

## Effect of Aeration Rate on the Biotransformation of Cortisolone Using *Cunninghamella elegans* in a Laboratory Scale Bioreactor

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**Abstract:** A single-step microbial transformation process for the production of cortisol and prednisolone from cortisolone (Reichstein's compound S) using *Cunninghamella elegans* cells in an aerated fermentor was investigated. The biotransformation process was carried out using 5 l stirred tank bioreactor. The effect of pH values, aeration rate, cell dry weight and cortisolone feeding strategy were investigated. At aeration rate of 1 v/v/m the total bioconversion efficiency (TBE) was 57%, at 48 h time course, cell dry weight 1.42 g/100 ml and the residual glucose was 3 g/l. On increasing the aeration rate up to 2 v/v/m, the products yields were increased up to 79.2%. The medium pH was maintained to constant 6.5 till the end of fermentation. The fermentation was influenced by oxygen transfer efficiency of the bioreactor and the cell dry weight was increased up to 3.58 g/100 ml after 48 h, accompanied by a sharp decrease of residual glucose to 4.14 g/l. At the first fed batch, the highest yields of cortisol and prednisolone (52.2% and 34.03%, respectively) were obtained.

**Key words:** Biotransformation • Cortisolone • *Cunninghamella elegans* • Fermentation • Steroids

### INTRODUCTION

The pharmaceutical industry has great interest in the biotransformation of steroids for the production of steroid hormones. Most of the important biotransformations are catalyzed by hydroxylases and dehydrogenases, which are relatively unstable and expensive. Therefore, intact microbial-cells are often used [1-3]. Microbial transformations of steroids are characterized by reaction substrate specificity, regiospecificity, stereospecificity, mild reaction conditions and has been drastically reduced the number of the required chemical steps in the production of steroid hormones and their derivatives [4, 5]. The filamentous fungus *Cunninghamella elegans* is a well known hydroxylator of cortisolone (Reichstein's compound S) at the 11 $\beta$ -position, which is one of the main steps during the production of therapeutically useful corticosteroid compounds. Manosroi *et al.* [6] tested several strains to perform the bioconversion of cortisolone to prednisolone but the highest prednisolone

yield was obtained on using free cells of *Cunninghamella echinulata* ATCC 8688a. Lisowska and Dlugonski [7] used *Cunninghamella elegans* IM 1785121 in the transformation of cortisolone yielding epihydrocortisone and hydrocortisone. Moreover, 11 $\beta$ -hydroxylation of cortisolone is an important reaction to produce cortisol (hydrocortisone, 11 $\beta$ , 17 $\alpha$ , 21-trihydroxypregn-4-ene-3, 20-dione) which is a principal pharmaceutical and also the precursor of the potent steroid prednisolone [8-10]. Transformation of cortisolone into prednisolone by mixed culture has been reported [11]. Fungal steroid 11 $\beta$ -hydroxylation was catalyzed by cytochrome P450 monooxygenases [12, 13]. Cytochrome P450 is responsible for the oxygen insertion in the steroid substrate molecule [14, 15]. According to Znidarsic and Plazl [4], the preferential use of whole cells over enzymes for this biotransformation is mainly resulting from necessity of co-factor regeneration and problems associated with the isolation of enzyme complex and its *in vitro* regeneration.

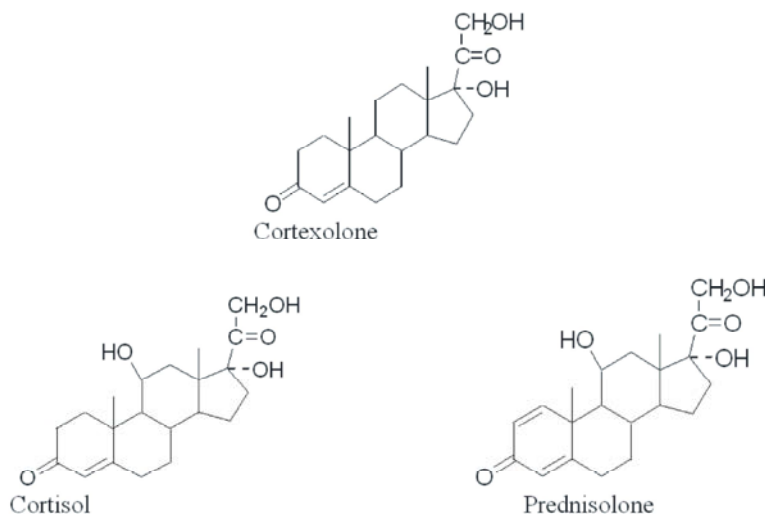


Fig. 1: Chemical structures of cortisolone and transformed products using *Cunninghamella elegans*.

The present work focused on the transformation of cortisolone to cortisol and prednisolone through one step transformation reaction (Fig. 1). Batch and fed batch processes of cortisolone 11 $\beta$ -hydrocortisone and cortisol dehydrogenation were studied in a laboratory-scale bioreactor using *C. elegans*. Effect of cell dry weight, medium pH, residual glucose and different aeration rates for improving the yield of cortisol and prednisolone were also studied.

## MATERIALS AND METHODS

**Reagents:** Cortisolone (Reichstein's compound S; 17 $\alpha$ -hydroxypregn-4-en-3,20-dione) was the substrate of steroid transformation; cortisol (hydrocortisone; Kendal's compound F; 11 $\beta$ , 17 $\alpha$ ,21-trihydroxypregn-4-en-3, 20-dione) and prednisolone (11 $\beta$ , 17 $\alpha$ , 21-trihydroxy-1, 4-pregnadiene-3, 20-dione) were the standards for the 11 $\beta$ -hydroxylation and  $\Delta$ 1,2- dehydrogenation products and were provided by Sigma Company, USA. Potato-dextrose agar (PDA) was obtained from Difeco Company, USA.

**Microorganism and Culture Medium:** *Cunninghamella elegans* was kindly obtained from regional center of fungi, Al-Azhar University, Egypt. The fungus was maintained on PDA slant with neutral pH value. After 6 days incubation the fungal spores ( $2 \times 10^6$  spores/ml) were inoculated into 250 ml Erlenmeyer flask containing 100 ml of cultivation medium (g/l): glucose 20, peptone 5, yeast extract 5, sodium chloride 5, K<sub>2</sub>HPO<sub>4</sub> 5, with pH adjusted to 6.5 and incubated for 24 h at 30°C on a rotary shaker at 200 rpm (this used for inoculation).

**Batch Fermentation Condition:** Fermentor experiments were carried out in 5l triple impeller bioreactor (New Brunswick Scientific Edison, N.J., USA) with a working volume of 2.5 l. The fermentation medium used was the same as that in flask cultivation. Fermentations were performed under controlled operational conditions as follows: temperature 30°C, initial pH 6.5, aeration rate (1 v/v/m) and agitation speed 200 rpm and inoculums 4% (v/v). Dissolved oxygen tension (DOT) was measured with a dissolved oxygen probe. The DOT level was adjusted using an on-off controller coupled to a solenoid valve that was connected to the compressed air (or pure oxygen) line. Cortisolone, 0.01 g/l, dissolved in 80% (v/v) ethanol was added to the culture for enzyme induction and expression after 16h of inoculation and continued for 8h cultivation. After that 0.1 g/l of cortisolone was added as a substrate.

**Fed-batch Fermentation:** Fed-batch fermentation was carried out in a 5 l fermentor initially containing 1000 ml of the cultivation medium. The fermentor was operated at 30°C, 200 rpm; pH was maintained at constant value of 6.5 and 2 v/v/m aeration. The fermentation was started on batch mode with the addition of the substrate to the culture medium. When the culture pH starts to decrease, fed-batch operation was activated by the addition of 1000 ml of culture medium supplemented with the substrate at 52 h and the culture pH was controlled at 6.5. The second fed-batch addition was performed at 98 h by the addition of 500 ml of culture medium supplemented with the substrate. The fed-batch fermentation was terminated with a final volume of 2.5 l [16].

**Dry Weight Estimation:** The fungus growth was separated by centrifugation, washed and dried at 60°C until constant weight. The culture filtrates were then analyzed for their contents of cortisol, prednisolone, residual corticosterone and residual glucose.

**Determination of Glucose:** Glucose was determined according to the method of Passing and Beblox [17]. A 10 µl of sample + 1 ml glucose oxidase kits (GOD) was incubated at 37°C for 15 min. The produced color was measured at 520 nm. A standard curve was prepared using a pure glucose standard solution.

**HPLC Analysis:** Sample preparation for HPLC analysis was carried out by extracting 2ml of reaction mixture with 2ml of chloroform [18]. After the clear separation of the organic phase from the aqueous phase, 1ml of organic fraction was analyzed by HPLC (LC Module, Millennium 2010, 50% Methanol, 50% water in 1mL/min, C-18 Novopak column, U.V. detector (SPD-10 AVP), at max 154 nm, using acetonitrile as the solvent):

$$\text{Product molar yield (\%)} = \frac{\text{Weight of product / MW of product}}{\text{Weight of substrate / MW of substrate}} \times 100$$

Total bioconversion efficiency (TBE) = Cortisol yield (%) + prednisolone yield (%).

Where MW is the molecular weight.

**Statistical Analysis:** The quality of fit was expressed by the coefficient of determination  $R^2$ . All experimental designs were randomized to exclude any bias. Experiments were performed in duplicate and the mean values were given using Microsoft Office Excel.

## RESULTS AND DISCUSSION

The primary aim of this biotransformation experiments was to produce cortisol and prednisolone from corticosterone in sufficient quantities by the filamentous fungus *Cunninghamella elegans*.

### Batch Biotransformation of Corticosterone to Cortisol and Prednisolone at Aeration Rate 1v/v/m:

Biotransformation of corticosterone was performed as batch cultivation in 5 l bioreactor under strictly controlled conditions, with working volume of 2.5 l. The added substrate concentration was 250 mg (0.7 mmol). At indicated time points, samples were taken and analyzed for products yield, glucose concentration, dissolved oxygen tension, pH values. The products amount is given as the percentage of the total substrate. Fig. 2 shows the incubation time course of the corticosterone bioconversion in the fermentor under aerobic conditions. *C. elegans* has the ability to transform corticosterone to cortisol and prednisolone in one step fermentation process. The yield of the formed products was found to be affected by the reaction time course at 8h the yields of cortisol and prednisolone were 13% and 7%, respectively of the added substrate. On increasing the time, the yield of the products gradually increased to reach its maximum peak at 48 h, with TBE of 57% of the added substrate, then they decreased by time to 42%. On the contrary, the added corticosterone gradually decreased by increasing the time course. Within the 48h of the experiment, about 78% of the added substrate was consumed. As shown in Fig. 3, glucose concentration, cell dry weight, pH values and DO were determined at regular time intervals. The glucose concentration in *C. elegans* biotransformation cultures (Fig. 3a) decreases rapidly with time, as could be expected.

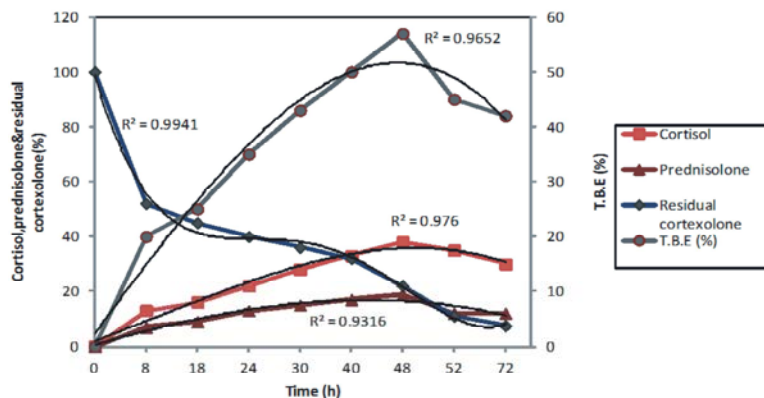


Fig. 2: Effect of incubation time on the biotransformation of corticosterone by *C. elegans* using batch cultivation at aeration rate of 1 v/v/m.

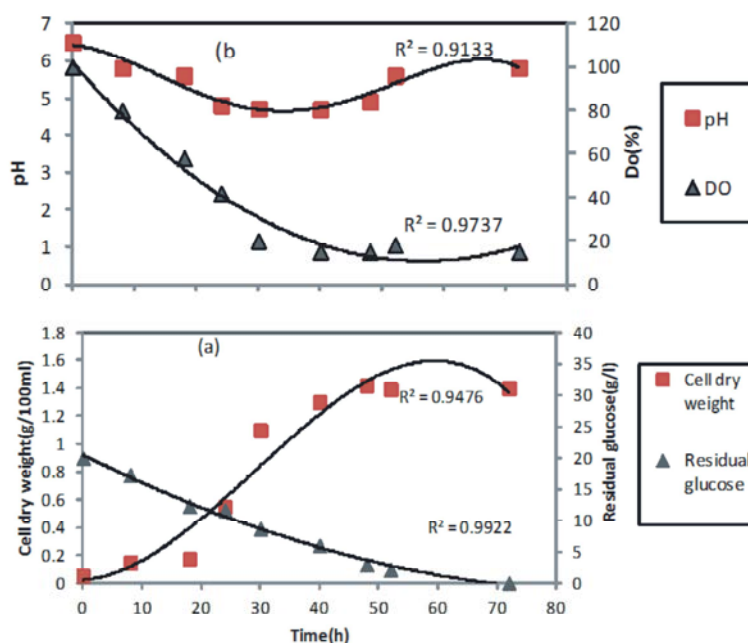


Fig. 3: The time profile of (a) determined cell dry weight, residual glucose and (b) medium pH, DO using batch cultivation at aeration rate of 1 v/v/m.

It was found that the biotransformation activity strictly depends on the presence of some glucose in the media. It can be assumed that the enzymes activity responsible for biotransformation process vanishes in the absence of glucose because in that case cofactor regeneration via microorganism metabolism is not sufficient. These results are in accordance with those obtained by Naumann *et al.* [15], who reported that, in cell metabolism the NADH/NAD<sup>+</sup> ratio is mainly increased by glycolysis as long as glucose is present. After a lag phase of around 8h, the enzyme systems related to cell growth and propagation began to form, from 12h, cells growth entered the logarithmic phase the propagated mycelia entered the exponential growth phase. The maximum biomass was reached after 48h of incubation and by increasing the transformation time the cell dry weight was nearly constant. Lu *et al.* [14] reported that cytochrome P450 monooxygenases which responsible for the oxygen insertion in the steroid substrate molecule increased steadily during the logarithmic growth phase.

Figure 3b shows the effect of pH regulation at different time of incubation (0-72h). The results showed that the pH of the reaction was almost unchanged during the bioconversion process at the maximum products yields (48h) the pH value was 4.9. According to Sukholdolskaya *et al.* [19] and Zhang [20] the constant pH was beneficial to the stability of cytochrome P450 activity and the 11 $\beta$ -hydroxylation. Through the first 24 h

a rapid decrease in the dissolved oxygen concentration was observed, the dissolved oxygen reached the lowest level due to the drastic growth of the mycelia, then it was steadily constant. Similar results were obtained by Chen and Wey [21]. Figures 2, 3a and 3b show the values of determination coefficients  $R^2$  for cortisol, prednisolone, TBE, pH, DO, cell dry weight and residual glucose. The closer the value of  $R^2$  to 1, the better is the correlation between the observed and the predicted values [22].

**Batch Biotransformation of Cortisolone to Cortisol and Prednisolone at Aeration Rate 2 v/v/m:** 11 $\beta$ -hydroxylation and  $\Delta^{12}$ -dehydrogenation of cortisolone are known to be affected by the pH and dissolved oxygen [23]. In this study, the amount of aeration was increased up to 2 v/v/m in the fermented cultures. Figure 4 shows the effect of DO% on the transformation of cortisolone. It is obvious that the maximum yield of cortisol and prednisolone, TBE%, was 79.22% shown after 48 h incubation and increased by 22.2% than that of 1v/v/m, as shown in Fig. 2 and this was accompanied by an increase in the cell biomass, 3.58 g/100ml (Fig. 5a). These higher biomass values have been attributed to the accumulation of the byproducts which easily produced due to the increasing of aeration rate [24]. Figure 5b shows the consumption of glucose in fermented cultures, the consumed glucose was in parallel with the increase of cell growth and maximum products yield. Kumar *et al.* [3] suggested that the supply

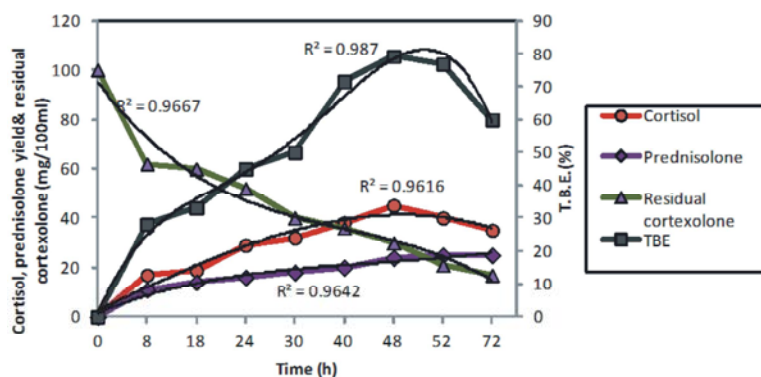


Fig. 4: Effect of time course on cortexolone biotransformation by *C. elegans* using batch cultivation at aeration rate 2 v/v/m.

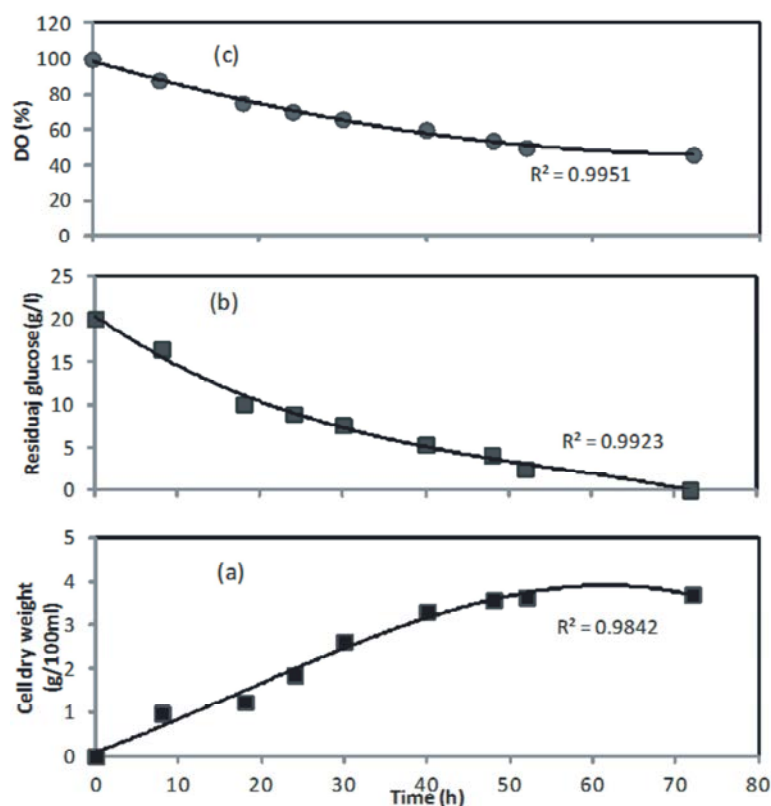


Fig. 5: The time course profile of (a) cell dry weight, (b) residual glucose and (c) DO% at aeration rate 2 v/v/m.

of reducing power, NADH, from the metabolism of glucose is necessary for the reduction of androst-4-en-3, 17-dione to testosterone in fermentation cultures of *Lactobacillus bulgaricus*. The results presented in Fig.5c shows the gradual decrease of DO% during the initial phase of the fermentation process until reached to 54% after 48h. Then it was nearly constant at the end of fermentation process. Thus, it can be proposed that *C. elegans* fermentation being aerobic, oxygen is necessary to obtain optimum enzymes activity. According

to Hu *et al.* [25], the aeration in the bioreactor provides the required oxygen for an aerobic microorganism and also mixing the fermentation contents. Proper aeration provides suitable gas holdup, a high residence time of the gas in the liquid and a high gas-liquid interaction area available for mass transfer. On the other hand, increasing the aeration rate up to 2v/v/m markedly increased the rate of cortexolone bioconversion, similar behavior was observed by Barberis and Segovia [24], that oxygen could enhance metabolite formation and the enzymatic reaction

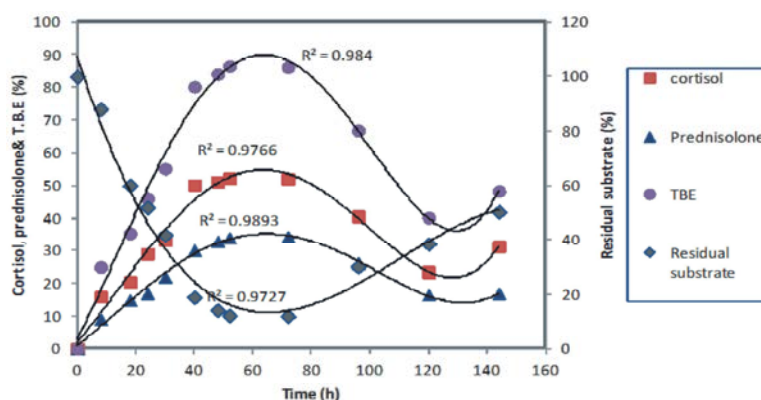


Fig. 6: Effect of time course on cortisolone biotransformation by *C. elegans* using fed batch cultivation at aeration rate 2 v/v/m.

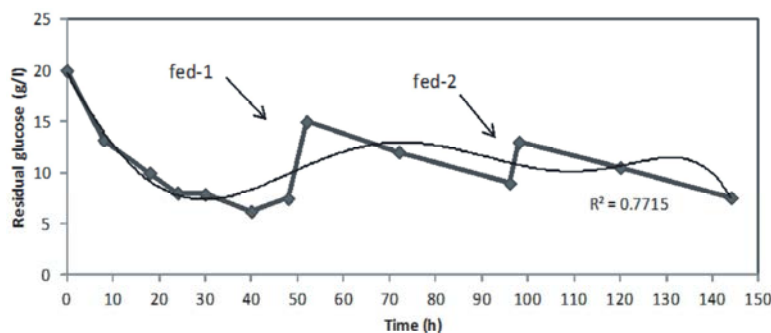


Fig. 7: Residual sugar measured at different time intervals at aeration rate 2 v/v/m.

of the product formation strongly depends on oxygen. Lu *et al.* [14] reported that the biotransformation can be regarded as three steps: substrate adapted into the cells, catalysis for oxygen insertion and cortisol secreted out of the cells.

#### Fed-batch Cultivation with Aeration rate 2v/v/m:

Fed-batch process of 11 $\beta$ -hydroxylation was performed to enhance cortisolone biotransformation. Figure 6 shows the time profile of the process. The process continued as usual, at 48 h the TBE was 84.11% increased up to 86.23% at the first fed-batch which accompanied by a highest decrease in substrate level to 12.22% and accumulation of residual glucose concentration at 15 g/l (Fig.7). At the second fed batch addition, the bioconversion efficiency decreased due to the excess amount of glucose in the medium which may not be consumed by the microorganism which causes substrate and/or product inhibition and a poor cell growth led to inefficient biotransformation [16]. The obtained values of  $R^2$  of the products also show high correlation. On the other hand,  $R^2$  for the residual sugar is 0.7715; some investigations find that confidence levels greater than 70% are acceptable [26]. As a result, the fed batch mode improved

cytochrome P450 expression and products concentration. The products were successfully excreted out of the cells which eliminated the quick accumulation of the transformed products in the cells and reduce the restraint of products.

#### CONCLUSION

Whole cells of the fungus *Cunninghamella elegans* were potently effective as a biocatalyst for the biotransformation of cortisolone for the production of two important steroidal drugs, cortisol and prednisolone, in a single-step. The effect of aeration rate, 1 v/v/m and 2 v/v/m, on the bioconversion process was studied in batch fermentations. The optimal conditions for producing a high concentration of products were at initial pH of 6.5, aeration rate of 2 v/v/m, cell dry weight 3.58 g/100 ml at 48 h. In fed batch fermentations, the results showed that the highest TBE was obtained at the second fed batch (86.53%) with residual substrate 12.22% and residual glucose of 15%. Thus whole-cell biotransformation process can be used for the production of industrially important steroidal compounds on a large scale.

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