Malaysian Journal of Microbiology, Vol 7(1) 2011, pp. 7-13
http://dx.doi.org/10.21161/mjm.22009

11β-Hydroxylation of cortexolone (Reichstein's compound S) to hydrocortisone using immobilized *Cunninghamella elegans* spores

Allam, R. F. [1], Shafei, M. S. [1]*, Refai A. H. El. [1], Ali, M. I. [2] and Mohamed, S. S. [1]

[1]Chemistry of Natural and Microbial Products, National Research Center, Cairo. Egypt.
[2]Faculty of Science, Cairo University, Cairo. Egypt.
E-mail: mona_sayed@hotmail.com

Received 8 November 2009; received in revised form 15 March 2010; accepted 29 April 2010

ABSTRACT

Two steps bioconversion of cortexolone (Reichstein's compound S) to its Δ^1-dehydro-11β-hydroxy derivatives and prednisolone, was successfully performed by the use of *C. elegans* for the production of cortisol and prednisolone from cortexolone. The combinations of sequential reactions, 11β-hydroxylation and Δ^1-dehydrogenation were performed by immobilized spores of *Cunninghamella elegans*. The immobilization technique was carried by either entrapment in sodium alginate or by adsorption on silver, 8 micron glass wool method. Maximum production of cortisol and prednisolone were obtained after 72 h transformation period using immobilized spores of *C. elegans* of concentration 2 x 10^7 spores/mL for both entrapment and adsorption. The highest transformation efficiency was recorded on using 1.6% w/v glass wool (92%) compared to that when the fungal spores were entrapped in 3% alginate (84%). Each immobilized microbial system was stable and could be used for the sequential reactions repeatedly (operational period, 18 days using entrapped *C. elegans* in alginate beads and 45 days using adsorbed spores on glass wool).

Keywords: hydroxylation, immobilization, cortexolone, cortisol, prednisolone

INTRODUCTION

The importance of microbial biotechnology in the production of steroid drugs and hormones was realized for the first time in 1952 when Peterson and Murray (1952) patented the process of 11α-hydroxylation of progesterone by a *Rhizopus* species. Since then, microbial reactions for the transformation of steroids have proliferated and specific microbial transformation steps have been incorporated into numerous partial synthesis of new steroids for the evaluation as drugs and hormones. These biotransformation have provided adequate tools for the large scale production of natural or modified steroid analogues (Asha and Vidyavathi, 2009).

Hydroxylations are possibly the most wide spread type of steroid bioconversions. Hydroxylations can be used to build intermediates for further chemical synthesis, by offering access to otherwise inaccessible sites of the steroid molecule, or to provide the steroid molecule with the adequate structure for therapeutic applications (Fernandes *et al*., 2003). 11β-Hydroxylation of steroids is an important reaction to produce hydrocortisone (11β, 17α, 21-trihydroxypregn-4-ene-3, 20dione, cortisol or substance F of Kendal) which is a principal pharmaceutical and also the precursor of the potent steroid like prednisolone (Naim *et al*., 2003; Lu *et al*., 2007). The 11β-hydroxylation of cortexolone by fungi belongs to transformation of biotechnological importance. It is a direct way to obtain cortisol, a pharmaceutical corticosteroid of considerable commercial value (Schmauder *et al*., 1991; Mahato and Garai, 1997; Sripalakit *et al*., 2006). Fungal steroid 11β-hydroxylation was catalyzed by cytochrome P450 monooxygenases (Suzuki *et al*., 1993; Paraszkiewiez and Dlugonski 1998). Cytochrome P450 is responsible for the oxygen insertion in the steroid substrate molecule (Lu *et al*., 2006).

Increasing interests and endeavours have been focused on the application of immobilized microbial cells for conducting biotransformations to produce useful compounds e.g 11β-hydroxylation of cortexolone by entrapment living mycelia of *Curvutaria lunata* (Sonomoto *et al*., 1981).

The protective microenvironment provided by the immobilized cells represents a major advantage, particularly when separate organic phase is present (Dias *et al*., 1994; Houng *et al .,*1994). Mass transfer limitation due to inadequate partition of the lipophilic substrates into hydrophilic gels have been reduced through the use of polyurea-coated alginate beads (Fernandes *et al*., 2003).Thus immobilized cell cultures may be efficient biocatalysts than free cells (Koshcheyenko *et al*., 1983).However, mycelial cells of fungi are fragile, and complex hydroxylation systems are liable to lose their activity.

The most obvious benefit of the immobilization technique is the capability of continuous cycling which provides a mean for using them in continuous cultures maintaining high cell population to achieve fast reaction rates (Koshcheyenko *et al*., 1983).

***Corresponding author**

The objective of the present study was to adopt two kinds of immobilization techniques (entrapping in calcium alginate gel and adsorption on glass wool) using *Cunninghamelta elegans* spores. The effect of some factors affecting the 11β-hydroxylation process such as sodium-alginate and glass wool concentrations, number of spores entrapped in the gel and that adsorbed on glass wool, the optimum bioconversion time for both and also the repeated use of the immobilized spores were also studied.

MATERIALS AND METHODS

Microorganism

Cunninghamella elegans RCMB 012001 was kindly obtained from the regional center of fungi, Al-Azhar University, Egypt.

Chemicals

Reichstein's compound S (cortexolone; 17α,21-dihydroxy-pregn-4-ene-3,20-dione) was the substrate, cortisol (hydrocortisone; Kendal's compound F; 11β,l7α,21-trihydroxy-4-pregnene-3,20-dione) and prednisolone (11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione) were the standards for the 11β-hydroxylation and Δ^{1-2}-dehydrogenation products and were provided by Sigma Company, U.S.A. Sodium alginate was a product of panreac Quimica (Espania). Glass wool was pyrex fiber glass, silver 8 micron coring glass work, Coring (U.S.A).

Preparation of immobilized spores in alginate beads

Sodium alginate solution 3% was prepared by dissolving 0.3 g in 7 mL distilled water. The resulting gel was autoclaved at 120 °C for 10 min then 3 mL of the fungal spores were added to the sterile alginate solution to obtain 3% final concentration. The previously prepared alginate-spores mixture was dripped into stirred 2% $CaCl_2$ solution (w/v). The alginate beads were cured by stirring in $CaCl_2$ for further 2 h at room temperature and then transferred to the refrigerator at 5 °C overnight in order to increase their stability.

The beads were then collected and washed thoroughly using sterile 0.5% NaCl solution (w/v) and then transferred to 50 mL fermentation medium (Fraser and Bickerstaff, 1997).The reaction was induced by adding 0.5 mg substrate for 8 h and then 5 mg of the steroid substrate was added and incubated for 48 h at 30 °C on a rotary shaker (120 rpm).

Preparation of immobilized spores adsorbed on glass wool

Immobilization of *Cunninghamella elegans* spores on glass wool was performed. Glass wool (0.8 g) was added to 50 mL of the fermentation medium unless otherwise stated. After sterilization, the flasks were left for 24 h on a shaking incubator at 200 rpm to form a thin, circular pad of glass wool network. Each flask was inoculated with 3 mL spore suspension and the biotransformation process was carried out as it will be subsequently mentioned. For repeated batch biotransformation, the fermentation medium was decanted at the end of each cycle and the glass wool pads were carefully squeezed and washed with 0.5% NaCl (w/v). The glass wool pads were then aspectically transferred to freshly prepared sterilized medium (Farid *et al.,* 1994).

Steroid transformation by immobilized spores

The immobilized spores of *C. elegans* (either using alginate beads or glass wool) were transferred to 50 mL of the fermentation medium (g/L): yeast extract, 5; bacto peptone, 5; glucose, 20; NaCl, 5; K_2HPO_4, 5; pH 6.0. The substrate (0.5 mg) was added as an inducer for 8 h followed by the addition of substrate to each flask (5 mg/50 mL) and incubated for 48 h at 30 °C on a rotary shaker (120 rpm) (Chinckolkar *et al*., 1995).

Extraction, qualitative and quantitative analysis of steroid compounds

The filtrates (collected after each transformation run) were extracted with chloroform (100 mL). The steroid substances present in the tested materials were estimated by thin layer chromatography with Kieselgel 60G. The amount of products were estimated by LC Module HPLC with software (Millenium 2010).The mobile phase used was 50% methanol and 5% water, mL/min with C-18 Novopak Column and U.V. detector (SPD- 10AVP/ 154 nm) (Houng *et al*., 1994).

RESULTS AND DISCUSSION

Effect of different concentrations of sodium alginate on cortexolone bioconversion to cortisol and prednisolone

Immobilization of fungal spores in gel matrices with subsequent germination and development into mycelia network overcomes the problems associated with the immobilization preformed by vegetative mycelia (Sonomoto *et al.*, 1983). The rigidity and size of the gel concentration seems to be critical for spore germination. In fact, the size of gel concentration markedly affected the growth and 11β- hydroxylation. Thus the gel concentration is an important factor for steroid 11β-hydroxylation activity.

The *C. elegans* spores were entrapped in sodium alginate at different concentrations (1%, 2%, 3%, 4%, 5%). Table 1 indicates that the 11β-hydroxylation capacity of the immobilized spores of *C. elegans* depended on the concentration of the entrapping agent. A 3% of sodium alginate proved to be the most suitable concentration for cortexolone bioconversion. Thus, the bioconversion capacities exhibited 1.2 folds increase as compared with the control (free spores).

Table 1: Effect of alginate concentrations on 11β-hydroxylation of cortexolone using entrapped spores of *C. elegans*

Na-alginate concentration (%)	Transformation mixture (mg/50mL medium)				
	Cortisol	Prednisolone	20β-hydroxy cortisol	Residual cortexolone	Transformation Efficiency (%)
1	1.3	1.2	0.8	1.4	52
2	1.6	1.5	0.9	0.8	61
3	1.8	1.6	1.0	0.4	68
4	1.7	1.6	0.7	0.7	61
5	1.6	1.5	0.5	1.1	59
Control*	1.5	1.4	0.9	0.9	59

- Control is the free spores of *C.elegans*.
- Cortexolone concentration = 5 mg/50 mL medium

Table 2: Effect of different inoculum sizes of entrapped spores of *C. elegans* on the conversion of cortexolone

Inoculum size (%)	Transformation mixture (mg/50mL medium)				
	Cortisol	Prednisolone	20β-hydroxy cortisol	Residual cortexolone	Transformation Efficiency (%)
5	1.3	1.2	0.6	1.7	50
10	1.6	1.5	0.7	0.9	62
15	1.9	1.7	1.1	0.3	72
20	1.7	1.6	0.8	0.7	66
25	1.5	1.4	0.7	1.0	59

- Cortexolone concentration =5 mg/50 mL medium
- Sodium alginate concentration = 3%

On the other hand the utilization of sodium alginate concentration more than 3% affected the transformation process adversely (Fukui *et al*., 1980). Similar results have been recorded by Wang *et al*. (1998) and Dlugonski *et al*. (1997).

Effect of different inoculum sizes of entrapped spores of *C. elegans* on the conversion of cortexolone

Different amounts of spore suspension were used (5, 10, 15, 20 and 25 mL) to prepare the alginate beads (Table 2). The 11β-hydroxylation process was affected by the amount of spores. The yields of cortisol and prednisolone increased with the increase of spore number up to l5 mL/50 mL medium which allowed maximal bioconversion of cortexolone (72%). On the other hand, higher concentrations of the spore suspensions exerted a deleterious effect on the enzymatic activity of fungal spores. This may be due to the bulky formation of the spores in the formed beads which may inhibit aeration and in turn inhibits the exchange of oxygen, which is an important limiting factor in the hydroxylation process (Martine, 1984; Lu *et al*., 2006; Manosroi *et al*., 2006). It is well known that cytochrom P450 acts as the key component in the steroid 11β-hydroxylation systems, which is responsible for the oxygen insertion into the steroid molecule (Lu *et al*., 2007). On the other hand, Manosroi *et al*. (2007) found that 1 x 10^3 initial cell number was the best concentration to get hydrocortisone acetate from cortexolone-21-acetate using *C. blakesleeana* ATCC 8688a.

Effect of transformation period on the bioconversion of cortexolone using entrapped *C. elegans* spores

The effect of the bioconversion time course as an effective factor in the 11β-hydroxylation of cortexolone using *C. elegans* spores entrapped in 3% alginate gel was investigated. Table 3 indicates that the best bioconversion estimates were reached after 72 h which was the optimal transformation period for attaining maximal favourable transformation activities (84%) using entrapped fungal spores. Increasing the transformation periods more than 72 h led to a decrease in the 11β-hydroxylation activity. This may be due to the lysis of beads containing spores and the level of the enzymic activity of the immobilized system can be controlled by changing the intensity of cell propagation within the gel (Lusta *et al*., 1989). It was previously stated that 63 h was the best time to get hydrocortisone using *Absidia archidis* (Wang *et al*., 1998). On the other hand, Yang *et al*. (2007) stated that rimexolone was obtained by microbial hydroxylation of l6α,l7α-dimethyl-17β-(1-oxopropyl) androsta-1.4-dien-3-one by *Curvularia lunata* after 30–44 h.

Table 3: Effect of the bioconversion time course on the transformation of cortexolone using entrapped spores of *C. elegans*

Bioconversion time course (h)	Transformation mixture (mg/50mL medium)				
	Cortisol	Prednisolone	20β-hydroxy cortisol	Residual cortexolone	Transformation efficiency (%)
12	**1.1**	**1.0**	**0.5**	**1.9**	**43**
24	**1.5**	**1.4**	**0.6**	**1.2**	**57**
48	**2.1**	**1.9**	**0.8**	**0.2**	**80**
72	**2.2**	**2**	**0.3**	**0.4**	**84**
96	**1.5**	**1.4**	**0.2**	**1.4**	**58**

- Substrate concentration = 5 mg/50 mL medium
- Sodium alginate concentration 3%.
- Spore concentration = 2 x 10^7 spores/mL

Table 4: Bioconversion of cortexolone using *C.elegans* spores adsorbed on different weights of glass wool

Weight of glass wool (g)	Transformation mixture (mg/50mL mixture)				
	Cortisol	Prednisolone	20β-hydroxy cortisol	Residual cortexolone	Transformation efficiency (%)
0.2	0.9	0.8	0.7	2.2	35
0.4	1.5	1.4	0.9	0.9	59
0.6	1.7	1.5	1.0	0.5	66
0.8	1.8	1.7	0.8	0.3	70
1	1.7	1.6	0.8	0.6	66
Control*	1.5	1.4	0.9	0.9	59

- Control* = free spores
- Concentration of substrate 5 mg/50 mL medium
- Spores concentration = 2 x 10^7 spores/mL

Reutilization of the entrapped *C. elegans* spores in alginate beeds for the biotransformation of cortexolone to cortisol and prednisolone

The immobilized *C. elegans* spores in alginate beads were reused in the biotransformation of cortexolone to cortisol and prednisolone. Figure 1 indicates that the immobilized spores can be reused for 6 runs with the optimum activity at the second cycle. The transformation capacity proved to decrease with the increase of cycle number, and it was also noticed that alginate beads almost dissolved at the sixth run (18 days). The decrease in the beads stability and high leakage of cells during the successive cycles may explain the gradual decrease of the products in the repeated batch cycles. The destabilization of the alginate beads had also been reported by Fraser and Bickerstaff (1997).

Effect of different concentrations of glass wool on the bioconversion of cortexolone

In the adsorption technique, different weights of glass wool (0.2, 0.4, 0.6, 0.8, Lg/50 mL) were used to adsorb *C. elegans* spores (2 x 10^7/mL). The good conversion yields, in favour of cortisol and prednisolone formation were recorded with fungal spores immobilized on 0.8 g glass wool (Table 4). The amount of the products was relatively higher as compared with free spores. The adsorption process involves three stages: reversible adhesion, irreversible adhesion and micro-colony formation (Marshall *et al*., 1971). Increasing the glass wool weight more than 1.6% (w/v) was accompanied by a decrease in the products due to the less availability of the fungus spores to the transformation medium. Amin (2005) found that the optimum weight of the glass wool used for the biotransformation of glycyrrhizin was 1.2% (w/v).

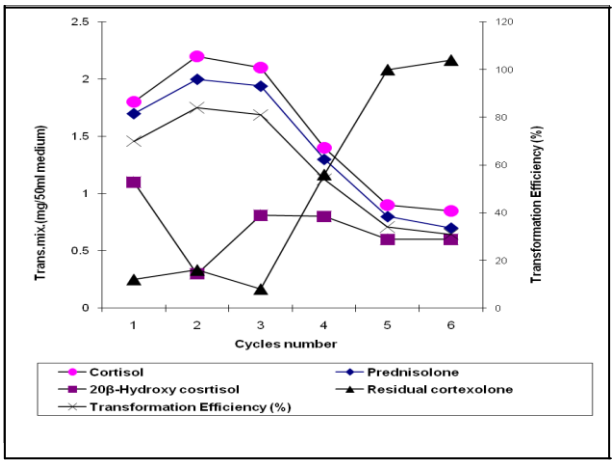

Figure 1: Reutilization of the entrapped spored of *C. elegans* for the biotransformation of cortexolen to cortisol and prednisolone

Table 5: Biotransformation of cortexolone as influenced by the loaded inoculum size of *C.elegans* spores on glass wool carrier

Inoculum size (mL)	Transformation mixture (mg/50mL mixture)				
	Cortisol	Prednisolone	20β-hydroxy cortisol	Residual cortexolone	Transformation efficiency (%)
5	1.1	1	0.5	2.1	42
10	1.8	1.7	0.3	0.9	70
15	2.1	2	0.3	0.4	82
20	2.1	1.9	1.0	0.4	80
25	1.7	1.6	0.8	0.6	66

- 1mL = 2 x 10^7 spores
- Cortexolone concentration = 5 mg/50 mL medium
- Glass wool wt. = 1.6% (wt/v)

Table 6: Effect of the bioconversion time course on transformation of cortexolone using adsorbed spores *C.elegans* on glass wool

Bioconversion time course (h)	Transformation mixture (mg/50mL mixture)				
	Cortisol	Prednisolone	20β-hydroxy cortisol	Residual substrate	Transformation efficiency (%)
12	1.1	0.9	0.3	2.3	42
24	1.2	1.1	0.7	1.7	46
48	2.1	1.9	0.9	0.1	81
72	2.4	2.2	0.2	0.01	92
96	2.3	2.16	0.4	0.13	89

- Cortexolone concentration = 5 mg/50 mL medium
- Glass wool = 1.6% (wt/v)
- Spores concentration = 2 x 10^7 spores/mL

Effect of inoculum size of *C.elegans* adsorbed on glass wool on the bioconversion of cortexolone

As it can be seen from Table 5, the volume of fungus spores loaded on glass wool affected the bioconversion activity. The maximum efficiency (82%) and the higher productivity of both cortisol and prednisolone were obtained with spore load of 15 mL (2 x 10^7 spores/mL). It was noted that the optimal spores loaded on glass wool was similar to that entrapped in alginate beads (15 mL i.e. 2 x10^7 spores/mL). By further increasing the concentration of the loaded spores the bioconversion process was retarded. Sonomoto *et al*. (1981) stated that the optimum cell concentration was 1.8 x 10^6 using *C. lunata* in order to attain good bioconversion yields.

Effect of transformation period on the bioconversion of cortexolone using *C. elegans* spores adsorbed on glass wool

To illustrate the rate of cortexolone transformation using immobilized *C. elegans* spores on glass wool, the products were measured in the fermentation mixtures at different time periods (12, 24, 48, 72 and 96 h). It is clearly evident from Table 6 that the bioconversion efficiencies of cortexolone linearly increased with the increase of transformation period. Evidence has been given that the maximum total production values (92%) were obtained after 72 h. Increasing the biotransformation period more than 72 h was accompanied by decreasing the bioconversion activity. It seems that the loaded spores need a definite time to grow and consequently producing the desired enzymes for the bioconversion process. Recently, Amin (2005) found that 96 h was the optimum incubation period to give maximum production of glycyrrhetic acid from glycyrrhizin using glass wool immobilized *A. niger* spores. Similarly, Manosroi *et al*. (1999) could obtain prednisolone from transformed cortexolone by mixed cultures of free *C. echinulata* ATCC 8688a with immobilized *B. sphaericus* ATCC 13805 after 96 h.

Reutilization of the adsorbed *C. elegans* spores on glass wool for the biotransformation of cortexolone to cortisol and prednisolone

On using the adsorbed fungal spores for cortexolone bioconversion for several batch cycles (Figure 2), the transformation efficiency reached about 94%, with highest productivity for hydrocortisone and prednisolone at the end of the second batch. These yields were of higher magnitude than that obtained by the free cells. However, the bioconversion estimates decreased gradually up to the 5th run. Obviously, reutilization of fungal spores adsorbed on glass wool appeared to be the most successful treatment wherein they maintained about 50% of their

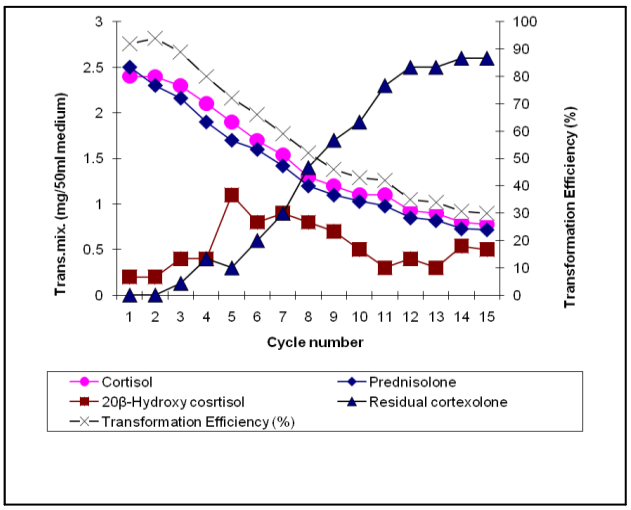

Figure 2: Reutilization of the adsorbed C. elegans spores on glass wool for the biotransformation of cortexolone to cortisol and prednisolone

productivity during 24 days, including 8 successive cycles. Moreover the adsorbed fungal spores still exhibited bioconversion activities (although of lower magnitudes) as the recyclization process was extended for further repeated 8 cycles. Similarly Wang *et al*. (1998) stated that cells can be used for six runs without any significant decrease in activity. However, from the seventh run the hydrocortisone yield began to decrease.

CONCLUSIONS

From this study, we can conclude that the transformation of cortexolone to cortisol and prednisolone using adsorbed *C. elegans* spores on glass wool was higher than that using the entrapment in calcium alginate gel technique. The optimum condition to obtain maximum transformation of cortexolone was 72 h using 2 x 10^7 spores/mL. The total amount of hydrocortisone and prednisolone formed during the repeated use of *C. elegans* spores entrapped in alginate beads were 9.25 mg and 8.44 mg respectively from 30 mg cortexolone (operational period, 18 days).

On the other hand, the total amount of hydrocortisone formed was 22.45 mg and prednisolone was 20.71 mg from 75 mg cortexolone (operational period 45 days) in the adsorption technique. Such immobilization method might be important from a practical view point for the production of useful compounds.

REFERENCES

Amin, H. A. S. (2005). Studies on the bioconversion of glycyrrhizin. Ph.D Thesis. Faculty of Science, Botany Department, Cairo University.

Asha, S. and Vidyavathi, M. (2009). *Cunninghamella* - A microbial model for drug metabolism studies - A review. *Biotechnology Advances* **27, 16-29.**

Bickerstaff, G. F. (1997). Immobilization of enzymes and cells. *In:* Immobilization of enzymes and cells: Methods in Biotechnology. Bickerstaff, G. F. (ed.). Humana Press, Inc., Totowa, New Jersey. **1-11.**

Chinckolkar, S. B., Laxman, R. S. and Wakharkar, R. D. (1995). Hydroxylation of progesterone by *C. blakesleeana* NCIM 687. *World Journal of Microbiology* and *Biotechnology* **11(3), 357-358.**

Dias, A. C. P., Cabral, J. M. S. and Pinheiro, H. M. (1994). Sterol side-chain cleavage with immobilized *Mycobacterium* cells in water immiscible organic solvents. *Enzyme Microbial Technology* **16, 708-714.**

Dlugonski. J, Paraszkiewicz, K. and Sedlaczek, L. (1997). Maintenance of steroid 11-hydroxylation activity in immobilized *Cunninghamella elegans* protoplasts. *World Journal of Microbiology and Biotechnology* **13, 469-473.**

Farid, M. A., El-Diwany, A. I. and El-Enshasy, H. A. (1994). Production of oxytetracycline and rifamycin Band SV with cells immobilized on glass wool. *Acta Biotechnologica* **14, 67-74.**

Fernandes, P., Cruz, A., Angelova, B., Pinheiro, H. M, and Cabral J. M. S. (2003). Microbial conversion of steroid compounds: Recent development. *Enzyme Microbial Technology* **32, 688-705.**

Fraser, J. F. and Bickerstaff, G. F. (1997). Entrapment in calcium alginate. *In:* Immobilization of enzymes and cells: Methods in Biotechnology. Bickerstaff, G. F. (ed.). Humana Press Inc., Totowa, New Jersey. **61-66.**

Fukui, S., Sonomoto, K., Itoh, N and Tanaka, A. (1980). Several novel method for immobilization of enzymes, microbial cells and organelles. *Biochimie* **62, 381-386.**

Houng, J. Y., Chiang, W. P. and Chen, K. C. (1994). 11α-Hydroxylation of progesterone in biphasic media using alginate entrapped *Aspergillus ochraceus* gel beads coated with polyurea. *Enzyme Microbiology and Technology* **16, 485-491.**

Koshcheyenko, K. A., Turkina, M. V. and Skryabin, G. K. (1983). Immobilization of living microbial cells and their application for steroid transformation. *Enzyme and Microbial Technology* **5, 14-21.**

Lu, W. Y, Du, L. X., Wang, M., Wen, J. P., Sun, B. and Guo, Y. W. (2006). Effect of two-steps substrate addition on steroids 11β- hydroxylation by *Curvularia lunata* CL-114. *Biochemical Bioengineering Journal* **32, 233-238.**

Lu, W., Du, L., Wang, M., Jia, X., Wen, J., Huang, Y., Guo, Y., Gong, W., Bao, H., Yang, J. and Sun, B. (2007). Optimization of hydrocortisone production by *Curvularia lunata*. Applied Biochemistry and Biotechnology **142, 17-28.**

Lusta, K. A., Starostina, N. G., Gorkina, N. B., Fikhe, B. A., Lozinskii, V. L., Vainermon, E. S. and Rogoezhin, S. V. (1989). Immobilization of *E. coli* in macroporous cryogels on polyacrylamide. *Biotechnology and Bioengineering* **24(2), 418-426.**

Mahato, S. B. and Garai, S. (1997). Advances in microbial steroid biotransformation. *Steroids* **62, 332-345.**

Manosroi, J., Saowakhon, S. and Manosroi, A. (2007). A novel one-step biotransformation of cortexone - 21 -acetate to hydrocortisone acetate using *Cunnighamella blakesleeana* ATCC 8688a. *Enzyme and Microbial Technology* **41, 332-325.**

Manosroi, J., Chisti, Y. and Manosroi A. (2006). Biotransformation of cortexolone to hydrocortisone by molds using rapid color- development assay. *Applied Biochemistry and Microbiology* **42(5), 479-483.**

Manosroi, J., Abe, M. and Manosroi, A. (1999). Biotransformation of steroidal drugs using microorganisms screened from various sites in Chiang Mai, Thailand. *Bioresource Technology* **69(1), 67-63.**

Marshall, K. C., Stout, R. and Mitchell, R. (1971). Mechanism of the initial events in the sorption of marine bacteria to surfaces. *Journal of General Microbiology* **68, 337-348.**

Martine, C. K. A. (1984). Cited from Biotechnology. Vol. 61. Rehm, H. J. and Reed, G. (eds.). Chap. 3. **79-95.**

Naim, N., Adham, N. Z., Abd El-Rhim, R. and Abd-El-Hady, A. (2003). Prednisolone production using *Pseudomonas fluorescens* cells immobilized with polymer carrier produced by radiation polymerization. *Process Biochemistry* **38, 1083-1089.**

Paraszkiewicz, K. and Dlugonski, J. (1998). Cortexolone 11β-hydroxylation in protoplasts of *Curvularia lunata. Journal* of *Biotechnology* **65, 217-224.**

Peterson, D. H. and Murray, H. C. (1952). Microbiological oxygenation of steroid at carbon 11. *Journal of American Chemical Society* **74, 1871-1872.**

Schmauder, H. P., Schlosser, D., Gunther, T., Hattenbach, A., Sauerstein, J., Jungnickel, F. and Augsten R. (1991). Application of immobilized cells for steroid transformation. *Journal* of *Basic Microbiology* **7, 187**-**236.**

Sonomoto, K., Hoq, M. M. D., Tanaka, A. and Fukui, S. (1983). 11β-Hydroxylation of cortexolone (Reichstein's compound S) to hydro cortisone by *Curvularia lunata* entrapped in photo-cross-linked resin gels. *Applied Environmental Microbiology* **45, 436-443.**

Sonomoto, K., Hoq. M. M. D., Tanaka, A. and Fukui, S. (1981). Growth of *Curvularia lunata* spores into mycelial form with various gels, and steroid 11β-hydroxy1ation by the entrapped mycelia. *Journal of Fermentation Technology* **59(6), 465-469.**

Sripalakit, P., Wichai, U. and Sara Phanchotiwitthaya, A. (2006). Biotransformation of various natural sterols to androstenones by *Mycobacterium* sp. and some steroid-converting microbial strains *Journal of Molecular Catalysis B: Enzymatic* **41, 49-54.**

Suzuki, K., Sabga, K., Chikaoka, Y. and Itagaki, E. (1993). Purification and properties of cytochrome P 450 catalyzing steroid 11β-hydroxylation in *Curvularia lunata*. *Biochimika Biophysika Acta* **1203, 215-223.**

Wang, J., Chen, C., Li, B., Zhang, J. and Yu, Y. (1998). Production of hydrocortisone from cortexolone-21-acetate by immobilized *Absidia orchidis* in cosolvent containing media. *Enzyme Microbial Technology* **22, 368-373.**

Yang, J., Yang, S., Yang. Y., Zheng, H. and Weng, L. (2007). Microbial hydroxylation of 16α,17α-dimethyl-17β-(l-oxopropyl) androsta-l,4-dien-3-one to rimexolone by *Curvularia lunata* AS 3.4381. *Journal of Molecular Catalysis B: Enzymatic* **47, 155-158.**