

Optimization and Scaling up of Prednisolone Production Using *Pseudomonas fluorescens* Cells Immobilized with Polymer Carrier Produced by Radiation Polymerization.

Nehad Z. Adham*, Abeer Abd El-Hady and Nadia Naim

National Research Center, Dokki, Giza, Egypt

Abstract

Pseudomonas fluorescens cells, which convert hydro-cortisone to prednisolone, have been entrapped in poly-hydroxy ethyl methacrylate (Poly-HEMA) which was prepared by radiation polymerization at low temperature. The Δ^1 -dehydrogenase of poly-HEMA immobilized cells were affected by biomass of incorporated cells on the biocatalyst. The role of some important additive compounds on hydrocortisone bioconversion was evaluated. The Δ^1 -dehydrogenase of poly-HEMA immobilized *Ps. fluorescens* was enhanced (1.3 fold) in the presence of 0.6g/L H₂O₂.

Production of prednisolone by using a commercially available fermentor was investigated. Maximum prednisolone yield and productivity (84.9%, 9.4mg/L/h) was obtained after 18h transformation period in the presence of H₂O₂ in 2L fermentor compared with 24h in shaking technique.

Key words: Steroid biotransformation, Δ^1 -dehydrogenation, *Pseudomonas fluorescens*, polyhydroxy-ethylmethacrylate, gamma-radiation, fermentor.

Introduction

The methods of microbiological transformation of steroid hormones based on enzymatic 1-2-dehydrogenation of pregnane derivatives are of considerable practical importance because dehydrogenated derivatives of corticosteroids are usually more effective than their precursors in treating such diseases as rheumatism, unspecific infectious polyarthrititis, and bronchial asthma. (Fokina, *et al* 1995).

Immobilized different microbial cells using different conditions of immobilization processes were used for 1,2-dehydrogenation of steroid substrate. This technique has certain technological advantages and facilitates the regulation of the enzymatic activity (Mosbach and Larsson, 1970 ; Constantinides, 1980 ; Lusta *et al.*, 1989 ; Gemeiner, *et al.*, 1994 ; Fokina *et al.*, 1995 and El-Hadi, 2002).

Particularly *Pseudomonas fluorescens* can be immobilized for the conversion of hydrocortisone to prednisolone using different techniques including incorporation into the gels of linked polyacrylamide ; calcium alginate (Adham *et al* 2002A) and photo linked poly-HEMA (Naim, *et al* 2002A). A comparison of these variants showed that the best results (in terms of stability of dehydrogenase activity) were produced using incorporation in poly-HEMA which was prepared by radiation polymerization at low temperature.

For industrial processes, economic aspects such as short reaction time, increased substrate concentrations, elimination of side reactions and low energy consumption have to be considered.

* Corresponding author

Further more, technical development requires the elaboration of appropriate reactor design, process control and scaling-up to industrial levels (Banks, 1979).

The cryogel (poly-HEMA) structure, cell distributions, and dehydrogenase activity could depend on the regime of immobilization procedures and the concentration of immobilized cells. In addition the high rate of retention of immobilized cells in the cryogel granules permitted repetitive transformation procedures over a prolonged time.

The mechanical strengths of cryogel particles were another important condition (Fokina *et al* 1995). The entrapment of *Ps. fluorescens* in poly-HEMA yielded preparation with good retention of Δ^1 -dehydrogenase activity, provided certain precautions taken during the polymerization process (our previous communication). (Naim *et al.*, 2003).

The purpose of the present work was

To report on some of the properties of poly-HEMA entrapped *Ps. fluorescens* cells including the concept of activation, i.e, an absolute increase in their steroid Δ^1 -dehydrogenation.

To study the operational stability of immobilized poly-HEMA cells in commercially available fermentor.

Materials and methods

Micro-organism: *Pseudomonas fluorescens* cells were kindly obtained from the Navy American Medical Research Unit III, Ramsis St., Abassia, Cairo, Egypt.

Chemicals: Cortisol (hydrocortisone), prednisolone and 20 β -hydroxy prednisolone (authentic), were obtained from Sigma Chemicals Company. Hydroxy ethylmethacrylate (HEMA) was purchased from Merck. All other chemicals used were of fine analytical grade.

Preparation of inoculum: 2 ml cell suspension of a 7 days old culture was used to inoculate 50 ml of the nutritive media (Sallam *et al.*, 1995), incubated for 24h at 30°C using a reciprocal shaker (200 rpm), the inoculated medium was centrifuged using a cooled centrifuge (3000 rpm) at 10°C for 30 min. the harvested wet cells were washed with 0.01M sodium phosphate buffer (pH 7.0) and were used as inoculums for immobilization techniques.

Preparation of the carrier: The monomer hydroxy ethylmethacrylate (HEMA) used in this work was mixed with 5ml distilled water. The mixture was irradiated at -78°C with γ -rays from a ^{60}Co source for 8h with dose rate of 2 mega (20KGY) (Naim, *et al.*, 2002). The resultant polymer carrier was cut to small pieces, approximately 5 mm in diameter, and shaken with excess amount of water for 2 days in order to be fully swollen. The swollen carrier was sterilized by autoclave at 121°C for 40 min. the sterilized carrier was immersed into the nutritive medium for 2 days to be filled with the nutrient medium. 2g of wet slurry cells of *Pseudomonas fluorescens* (prepared as previously described) was used as inoculums of 10 discs of polymer carrier in erlenmeyer flasks containing 50 ml of the transformation medium (g/L : glucose 10 beef extract 3 and peptone 5).

Evaluation of immobilized cells bioconversion: The polymer carriers of immobilized cells were washed well with the nutrient medium. The washed immobilized cells were put into 50ml of the transformation medium containing 5.0 mg of cortisol dissolved in 1ml 96% ethanol. Bioconversion was extended to 24h in an incubator at 30°C under gentle rotary shaking (unless otherwise stated).

Analysis of steroid conversion: At the end of the transformation period, the filtrate of each flask was extracted and treated to give a semi-solid residue "test material" (Naim *et al.*, 2002 A ; Sallam *et al.*, 1995), that was analyzed, and the transformation products were identified using high pressure liquid chromatography (HPLC) (Kloosterman and Lilly, 1984), in which 1ml of the previously extracted sample containing the steroid was mixed with 1ml methylene chloride, vortexed for 1min, and the steroids allowed to separate. A 10 μ l sample of the methylene chloride phase was injected into an HPLC, using a lichrosorb Si-60 5- μ m column, methylene

chloride as a mobile phase containing 5% methanol and 0.55% acetic acid, 1ml/min. flow rate, and a UV detector at 254 nm.

Repeated batch enzyme reaction of poly-HEMA immobilized cells in 2L fermentor: The poly-HEMA immobilized cells were repeatedly used, to transform different batches of equal volumes (1300ml) of transformation medium in 2L fermentor containing 0.26 gm of hydrocortisone [optimum substrate concentration from our previous communication (Naim, *et al.*, (2002B)]. Δ^1 -dehydrogenation of cortisol was allowed to proceed for 18h, thereafter the transformation medium was withdrawn and the necessary analyses were carried out. Poly-HEMA immobilized discs were washed with 85% NaCl and with nutritive medium, and used for further transformation procedure.

Results and discussion

We studied the capacity of *Pseudomonas fluorescens* cells immobilized by poly-HEMA to catalyze 1,2-dehydrogenation of hydrocortisone. The effect of the biomass of incorporated cells on the biocatalyst activity was studied. The biomass of incorporated cells was controlled by concentration of cell suspension used in biocatalyst preparation. The process of dehydrogenation of 0.1g/L hydrocortisone by increasing amounts of cells immobilized in 5mm diameter granule (disk) is illustrated in (Fig.1).

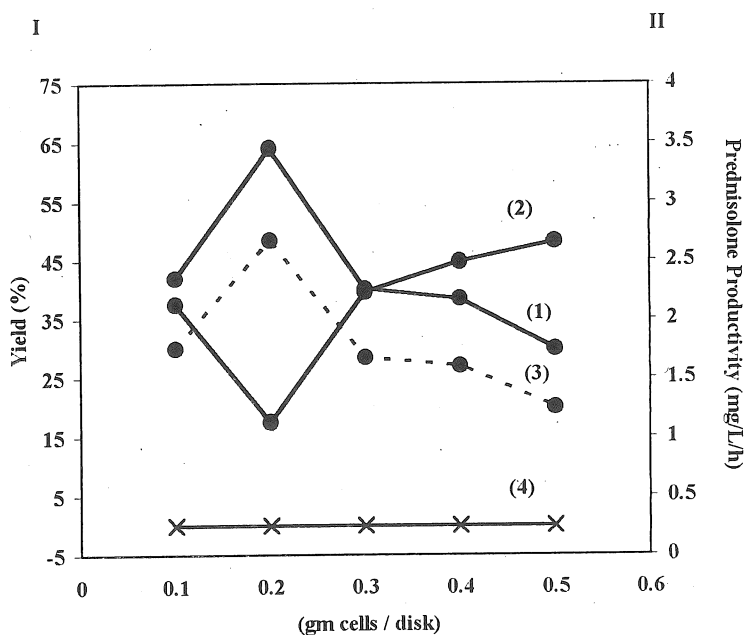


Figure 1. The effect of incorporated cell biomass on the activity of *Ps. fluorescens* cell immobilized in poly-HEMA.

- Curve (1) Prednisolone yield, Scale I %
- Curve (2) Residual Cortisol, Scale I %
- Curve (3) Prednisolone Productivity, Scale II
- Curve (4) Hydroxyprednisolone, Scale II

Results clearly indicated that 0.2g/disk was the best cell concentration for maximal prednisolone yield (64.1%) and cortisol bioconversion (82.55%) (curves 1 and 2 respectively). On the other hand increasing in cell concentration /disk lead to a gradual decrease in

productivity (2.67 – 1.25) which may be due to the competition between the cells with each other (inside the polymer pores) on nutrient requirements and oxygen needed for Δ^1 -dehydrogenase (Curve 3). Also multiplication to intact cells, which ensures dehydrogenase stability, were decreased and maintenance of cells density with good viability was not possible. In addition immobilization of cells often tend to aggregate within a hydro-gel matrix when there is a high loading of cells and such aggregates created diffusional resistances of substrate and oxygen to the immobilized cells located in the core region of such aggregates (Park and Hoffman, 1990) Furthermore the increase in the amount of cells may have effected the poly-HEMA structure, which can be a determinant of the entire kinetics of the process. Fokina, *et al.* (1995) showed that maximal specific activity of immobilized cells was observed with minimum concentrations of the cells in the cryogel. Next, we studied the effect of disc numbers of poly-HEMA immobilized *Ps. fluorescens* on hydrocortisone bioconversion. Different numbers of poly-HEMA immobilized disks (0.2gm cells/disk) were used to transform 5mg hydrocortisone in 50ml media. The results depicted in (Fig.2) indicated that bioconversion efficiencies of cortisol in favor of prednisolone production were maintained upon using 10 immobilized poly-HEMA disks/flask, by increasing number of disks/flask prednisolone yield and productivity showed only a tendency to decrease. These findings suggest the competition of immobilized microbial cells for oxygen which probably occurred at cell concentrations more then 3 gm/flask (15 disk). Decreasing the number of disks to 5 disks/flask, result lower inoculums suggesting lower rates of substrate utilization affecting prednisolone productivity.

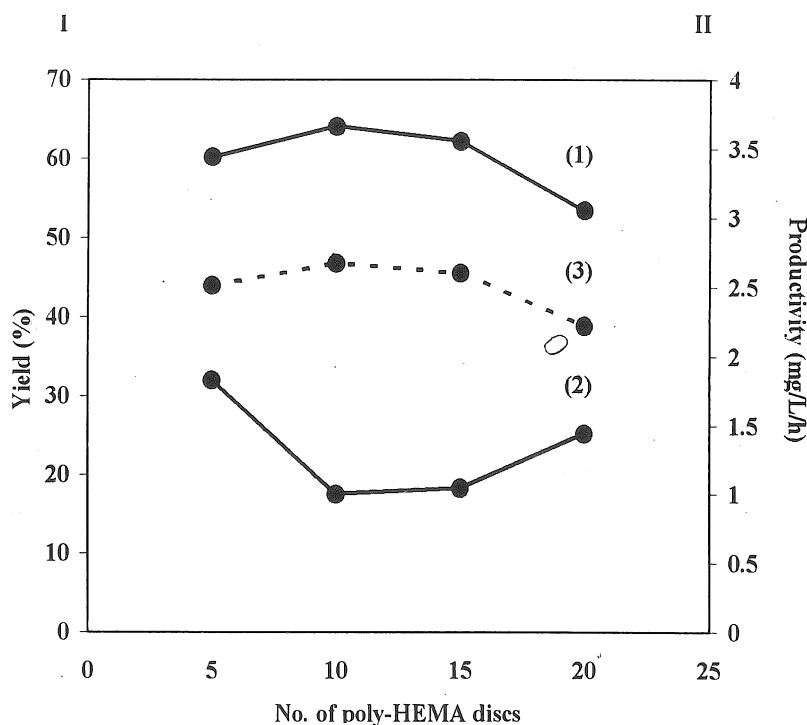


Figure 2. Reaction rate or prednisolone formation with poly-HEMA immobilized *Ps. fluorescens* on prednisolone yield.
 Curve (1) Prednisolone yield, Scale I %
 Curve (2) Residual Cortisol, Scale I %
 Curve (3) Prednisolone Productivity, Scale II

In a previous communication, we reported that according to the available literatures, nothing had been reported for the conversion of hydrocortisone to prednisolone by our selected strain of

Ps. fluorescens, the physiological and biochemical aspects of hydrocortisone bioconversion using free cells were evaluated [Naim, *et al.* (2002 A) ; Adham, *et al.*(2002B)]. In the present study some of the optimum conditions which had been reported in the previous communication were tested in the bioconversion of hydrocortisone using poly-HEMA immobilized *Pseudomonas* cells. The effect of some biologically active compounds including oxidiz agents (0.6ml/L H₂O₂) ; macro elements (0.6g/L NaCl) ; microelements (0.06g/L MnSO₄.7H₂O) ; chelating agents (5×10⁻⁵ M EDTA) and inducer agents (0.4g/L cholesterol) have been studied [all compounds were added separately during the conversion phases and only cholesterol was added 12h prior to the conversion phases]. (Fig.3) clearly indicates that all treatments have highly affected the induction of cortisol bioconversion and increased prednisolone yield by 1.26 – 1.33 fold when compared to untreated poly-HEMA immobilized cells as control. The highest prednisolone yield (84.9%) was obtained by the addition of minute amount of H₂O₂ (0.6ml/L) as an oxidizing agent and this result revealed us to the use of H₂O₂ as an oxidizing agent encourage the dehydrogenation reaction (Martine, 1984 ; Sallam, *et al.* 1995).

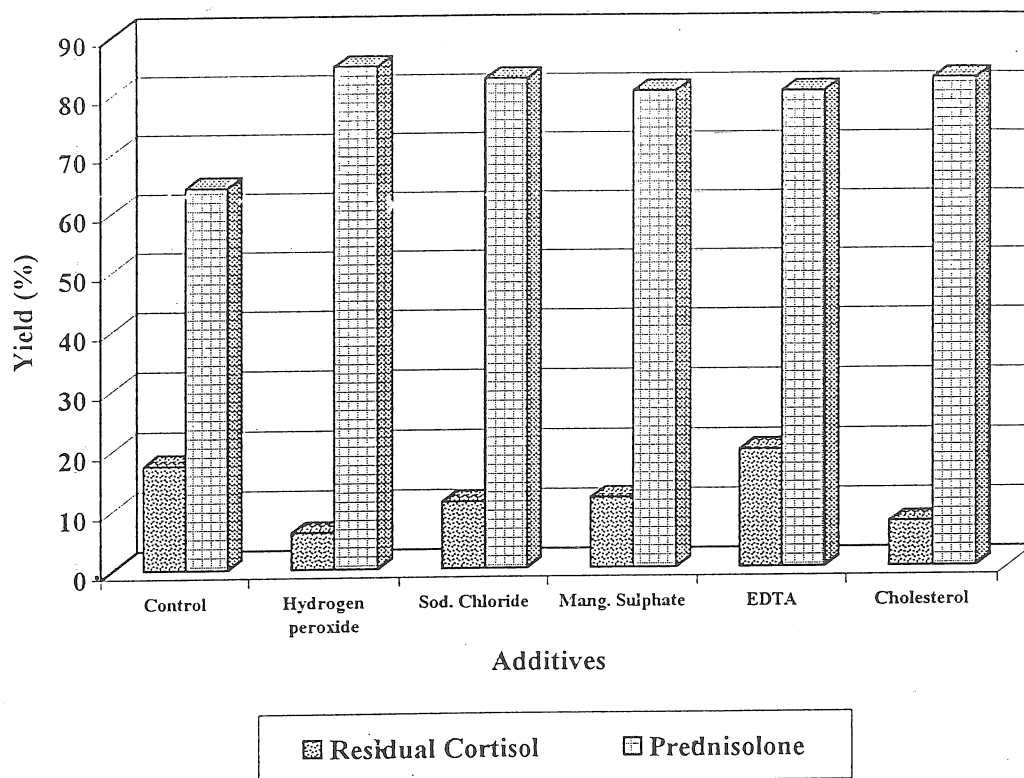


Figure 3. Bioconversion of cortisol to prednisolone by poly-HEMA immobilized *Pseudomonas fluorescens* in present of biological active compounds.

Scaling up of dehydrogenation system of poly-HEMA Ps. flourescens cells to 2L fermentor :
 The bioconversion of hydrocortisone was recorded in the presence of H₂O₂ in a commercially available fermentator (New Brunswick Scientific Co., INC. Edison, NJ., USA). This fermentator was loaded with 260 disks of poly-HEMA biocatalyst. Substrate 0.26gm/1300ml of modified production medium (gm/L: beef extract, 3 , peptone 5, glucose 10) pH 6.5, agitation was performed using a three-6 bladed rushton turbine impellers di=40 mm at 200 rpm, aeration rate was 1 volume of sterile air per volume of medium/minute (v/v/min). Exhaust gas expacing was applied under gauge pressure of 11.5cm water column. Temperature was adjusted to 30°C and foaming was hindered by the addition of 1ml paraffin oil. Samples were taken for analysis

at different time intervals. From data shown in table 1 gradual increase in cortisol bioconversion is obvious (5.7 – 90.8%). Highest prednisolone yield (84.9) and productivity (9.4) were obtained after 18h (Fig 4). This conspicuous increase in hydrocortisone bioconversion, prednisolone yield and productivity after 18h was clearly achieved by the application of optimal physiological and biochemical parameters and also the utilization of fermentor that offer good aeration. By increasing the bioconversion time (48 –72h), the undesired 20 β -hydroxy prednisolone form increased gradually (10.1 –17.1%) This was due to the formation of the reductase system which transformed prednisolone to its 20 β -hydroxy derivative (Table 1).

Table 1. Hydrocortisone dehydrogenation by poly-HEMA immobilized *Pseudomonas fluorescens* cells at different stages of fermentation using 2L-fermentor.

Transformation time (h)	Residual Cortisol (%)	Prednisolone Yield (%)	20 β Hydroxy prednisolone (%)
2	94.3	3.2	-
4	87.2	8.1	-
6	61.2	16.9	-
12	12.2	47.0	-
16	11.5	68.8	-
18	9.2	84.9	-
24	35.1	78.8	-
48	47.4	38.4	10.1
72	76.1	5.2	17.1

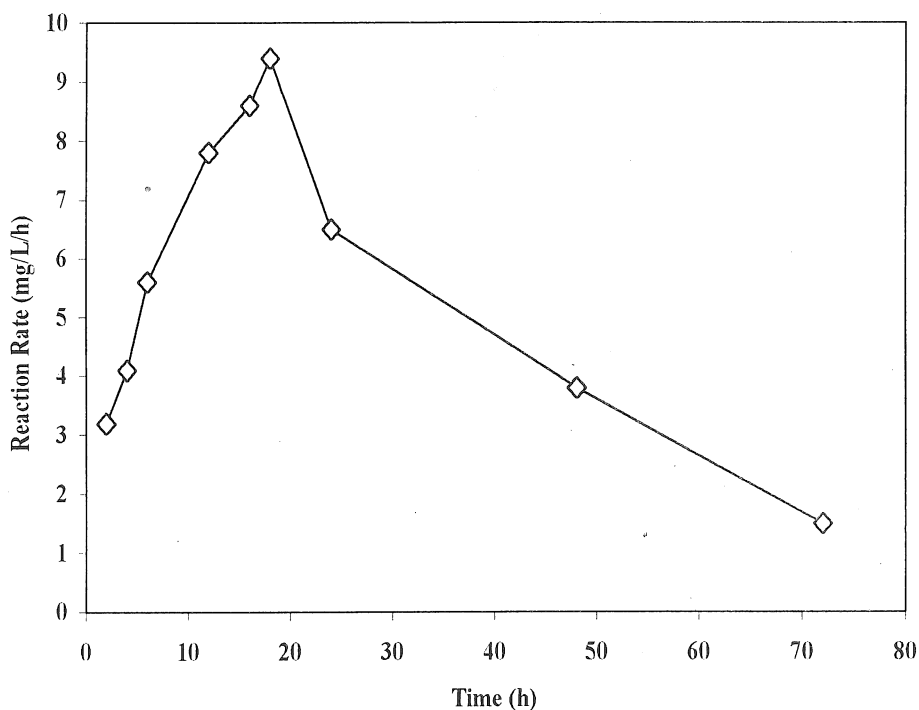


Figure 4. Reaction rate of prednisolone formation with poly-HEMA *Ps. fluorescens* cell in 2L-fermentor.

Semi continuous hydrocortisone dehydrogenation using poly-HEMA immobilized Ps. Fluorescens: Results in table 2 indicated that the poly-HEMA immobilized cells can be

repeatedly used for 12 times to perform Δ^1 -dehydrogenation of hydrocortisone to prednisolone in a commercially available fermentor. Prednisolone productivity by semi continuous fermentation technique was gradually increased (9.4 – 10.7mg/L/h) for 9 times (Fig.5). The restoration of activity was probably due to growth of microorganism in the gel. Similar results were obtained by Ohlson *et al.* (1978), Constantinides (1980) and Goetchel and Bar (1992). Using poly-HEMA immobilized cells more than 9 times resulted with a gradual decrease in the productivity (8.5 – 2.3mg/L/h) which may be due to the lysis of cells. (Siibigerd and Freeman 1991). On the other hand, it was obvious that 20 β hydroxy prednisolone not form in all the recycle.

Table 2 : Semi-continuous fermentation of immobilized poly-HEMA *Pseudomonas fluorescens* cells using 2L-fermentor.

Number of Cycle	Residual Cortisol (%)	Prednisolone Yield (%)	20 β Hydroxy prednisolone (%)
1	9.2	84.9	-
2	8.8	85.2	-
3	7.2	87.6	-
4	6.5	89.7	-
5	5.3	90.1	-
6	5.0	91.1	-
7	4.3	92.6	-
8	3.1	96.1	-
9	3.1	96.7	-
10	17.6	76.1	-
11	38.3	40.1	-
12	67.5	21.1	-

The analyses were calculated for 50 ml fermentation medium.

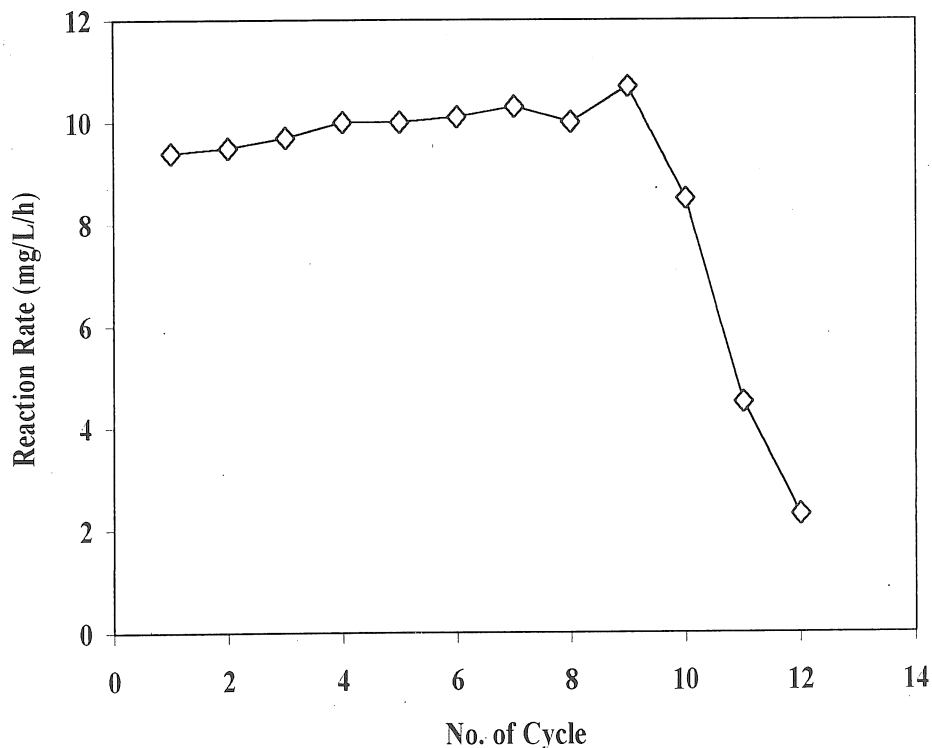


Figure 5. Reaction rate of prednisolone formation with poly-Hema *Ps.fluorescens* cell in semi-continuous fermentation using 2L-fermentor.

Isolation and identification of the transformation products: Transformation products of cortisol bioconversion from the broth of the fermentor were separated and identified. Thin layer technique (TLC) of the extracted residue (see material & methods) indicated that the product was identical with that of authentic sample of prednisolone. HPLC also confirmed that the product sample was prednisolone. The product sample was confirmed from the basis of its mp and mmp in addition to its spectral data (IR, ¹H NMR, X-ray and DSC). IR spectrum of prednisolone shows a bands at 3300-3000 cm⁻¹ (OH), 1710 cm⁻¹ (COOH), 1690 cm⁻¹ (C=O) and 1600 cm⁻¹ (C=C). Its ¹H NMR spectrum reveals a pair of doublet at δ 6.3 and 7.3 ppm which not present in the starting material. From the X-ray the diffractograms of CP (sample) is quite similar to that of C+P (authentic). The proximity of the endothermic melting points of C (206.7 °C); P (232.9 °C) and artificial mixture CP (217.8 °C).

In conclusion, we observed that hydrocortisone dehydrogenation process was highly effected by biomass on poly-HEMA biocatalyst and also addition of H₂O₂ in this process allowed for significant prednisolone yield enhancement.

The use of 2L fermentor allowed for caring out semi continuous dehydrogenation with relatively short cycle (18h) and good prednisolone yield,(84,9%) also, the high mechanical strength of poly-HEMA allowed the repeated use of immobilized cells up to 9 times with an increase in prednisolone production without the formation of 20β-hydroxy derivatives.

Acknowledgements

The authors thank to Dr. Hassan Abd El-Reheem [National Centre For Radiation Research And Technology (NC RRT), Egypt] for technical help.

References

- Adham, N.Z., EL-Hady, A.A., and Naim, N.(2003). Biochemical studies on the microbial Δ dehydrogenation of the cortisol by *Pseudomonas fluorescens*. *Process Biochem.*38: 897-902.
- Adham, N.Z., EL-Hady, A.A. and Naim, N.(2002B). Δ¹-dehydrogenation of cortisol by immobilized *Pseudomonas fluorescens* cells entrapped in calcium alginate or poly acrylamide gels. *A.M.S.E. J.* In press.
- Banks, G.T.(1979). Scale-up of fermentation processes in " Topics in Enzyme and Fermentation Biotechnology" (A. Wiseman, ed.), Vol.3.pp 170 – 266. Ellis Horwood Ltd., Chi-Chester.
- Constantinides, A.(1980). Steroid transformation at high substrate concentration using immobilized *Corynebacterium simplex* cells. *Biotechnol. Bioeng.* 22: 119-123.
- EL-Hadi, A.(2002). Production of Prednisolone by *Bacillus Pumilus* E 601 cells incorporated in Radiation Induced Poly (Vinyl Alcohol g-2 Hydroxy Ethyl Methacrylate) Cryogels. *Process Biochem.* In press.
- Fokina, V.V., Arinbasarova, A. Yu., Zubov, L.A., Loziinsky, V.L. and Koshcheenko, A.K.(1995). Dehydrogenation of steroid substrate by the cells of *Arthrobacter globiformis*. 193 incorporated in poly (vinyl alcohol) cryogels. *Appl. Biochem. Microbia.*, 31(2): 184-189.
- Gemeiner, P., Rexova-Benkova, U., Svec, F. and Norrlov, O. (1994). Natural and synthetic carriers suitable for immobilization of viable cells, active organelles and molecules. In: *Immobilized Biosystem. Theory and Practical Applications*, pp. 1-128. (Eds.) I.A. Veliky and R.J.C. McLean. CHAPMAN & HALL. London, Glasgow, New York, Tokyo.
- Goetschel, R. and Bar, R.(1992). Formation of mixed crystals in microbial conversion of sterols and steroids. *Enzyme Microb. Technol.* 14: 462-469.

- Kloosterman, J.I.V. and Lilly, M.D.(1984). Effect of supersaturated aqueous hydrocortisone concentrations on the Δ^1 -dehydrogenase activity of free and immobilized *Arthrobacter Simplex*. *Enzyme Microb. Technol.* 6: 113-116.
- Lusta, K.A., Starostina, N.G., Gorkina, N.B., Fikhte, B.A., Lozinskii, V.L., Vainerman, E.S. and Rogozhin, S.V. (1989). Immobilization of *E. coli* cells in macroporous cryogels based on polyacrylamide. *Biotechnol. Bioeng.* 24(4): 418-426.
- Martine, C.K.A.(1984) Sterols Biotechnology, Vol. 6a, edited by H.J. Rehm and G. Reed, Chapter 3, pp.79-95.
- Mosbach, K. and Larsson, O.P.(1970). Preparation of polymer entrapped enzymes and micro organisms in microbial transformation process with special reference to steroid 11β -hydroxylation and Δ^1 -dehydrogenation. *Biotechnol. Bioeng.* 12: 19-27.
- Naim, N., Adham, N.Z., Abd El-Reheem, H, and Abd EL-hady, A.(2002B). Prednisolone production using *Pseudomonas fluorescens* cells immobilized with polymer carrier produced by radiation polymerization. *Process Biochem.* In press.
- Naim, N., Adham, N.Z., El-Hadi, A.A. (2002B). Microbial dehydrogenation of cortisol. *Bull-NRC*, Egypt. In press.
- Ohlson, S., Larsson, P.O. and Mosbach, K.(1978). Steroid transformation by activated living immobilized *Arthrobacter Simplex* Cells. *Biotechnol. Bioeng.* 25: 1267-1284.
- Park, T.G. and Hoffman, A.S.(1990) Immobilization of *Arthrobacter Simplex* in a thermally reversible hydrogel: Effect of temperature cycling on steroid conversion. *Biotechnol. Bioeng.* 35: 152-159.
- Sallam, L.A.R., El-Abyad, M.S., El-Refai, A.H.; El-Menofi, H.A. and Adham, N.Z.(1995). Bioconversion of 19-nortestosterone by *Rhodococcus* sp. DSM 92-344.1: Optimization of transformation parameters. *Process Biochem.* 30(1) : 25-34.
- Siibigerd,E. and Freeman, A.(1991). Continuous Δ^1 -hydrocortisone dehydrogenation with in situ product recovery. *Enzyme Microb. Technol.* 13: 869-872.

Accepted:18.09.2002