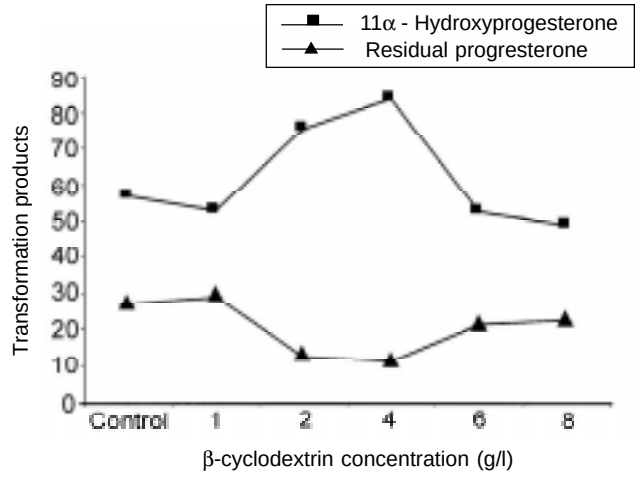

Fig 7. Effect of different β-cyclodextrin concentrations on the bioconversion of progesterone by *A-ochraceus* NRRL 405.

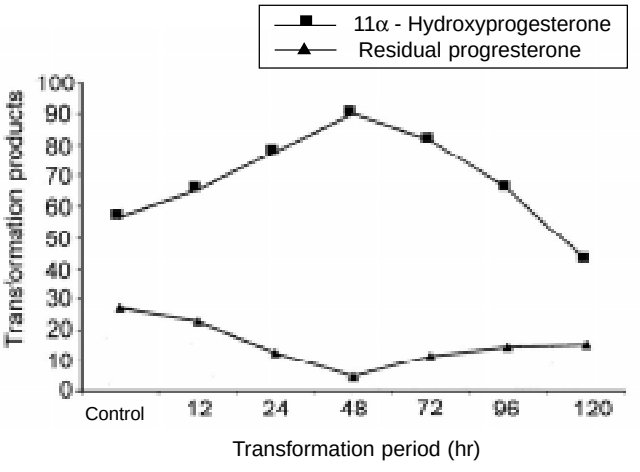

Fig 8. Effect of β-cyclodextrin addition on the bioconversion time course of progesterone to 11α-hydroxyprogesterone.

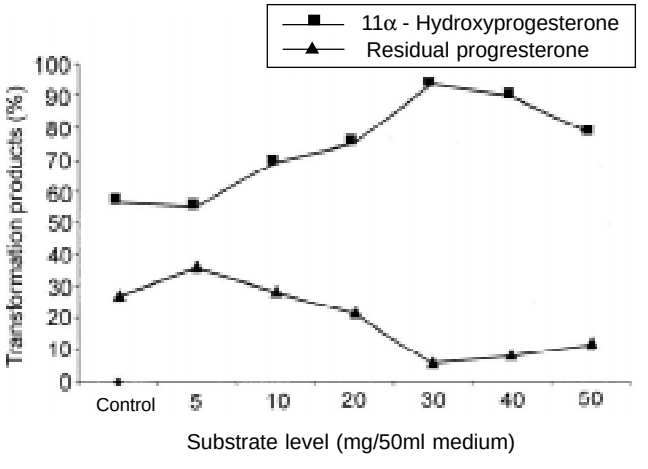

Fig 9. Bioconversion of progesterone at high substrate levels in the presence β-cyclodextrin.

mation of progesterone was investigated (Fig 7). The results showed that 34% increase in the yield of 11α-HP was obtained at conc. 4 g/1. The addition of β-cyclodextrin led to higher partition coefficient and thus better availability of progesterone in comparisons with the control culture (Paul *et al* 1989; (Schlosser *et al* 1993).

Effect of β-cyclodextrin on the bioconversion time course. Effect of β-cyclodextrin addition on the bioconversion time course of progesterone was studied (Fig 8). The results showed that the maximum yield of 11α-HP (89.7%) was obtained at 48 h after incubation. The transformation period was decreased and these results indicated that the presence of β-cyclodextrin in the fermentation medium increase the progesterone solubility S_0 and thus led to better availability to the hydroxylating enzymes which resulted in lower bioconversion time.

Bioconversion process at high substrate levels. The transformation reaction was carried out at different progesterone concentrations 5-50 mg/50 ml medium in the presence of β-cyclodextrin (Fig 9). The results indicated a noticeable steady increase in 11α-HP formation as progesterone concentration was increased and reached to the maximum (93.10%) at 30mg/50ml medium. The addition of β-cyclodextrin to the culture medium had not only led to better progesterone solubility as stated above but also decreased the toxic effect of higher substrate concentrations. Thus the microorganism tolerated high substrate levels and led to better transformation activity.

Conclusion

The mould culture *A. ochraceus* NRRL 405 transformed progesterone to 11α-hydroxyprogesterone efficiently in the presence of β-cyclodextrin. The maximum yield of 11α-HP (93.1%) was obtained as compared to the control culture (56.87%). These results indicated that the presence of β-cyclodextrin increased the progesterone solubility. Hence, its availability to the growth cells, which allowed to perform the 11α-hydroxylation reaction at high progesterone levels and shorter transformation period were compared to the control.

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