PRAJÑĀ-Journal of Pure and Applied Sciences, Vol. 19: 4 - 7 (2011)
ISSN 0975 - 2595

BIOTRANSFORMATION OF 11β, 17α-DIHYDROXY-4-PREGNENE-3, 20-DIONE-21-O-SUCCINATE TO A 17-KETOSTEROID BY *PSEUDOMONAS PUTIDA* MTCC 1259 IN ABSENCE OF 9α-HYDROXYLASE INHIBITORS

Rahul Patel* and Kirti Pawar

Ashok and Rita Patel Institute of Integrated Study and Research in Biotechnology and Allied Sciences, New Vallbh Vidyanagar, Anand 388 121, Gujarat, India

ABSTRACT

Growing cells of *Pseudomonas putida* MTCC 1259 were used for biotransformation of a steroid with cortical side chain, 11β,17α-dihydroxy-4-pregnene-3,20-dione-21-O-succinate. Separation of the steroidal components of fermentation extracts was done by TLC with an optimized solvent system. The precursor and different product steroids were found to separate satisfactorily in a 2:4 mixture of benzene:ethyl-acetate. A 17-ketosteroid was found to accumulate in the fermentation medium after 120 h incubation at 37 ºC under aerobic incubation without addition of any ring cleavage inhibitors which suggested a desirably low 9α-hydroxylase activity in the strain. The biotransformation product was characterized to be Adrost-4-ene-3,17-dione on the basis of FTIR spectroscopic analysis. The pathway of corticosteroid side chain cleavage by the organism was worked out. The absence of C-1(2)-dehydrogenated product in the fermentation broth indicated a low activity of C-1(2)-dehydrogenase enzyme in the strain. The strain is found suitable for production of 17-ketosteroids from precursors having cortical side chains.

Key words : *steroid bioconversion; 17-ketosteroid production; 9α-hydroxylase inhibitors; chromatographic separation of steroids*

INTRODUCTION:

Steroid modification or synthesis is one biotechnological process having potential applications in pharmaceutical industries due to the complex structure of steroid molecule and requirement of complicated, multi-step schemes for the chemical synthesis of steroid compounds. The basic ring structure of some steroid derivatives is sensitive to cleavage by a wide variety of chemicals. Chemical synthesis also requires the use of reagents such as pyridine, sulfur trioxide or selenium dioxide which are hazardous to the health of production staff and constitute a serious environmental disposal problem. There are plenty of steroid drugs available in market, and much research is on going to modify steroid drugs to more potent derivatives with longer half-lives in the blood stream, having simpler delivery methods, more specific and one having no or less side-effects. This can be achieved by microbial bioconversion [1].

Microbial cleavage of steroid side-chains has been an extensively researched subject because of the potential utility of this reaction for the production of high value therapeutic pharmaceuticals. Side-chain degradation has been studied by first blocking ring-degradation. It has been found in several studies using *Mycobacteria* that hydroxylation of the terminal carbons is the first step [2]. Wide varieties of fungi are capable of side-chain cleavage of progesterone [3]. During side-chain cleavage of progesterone, usually 17-acetate is formed by introduction of oxygen between C-17 and C-20 then an esterase cleaves the acetate leaving the 17-hydroxy steroid, testosterone. Then the 17-hydroxyl group is oxidized to 17-ketone, some of the organisms may form the 1-dehydrogenated derivatives. Adrost-4-ene-3,17-dione [AD], and Androsta-1,4-diene-3,17-dione [ADD] are among several important compounds obtained by degradation of steroid side-chain which may be useful for the production of androgens, estrogens and other compounds by further chemical modification. 1-dehydrotestolactone may also be produced which is approved for the treatment of mammary cancer. Androst-4-ene-3,17-dione can be chemically converted to spironolactone which is an important drug in the treatment of hypertension. Androst-4-ene-3,17-dione may also be chemically reduced to give testosterone and some derivatives that have important medicinal uses [3].

Pseudomonads are able to grow in different environments and have broad metabolic versatility and genetic plasticity. Also some important reactions for steroid bioconversion are reported with these groups of organisms. Screening of microorganisms able to grow on bile acids and in the presence of organic solvents led to the isolation of *Pseudomonas putida* ST491 [1]. When grown in 5 g l^{-1} litocholic acid in the presence of diphenyl ether, 60% of the substrate was cleaved to AD (75% yield), with ADD (17% yield) and pregna-1,4-dien-3-on-20-al (8% yield) as secondary products. If the organic solvent was replaced by Triton X-100 in the aqueous conversion medium, 40% of the substrate was converted to ADD alone [1]. *Pseudomonas* mutant had converted compound-S directly to prednisolone, showing that organism is capable of 1-dehydrogenation along with 11β-hydroxylation [4]. According to one report, [5] 19-hydroxyandrostenedione was converted to estrone by a *Pseudomonas*. They proposed that the organism 1-dehydrogenated the steroid and then the hydroxymethyl group was removed by aldol cleavage.

The study was initiated with an aim to cleave side chain of a corticosteroid using *Pseudomonas putida* MTCC 1259 which was known to successfully degrade side chain of cholesterol [6]. Emphasis was to accumulate 17-ketosteroids without addition of inhibitors for enzymes responsible for cleavage of steroid ring system.

MATERIALS AND METHODS:

Materials: The general chemicals and media components required for the study were purchased from Hi Media, Glaxo, Qualigens, Merck and S D fine Chemicals.

Microorganisms: The strain *Pseudomonas putida* MTCC 1259 was purchased from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India. The strain was maintained on nutrient agar slants supplemented with appropriate inducer steroid (1 mg l^{-1}) and stored in a refrigerator at 4 ºC. The organisms were sub-cultured every month.

Bioconversion of 11β,17α-dihydroxy-4-pregnene-3,20-dione-21-O-succinate by growing cells: Medium containing g L^{-1}, Beef Extract (1.5), Peptone (5), NaCl (5), Yeast Extract (1.5), Inducer steroid (1 mg L^{-1}) was prepared and pH adjusted to 7. Medium (20 ml) was dispensed in series of 100 ml conical flasks and sterilized at 121 ºC for 15 mins and allowed to cool to room temperature. Three flasks were inoculated with actively growing culture of *Pseudomonas putida* MTCC 1259 in the same medium. After overnight growth at 37 ºC on a 110 rpm shaking platform, steroid substrate (4 mg) dissolved in 0.5 ml ethyl acetate (or as specified) was added in each flask. Sample (2 ml) was withdrawn every 24 hours and extracted twice with equal volume of ethyl acetate. The organic layers were decanted, pooled, dried over sodium sulphate and solvent evaporated by

*Corresponding author : r.jpatel@rediffmail.com

keeping the tubes in boiling water bath and residual steroid re-dissolved in 0.1 ml ethyl acetate. Aliquots were taken for qualitative analysis by thin layer chromatography.

Thin Layer Chromatography of steroids: Method described by [7] was used for qualitative analysis of the bioconversion products. Slurry of silica gel (5%) was prepared in distilled water and 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 ºC for 30 mins and allowed to cool to room temperature. Extracted steroid samples were spotted along with the authentic precursor with the help of capillary tube. The plates were developed in a mobile phase having mixture of benzene: ethyl acetate optimized for better separation of steroid samples. The solvent was allowed to evaporate at 50 ºC for 15 mins and plates were sprayed with 60% sulphuric acid for preparing steroid chromogens. The plates were heated in a hot air oven for 110 °C for 30 mins for visualization of chromogens.

Identification of Steroids: Identification of the steroids was done by comparing the color and Rf of the spots with the authentic samples. Confirmation of identification was done by FTIR spectroscopy. For isolation of steroids for IR spectroscopy, preparative TLC as described by [8] using the solvent system optimized for separation of the desired steroids. The separated steroids were visualized in a chamber of iodine vapors, positions of steroids marked; silica gel was scrapped from the marked area and extracted thrice with ethyl acetate. Ethyl acetate was recovered, steroid sample concentrated in a powder form, recrystallized from either methanol or acetone and submitted for FTIR analysis using **FTIR spectrometer, Perkin Elmer make, model Spectrum GX, wave number range:** 30-15600 cm^{-1} suitable for liquid, solid and gas samples, operating mode NIR and MIR with NIR source optical system 15,200 – 1,200 cm^{-1}, beam splitter KBr: 7,800 - 370 cm^{-1} with optimum range: 7,800 - 1,200 cm^{-1} and detector MIRTGS: 10,000 - 220 cm^{-1}.

RESULTS AND DISCUSSION:

Bioconversion products of 11β,17α-dihydroxy-4-pregnene-3,20-dione-21-O-succinate by *P. putida* MTCC 1259 have been shown in table 1. A prominent bluish green spot appeared on the TLC plate along with the precursor that showed reddish brown spot. Product forming bluish green spot was supposed to be accumulating as intensity of the spot increased with increasing time of incubation.

Although it was possible to identify the product steroids from the precursor steroids using 5 : 1 benzene : ethyl acetate as a mobile phase, to obtain the individual compounds with highest purity using preparative TLC, it was necessary to attain maximum possible separation of product steroids. Hence, it was decided to optimize the mobile phase composition for 11β,17α-dihydroxy-4-pregnene-3,20-dione-21-O-succinate and its bioconversion products. During chromatographic separation of steroids choice of proper mobile phase and optimization of mobile phase are important as the product steroids are closely related compounds which makes their separation difficult. In first step 1 : 1 mixture of acetone : ethyl acetate and 5 : 1 mixture of benzene : ethyl acetate as a mobile phase solvent system was screened for separation of precursor steroid and its bio-conversion product.

Although visualization of the product was possible, separation of the product from precursor was very low. So it was decided to optimize the composition of mobile phase for which, the ability of different composition of mobile phase (5 : 1, 4 : 2, 3 : 3, 2 : 4, 1 : 5 benzene : ethyl acetate) to separate precursor from product was checked by spotting steroids extracted from 2 ml bioconversion medium of *P. putida* MTCC 1259 along with authentic precursor.

Table-1: Bioconversion products of hydrocortisone succinateby *P. putida* MTCC 1259 as seen on TLC analysis

Time	192 hrs	240 hrs		336 hrs
Spot	Green	Green	Golden	Green
Rf	0.0967	0.0793	0.174	0.079
Spot intensity	+	+++	++	++++

+ Score of spot intensity

Table 2: Effect of mobile phase composition on Rf of bioconversion products of *P. putida* MTCC 1259 on TLC plate

Ratio of Benzene: Ethylacetate	**Precursor (Spot 1)**	**Rf of Golden spot (Spot 2)**	**Rf of Green Spot (Spot 3)**
5: 1	At origin	0.1	0.079
4 : 2	At origin	0.36	0.45
3 : 3	At origin	0.36	0.72
2 : 4	At origin	0.45	0.87
1 : 5	At origin	0.63	0.90

It is clear from Table 2 that 2 : 4 mixture of benzene : ethyl acetate shows maximum separation of bioconversion products. It was also obvious that two major products were formed golden (spot-2) and Green (spot-3). Authentic precursor did not move past the origin in all the tested combinations of benzene and ethyl acetate. It was also observed that authentic hydrocortisone succinate was poorly soluble in ethyl acetate. Also, products solubility in ethyl acetate was higher than in benzene. Hence it was decided to use 2 : 4 benzene : ethyl acetate mixture as the developing solvent for TLC during future experiments.

Although product steroids can be identified by the color and Rf of the spot, FTIR spectra provides an important tool for further confirmation of this analysis. Figure 3 represents FTIR spectrum of authentic precursor used for bioconversion. Figure 4 and 5 depict the FTIR spectra of spot-2 (golden) and spot-3 (green) respectively. Upon inspection of FTIR spectrum of spot-2, prominent peaks were observed at 1647 and 1711 cm^{-1} indicating the presence of 3-keto group. Absence of peak at 1730-1750 cm^{-1} suggests absence of 17-keto group. Broad absorptions in the range of 2800-3400 cm^{-1} indicate presence of aliphatic stretch, indicating that the succinate side chain of the precursor has not been cleaved. Absorbance at 2850 and 2981 cm^{-1} indicated the presence of methyl or methylene groups. The comparison of IR spectra of hydrocortisone succinate and spot-2 indicates only minor changes in the steroid ring (additional peak at 1711.23 cm^{-1} in spot-2). Similar spectral analysis have been suggested while explaining 1,2-dehydrogenation of 17,21-dihydroxypegn-4-ene-3,20-dione. Their product showed had I.R. spectrum bands (KBr) at: 3400 cm^{-1} (OH); 2958 cm^{-1} (CH); 1718 cm^{-1} (CO); 1666 cm^{-1}, 1620 cm^{-1}, 1607 cm^{-1} (Δ1,4-3-one) indicating 1,2dehydrogenation of hydrocortisone to prednisolone. As observed from the suggestions in the IR spectra

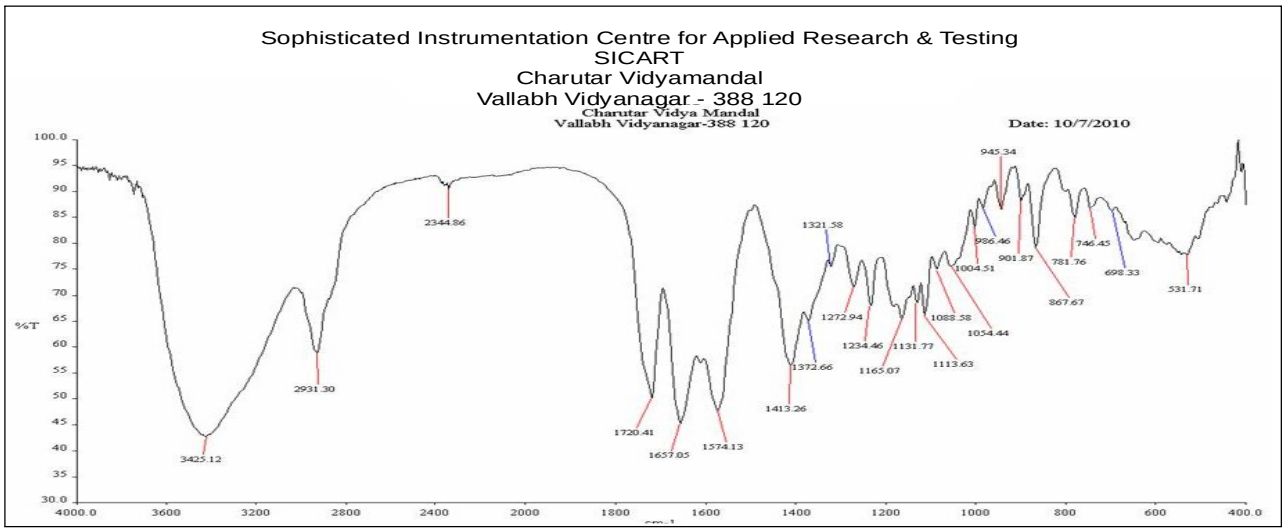


Figure-3 : FTIR spectra of Hydrocortisone succinate – sodium salt, copied from [11] with permission

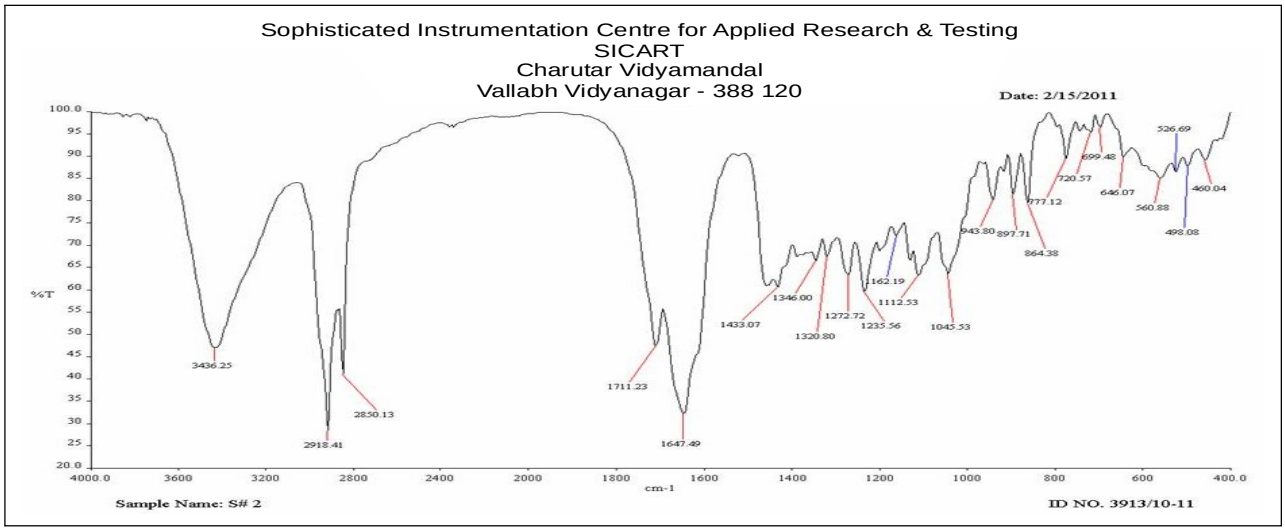


Figure-4: FTIR spectrum of bioconversion product of hydrocortisone succinate by *P. putida* MTCC 1259 (Golden spot or Spot-2)

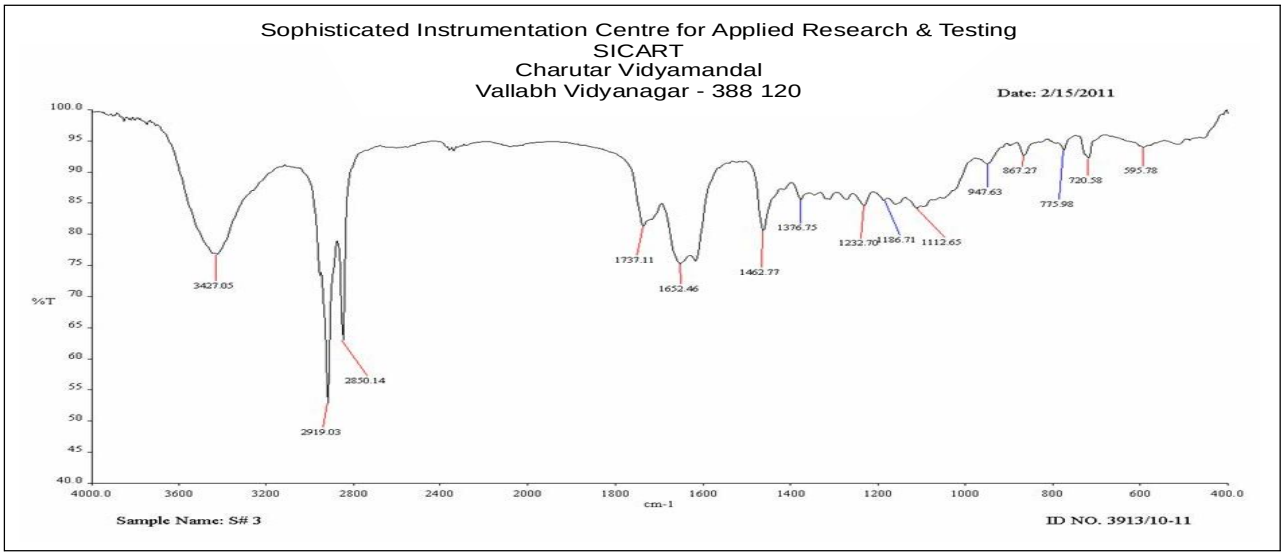
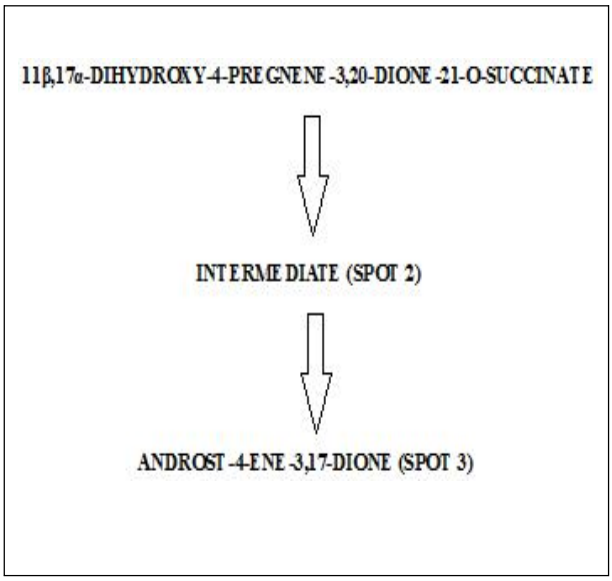


Figure-5: FTIR spectrum of bioconversion product of hydrocortisone succinate by *P. putida* MTCC 1259 (Greenspot or spot-3)

analysis by [9] where the C-H stretch of an aliphatic compound (or fragment) have been analysed. He observed the asymmetric C-H stretch of the methyl and methylene groups (2960 and 2930 cm^{-1}, respectively) occurring at slightly higher frequency than symmetric vibrations (2875 and 2855 cm^{-1}, respectively for methyl and methylene). He further suggested that most part, this simple rule holds true for most common sets of vibrations. Naturally there are always exceptions, and a breakdown of the rationale may occur when other effects come into play, such as induced electronic, spatial or entropy-related effects. The identification peaks suggested for prednisolone by [10] during bioconversion of hydrocortisone are IR ν_{max} (KBr, cm^{-1}) 3050, 2980, 1658, 1624, 1600 cm^{-1} supporting the inferences of the present investigation.

Whereas, observing the FTIR spectra of spot-3 a prominent peak at 1737 cm^{-1} indicated the presence of 17- keto group, hence, side –chain of this product steroid had been cleaved. Absorbance at 1652 cm^{-1} indicate the presence of 4-ene,3-one. Absence of prominent peaks in 1600 cm^{-1} suggests absence of C1-2 double bond. Hence the compound may be Androst-4-ene-3,17-one. Presently it can be concluded that the side-chain of hydrocortisone succinate was successfully cleaved. The pathway for side chain cleavage of can be stated as:



ACKNOWLEDGEMENTS:

The authors thank Charutar Vidyamandal, Vallabh Vidya Nagar, Anand, Gujarat, India for extending all the facilities for successful completion of this work. The authors like to acknowledge Microbial Type Culture Collection, Institute of Microbial Technolgy, Chandigarh, India for providing the strains used for the study and Sophisticated Instrumentation Centre for Applied Research and Training (SICART), Vallabh Vidya Nagar, Anand, Gujarat, India for the IR Spectral analysis of steroid samples.

REFERENCES:

[1] Fernandes, P., Cruz, A., Angelova, B., Pinheiro, H. M. and Cabral J. M. S. (2003) Microbial conversion of steroid compounds: recent developments. *Enzyme and Microbial Technology*, **32**: 688–705.

[2] Zaretskaya, I. I., Kogan, L. M., Tikhomirova, O. B., Sis, J. D., Wulfson, N. S., Zaretskii, V. I., Zaikin, V.G., Skryabin, G. K. and Torgov, I. V. (1968) Microbial hydroxylation of the cholesterol side chain. *Tetrahedron*, 24: Issue 4: 1595-1600.

[3] Miller, T. L. (1972) Steroid Fermentations. In *Comprehensive Biotechnology* (Murrey, M. and Young, E.), Pergamon Press, USA, Vol 4. pp. 297-316.

[4] Takeda, R., I., Nakanishi, J. Terumichi, M. Uchida, M. Katsumata, M. Chibayahsi, and H. Nawa. (1959) Transformation of Reichstein's Substance S to prednisolone by Pseudomonas. Tetrahedron Letters No. 18:17-19.

[5] Dodson, R. M., and R. D. Muir. 1961. Microbiological transformations. VI. The microbiological aromatization of steroids. J. Amer. Chem. Soc. **83**:4627-4631.

[6] Pawar, K. and Bhatt, M. (2011) Accumulation of a Pharmacologically important 17-ketosteroid during Side Chain Cleavage of Cholesterol by *Pseudomonas putida* MTCC 1259. *World Journal of Science and Technology*, 1: No 5: 62-65.

[7] Shinde K (2000): Biotransformation of some steroid drug precursors to 17-ketosteroids by mixed bacterial culture. Ph. D Thesis, Devi Ahilya Vishwavidyalaya, Indore (MP) India.

[8] Coates, J. (2000) Interpretation of Infrared Spectra: A practical approach. In *Encyclopedia of analytical chemistry* (Meyers, R. A., Meyers) John Wiley & Sons Ltd Press, USA, pp. 10815-10837.

[9] Ghasemi, Y., Sara, R. A., Mohammad, H. M., Mohammad, J. R., Mohammad, B. G., Fatemeh, N., Narges, N., Rezvan, P., Seyed, B., Mosavi, A. and Shadman, S. (2008) Dehydrogenation, C-20 Ketone Reduction and Side-chain Cleavage of Hydrocortisone in *Chlamydomonas reinhardtii* Cultures. *Molecules*, 13*: 2416-2425.*

[10] Lad N. (2010) *Optimization of parameters for hydrocortisone succinate bioconversion*. M. Sc. Microbiology Dissertation thesis, Sardar Patel University, Vallabh Vidya Nagar, Anand, Gujarat, India.