



BIOCONVERSION OF 11- β , 17- α , 21-TRIHYDROXY-4-PREGNENE-3, 20-DIONE, 21-O-SUCCINATE BY ACTIVELY GROWING CULTURE OF *PSEUDOMONAS PUTIDA* MTCC 1259

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ABSTRACT: Steroids constitute a particular class of lipids characterized by a typical tetracyclic skeleton, composed of one five-member ring and three fused six-member rings. Steroids are widely used as therapeutic agents. The present work is on corticosteroid. The corticosteroid used in present research work was 11- β , 17- α , 21-trihydroxy-4-pregnene-3, 20-dione, 21-O-succinate (Hydrocortisone sodium succinate). An attempt was made to cleave the side chain of the precursor corticosteroid by actively growing culture of *Pseudomonas putida* MTCC 1259. Growing cells of *Pseudomonas putida* MTCC 1259 were found to effectively convert hydrocortisone succinate in nutrient broth, at pH 7.5, temperature 37 °C when 0.25 to 0.50 mg l⁻¹ substrate was added after 24 hr of inoculation. Higher substrate concentration was found to be inhibitory for the bioconversion of the precursor steroid.

Key-words: corticosteroid, Hydrocortisone sodium succinate, 1, 2-dehydrogenation, side-chain cleavage

INTRODUCTION

The steroids vary by the functional groups attached to their four ring core and by the [oxidation state](#) of the rings. To produce functionalized compound with therapeutic use and commercial value, highly specific reaction are required. The complexity of steroid molecules renders the use of biocatalysts particularly interesting, due to high region- and stereo-selectivity of the reaction to be performed. These characteristics together with mild condition required for bioconversions, led to the development of high yield biological production processes, which are more environmentally friendly than their chemical synthesis counterparts. However, there are many well-established bioconversions but research efforts are going on to enhance the efficiency of the existing processes as well as to identify new potentially useful bioconversions. Numbers of microorganisms have been utilized for steroid transformation including varieties of bacteria, fungi, yeast and algae. A major problem in the biotransformation of steroid is the poor solubility of substrates in aqueous solution, which leads to extremely low productivity. A unicellular microalga, *Chlamydomonas reinhardtii*, was isolated from rice paddy-field soil and water samples and used in the biotransformation of hydrocortisone (1). Fermentation was carried out in BG-11 medium supplemented with 0.05% substrate at 25°C for 14 days of incubation. The products obtained were chromatographically purified and characterized using spectroscopic methods. 11 β , 17 β -Dihydroxyandrost-4-en-3-one (2), 11 β -hydroxyandrost-4-en-3,17-dione (3), 11 β , 17 α , 20 β , 21-tetrahydroxypregn-4-en-3-one (4) and prednisolone (5) were the main products of the bioconversion. The observed bioreaction features were the side chain degradation of the substrate to give compounds 2 and 3 and the 20-ketone reduction and 1,2-dehydrogenation affording compounds 4 and 5, respectively. (Ghasemi et al 2008).

A spectrophotometric method for simultaneously estimating 11 β , 17 α -dihydroxy-4-pregnene-3, 20-dione 21-succinate and its 1(2)-dehydrogenated product has been developed using sulphuric acid chromogens by Pawar and Lad (2011). The method was used to follow the progress of bioconversion during C-1(2)-dehydrogenation by *Comamonas acidovorans* MTCC 3364. Two corticosteroids, 11 β , 17 α -dihydroxy-4-pregnene-3,20-dione-21-succinate (hydrocortisone succinate) and its dehydrogenated product 11 β ,17 α -dihydroxy-1,4-pregnadiene-3,20-dione-21-succinate(prednisolone succinate) are important anti-inflammatory agents used in variety of dosage forms. Simultaneous estimation of precursor and products during steroid 1(2)-dehydrogenation was done by this method. The work represented rapid and cost effective spectrophotometric method for routine estimation of precursor and product during C-1(2)-dehydrogenation of hydrocortisone succinate by *Comamonas acidovorans* MTCC 3364. The method was based on characteristic chromogen formation by the steroids which showed concentration dependent difference in absorption at the selected wavelength. Several derivatives of hydrocortisone (11 beta, 17 alpha, 21-trihydroxy-4-pregnene-3, 20-dione) have been prepared in order to increase the solubility of the steroid in aqueous media. Bioconversion of hydrocortisone is frequently used as a model system to investigate the alternative bioconversion possibilities. The results are generally extrapolated to be applicable on complex bioconversions like side chain cleavage. The present work was initiated with the aim to study the effect of medium composition, various pH, temperature of incubation, substrate concentration and time of substrate addition on the bioconversion of hydrocortisone sodium succinate by *Pseudomonas putida* MTCC 1259.

MATERIALS AND METHODS

Materials: The general chemicals and media components required for the study were purchased from Hi media, Glaxo, Qualigens, Merk and S D fine chemicals. The marketed injection of hydrocortisone sodium succinate was used as precursor steroid for the study.

Microorganism: The following strain of microorganism was purchased from MTCC, Institute of Microbial Technology, Chandigarh, India.

•*Pseudomonas putida* MTCC 1259

The strain was maintained on nutrient agar slant supplemented with appropriate inducer steroid (1mg/litre) and stored in refrigerator at 4°C. The organism was sub-cultured every month.

Bioconversion by growing cells: Medium E of composition shown in table 1 was prepared and pH adjusted to 7 with the help of 0.1N NaOH or HCl. Medium (20 ml) was dispensed in a series of 100 ml conical flasks and sterilized at 121 °C for 15 min and allowed to cool to room temperature. The flasks were inoculated with actively growing culture of the strain of microorganism in the same medium. After overnight growth at 37 °C on a shaking platform at 120 rpm, 2 mg precursor steroid was added. Samples were drawn after 96 hrs, 144 hrs and 192 hrs and extracted twice with equal volume Benzene:ethyl acetate (1:2) or ethyl acetate. The organic layers were decanted, pooled and solvent evaporated by keeping the tubes in a boiling water bath. The residues were dissolved in 3 ml sulphuric acid and chromogen was developed by keeping the tubes in a boiling water bath for 30 min and absorbance of chromogen determined at selected wavelength. Various parameters like effect of pH, incubation temperature, substrate concentration, time of substrate addition and medium composition on bioconversion of hydrocortisone sodium succinate were optimized. All the experiments were conducted in triplicate.

Thin Layer Chromatography of steroids: Slurry of silica gel (25%) was prepared in water, allowed to swell for 5 min, layered on a clean glass plate and allowed to air dry. The plates were activated in a hot air oven at 110 °C for at least 30 min and allowed to cool to room temperature. Extracted steroid samples were spotted along with authentic precursor and product steroids. The plates were developed in 1: 2 mixture of Benzene: ethyl acetate till the solvent front reached the top of the plate. The solvent was allowed to evaporate and plates sprayed with 98% sulphuric acid. The plates were then heated in a hot air oven at 110 °C for 5-10 min for visualization of steroids.

Quantitative estimation of steroids: Aliquots of extracted steroids were taken in separate tubes and solvent evaporated by keeping the tubes in boiling water bath. The residue were dissolved in 3 ml sulphuric acid and chromogen developed by heating the tubes in a boiling water bath for 30 minutes. Amount of steroid in the sample was determined from the calibration curve of the steroidal product.

RESULTS AND DISCUSSION

Simultaneous estimation of precursor and product steroids has always been a problem during steroid bioconversions since these compounds show similar absorption patterns under UV light. Hence an appropriate estimation method for determination of precursor and product concentrations in the fermentation extracts has been a difficult task (Lad 2011). Sulphuric acid chromogens have been effectively used for simultaneous estimation of steroidal compounds. Hence, it was decided to prepare sulphuric acid chromogens of hydrocortisone sodium succinate and its degradation product(s) by *Pseudomonas putida* MTCC 1259 and study their absorption spectra.

Bioconversion medium was extracted using equal volume of ethyl acetate and evaporated product(s) were loaded on thin layer chromatography plates. Different products were accumulated at different hours. After 96 hrs three spots were observed but only one degradation product obtained after 120 hrs and every 24 hrs after that on TLC plates. The compound was obtained in high concentration and was major degradation product.

As reported by Patel R. (2011), the precursor hydrocortisone sodium succinate showed minimum mobility when TLC plates were run with 1: 2 benzene : ethyl acetate. Only products moved with the solvent and precursor was found at the spotted position. It was decided to extract the fermentation medium with 1: 2 benzene : ethyl acetate to minimize the interference of substrate in extracted product. Extracted samples were spotted on TLC plates and it was observed that only product was present in extracts which showed mobility along with the solvent front and showed brown coloration when sulphuric acid was sprayed and charred at 110 °C. Spot of precursor was not observed. Preperative TLC was done and product isolated from the TLC plates and recrystallized using acetone. The crystallized product was weighed and different aliquotes were prepared after dissolving in appropriate solvent. Then, solvent was evaporated, sulphuric acid chromogens were prepared from the aliquotes and absorbance were taken at different wavelengths. The product steroid showed linear increase in absorption with increasing concentration at 260 nm and 400 nm (Jay Jethwa, 2011). Hence, it was decided to follow the progress of bioconversion of hydrocortisone succinate by *Pseudomonas putida* MTCC 1259 by monitoring the absorption of sulphuric acid chromogen at 260 nm and 400 nm.

Bioconversion of steroids has been shown to be an oxygen requiring process. Further, the enzymes required for catalysing chemical modification of steroid molecule are inducible and appear in the medium in sequential manner. The product of one step acts as inducer as well as substrate for the next enzyme in sequence (Lad, 2011). Hence, growing cells show a better conversion of precursor steroids. Side chain cleavage of cortical compounds is considered one of the important steroid bioconversions (Marshak, et al., 1972). Hydrocortisone sodium succinate salt is easily available and economically feasible precursor. *Pseudomonas putida* MTCC 1259 was shown to effectively cleave the side chain of variety of corticosteroids (Patel R, 2011) and hence, was selected to study the effect of various parameters on bioconversion of hydrocortisone succinate.

Various parameters for the bioconversion of hydrocortisone succinate were optimized. Table 2, shows the effect of composition of medium used for the cultivation of the organism. It can be apparently seen from the table that maximum bioconversion was observed in medium E (Table 1). Similar medium with addition of glucose was used by Abeer Abd El-Hady, et al., (2004) for bioconversion of hydrocortisone to prednisolone by *Pseudomonas oleovorans* cells and achieved 51 % of prednisolone yield.

Table 1: Composition of media used for culture of microorganisms.

Medium components (g/l)	A	B	C	D	E
Ammonium Sulphate			3.0	3.0	
Beef Extract					1.5
CaCl ₂			0.02	0.02	
FeSO ₄			0.1	0.1	
Glucose			20.0	20.0	
K ₂ HPO ₄			3.0	3.0	
KH ₂ PO ₄			1.0	1.0	
MgSO ₄			0.2	0.2	
Peptone	5.0	10.0			5.0
Sodium Chloride		5.0			5.0
Yeast Extract		5.0		10.0	1.5

Table 3, illustrates the effect of pH on bioconversion of hydrocortisone succinate. It is obvious from the table that maximum activity was observed at pH 6.5 to 7.5 with a maximum of about 71 % conversion after 96 hr incubation at pH 7.5. There was decrease in activity observed at higher or lower pH. The same range of pH was used by Nobile (1958), for the cultivation of *A. simplex*. The results also act in accordance with the observation of Miller (1972). He found that enzyme involved in bioconversion of hydrocortisone shows the maximum activity between the pH 7 to 8.

Table 2: Effect of medium composition on hydrocortisone succinate bioconversion

Medium	% conversion after incubation time(Hr) *		
	96	144	192
A	18.16± 0.37	15.38 ±0.25	15.04 ±0.36
B	15.80± 0.53	16.13 ±1.69	15.58 ±1.60
C	15.66 ±0.87	17.25± 0.11	16.61± 0.17
D	19.03± 1.85	23.73± 1.44	22.18 ±1.64
E	29.05± 0.23	29.88± 2.58	31.24± 3.89

* Data are mean of three replicates ± SD;

Table 3: Effect of pH on hydrocortisone succinate bioconversion by growing cells of *Pseudomonas putida* MTCC 1259.

pH	% conversion after incubation time (Hr)*		
	96	144	192
6	53.82± 1.60	51.35± 2.71	56.93± 6.33
6.5	54.80± 1.81	51.91± 5.0	54.97± 6.46
7	59.85± 1.61	61.89 ±5.0	56.45± 4.62
7.5	71.29± 4.59	70.25± 4.11	70.87± 3.13
8	23.22± 4.18	33.13± 1.88	35.65± 4.93
8.5	52.94± 3.084	49.77± 0.16	54.13± 2.75

*Data are mean of three replicates ± SD;

Table 4, manifests that maximum bioconversion of hydrocortisone succinate was observed at 37 °C after 144 hr incubation. The organism failed to grow at 47 °C hence no bioconversion was observed. The similar range of temperature was used by Herzog (1967). He observed that fermentation temperature for dehydrogenation usually higher than for hydroxylation, ranging from 25 to 37 °C. The results also comply with the studies of the Kominek, et al. (1988), who used similar temperature range for cultivation of *A. simplex*, to study the effect of scavenger of toxic oxygen on microbial 1-dehydrogenation.

Table 4: Effect of incubation temperature on hydrocortisone succinate bioconversion by growing cells of *Pseudomonas putida* MTCC 1259

Temperature (°C)	% conversion after incubation time (Hr)*		
	96	144	192
37	29.41± 2.35	36.32± 3.79	33.89± 2.04
42	20.63± 2.17	21.05± 5.49	19.94± 1.48
47	0	0	0

*Data are mean of three replicates ± SD;

Table 5: Effect of substrate concentration on hydrocortisone succinate bioconversion by growing cells of *Pseudomonas putida* MTCC 1259

Substrate Concentration (mg l ⁻¹)	% conversion after incubation time (Hr)*		
	96	144	192
0.25	21.80 ± 1.15	30.79± 1.16	24.50± 0.40
0.50	29.44 ± 0.64	34.01± 1.07	27.97± 2.85
1.0	4.18 ±1.35	4.30± 1.36	4.24± 0.54
1.5	6.39± 1.76	6.41± 1.61	5.99± 0.07
2.0	1.94± 0.03	2.07± 1.49	1.83± 0.05
3.0	2.04± 0.04	1.99± 0.92	1.68± 0.04

*Data are mean of three replicates ± SD;

Steroid bioconversions have been reported to be affected by substrate inhibition (Patil, et al., 2002). Aim of steroid bioconversions is to attain maximum product accumulation with high substrate addition, where split doses of substrate are added at different time intervals to circumvent the problem of substrate toxicity to the microorganism or the biocatalyst. The effect of substrate concentration on the bioconversion of hydrocortisone succinate has describe in Table 5, it is evident from the table that maximum bioconversion was observed at substrate concentration ranging from 0.25 to 0.50 mg l⁻¹. There was sharp decrease in bioconversion rate with the increase in substrate concentration indicating the inhibitory effect of higher concentration of substrate on enzymic activity. Evans, et al. (1988) used 0.5 mg l⁻¹ for the bioconversion of hydrocortisone to prednisolone using dried cells of *A. simplex*. Maximum bioconversion of hydrocortisone to prednisolone was achieved at substrate concentration 5 to 30 mg using *Pseudomonas oleovorans* cells (Abeer Abd El-Hady, et al., 2004). Many literatures suggest that in conversion of practical importance, substrate levels are generally 0.5 to 2.0 g l⁻¹ and conversion reaches to 80-90 % at completion (Fernandes, et al., 2003).

Table 6, depicts that maximum bioconversion of hydrocortisone succinate was observed after 24 hr of substrate addition. As much as 71 % conversion rate was achieved when substrate was added after 24 hr. Lower conversion rate was observed when substrate was added into the medium during inoculation or after 48 and 72 hr. Abeer Abd El-Hady, et al. (2004) added substrate at the time of inoculation in production of Prednisolone from hydrocortisone by *Pseudomonas oleovorans* cells and achieved maximum 65% yield of prednisolone. It seems that the precursor is toxic for the *Pseudomonas putida* MTCC 1259 selected for the present study and using longer incubation time leads to synthesis of degradative enzymes responsible for further degradation of the desired product. Jiradej, et al. (2003) added substrate after 24 hrs of inoculation in biotransformation of chlormadinone acetate to delmadinone acetate by free and immobilized *Arthrobacter simplex* ATCC 6946 and *Bacillus sphaericus* ATCC 13805 and achieved maximum 47 % yield of demadinone acetate. Gharaee (2006) added substrate at 0 hrs during biotransformation of hydrocortisone by *Neurospora crassa* and achieved 25 % conversion rate.

The results highlight the usefulness of the organism for bioconversion of 11- β , 17- α ,21-trihydroxy-4-pregnene-3, 20-dione,21-O-succinate.

Table 6: Effect of time of substrate addition on hydrocortisone succinate bioconversion by growing cells of *Pseudomonas putida* MTCC 1259

Time of substrate addition (Hr)	% conversion after incubation time (hr)*		
	96	144	192
0	37.33 \pm 2.24	40.22 \pm 3.07	43.48 \pm 4.04
24	71.29 \pm 4.59	70.25 \pm 4.11	70.87 \pm 3.13
48	40.69 \pm 3.33	42.71 \pm 3.50	54.38 \pm 1.51
72	46.20 \pm 4.74	48.22 \pm 4.29	50.17 \pm 0.68

*Data are mean of three replicates \pm SD;

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