Acta Pharmaceutica Turcica
47: 131 - 141 (2005)

Stability of the steroid Δ^1- dehydrogenation system of *Rhodococcus sp* DSM 92-344 in organic solvent water two liquid phase environment

Nehad Z. Adham* Ibrahim S. Abd –Elsalam and Mona S. Shafei

Chemistry of Natural and Microbial Products Department, National Research Center, Dokki, Cairo, Egypt

Abstract

The stability of Δ^1- dehydrogenation system of *Rhodococcus sp.* in organic solvent aqueous two liquid phase environments has been studied. A number of water-miscible or immiscible solvents has been examined, water immiscible organic solvents have been found superior. Solvents with high log P values such as hexane, toluene gave much higher conversions. The study emphasizes that 20 % solvent concentration was favorable for an increase in productivity which results from an increase in the accumulation of product (prednisolone) in using both n-hexane and toluene.
The optimal reaction time which gave maximum conversion yield was 18 h for n-hexane and 24 h for toluene. 58 % of the added substrate (20mg/100ml) has been converted to prenisolone using n-hexane or toluene, increase substrate concentration reduced prednisolone yield which was more significant in using n-hexane as organic phase. Inoculum size displayed great effect on prednisolone yield, at high substrate concentration (80 mg/100 ml) on increasing biomass 3 fold prednisolone productivity increase from 11 to 27.7 using toluene as organic phase.

Keywords: Steroid Δ^1- dehydrogenation, *Rhodococcus sp.*, Organic solvent, two liquid phase bioctalysis.

Introduction

The Δ-dehydrogenation of hydrocortisone by *Rhodococcus sp* is being used as a model to study the effects of organic solvents on microbial aqueous two liquid phase environments. Aqueous steroid bioconversion system very often lead to low productivity levels, mainly because of the low solubility (10^{-4}-10^{-5}) and toxicity of these substrate in aqueous media (Riva, 1991; Oda *et al.*, 2001)

Conventional approaches to minimize mass transfer limitation include the use of micronized substrates (Kutney *et al.*, 1999; Redikultsev *et al.*, 1995) and the feeding of substrates in the form of saturated solutions in water-miscible solvents such as methanol (Berrie *et al.*, 1999) acetone (Srivastava and Palil, 1995) ethanol (Adham *et al.*, 2002). The amount of co-solvent added usually has to be kept below 1.5-5 % (v/v).

A more radical approach to cope with the low water solubility of steroid-like compounds has been the use in bioconversion medium of a water immiscible organic phases as substrate carrier

* Author for correspondence

and for in –situ product recovery (Mahato and Mukherjee, 1984; Mahato and Garai, 1997; Leon *et al.,* 1998; Angelova and Schmauder, 1999 and Gruz *et al.,* 2001).

The use of water –immiscible organic solvents offers several potential advantages in biocatalysis such as the possibility of operating at high substrate and product concentrations whilst avoiding product and substrate inhibition. There are also a number of disadvantages, such as the need for adequate mass transfere between the two liquid phases and catalyst inactivation by the organic phase (Hocknull and Lily 1990; Van Sonsbek *et al.,* 1993; Geen *et al.,* 1988; Hocknull and Lilly 1987; Ramelmeier and Blanch, 1989).

Jaane *et al.,* (1985) proposed that " activity retention" of biocatalysts in organic solvent-water two liquid phase environments is related to the logarithm of the partition coefficient (log P) of the organic solvent. They concluded that activity retention was low in solvents of log $P< 2$ and high only in solvents of log $P>4$ solvents of log P in the range 2-4 may be used but authors suggest that harmful effect may result.

Microbial Δ^1-dehydrogentation is usually performed with whole cells, since this is a co-factor – dependent reaction the continuous regeneration of the necessary co-factor is ordinary accomplished by the active cell machinery (Fernandes *et al.,* 2003).

Activity retentions are essentially productivity measurements and thus give little information about the stability of the biocatalyst in the two-liquid phase environments.

Therefore, it is important to assess the intrinsic stability of the tested microorganisms with respect to solvents, and the optimum conditions at which the maximum Δ^1-dehydrogentation was carried out.

Materials and Methods

Microorganism

The experimental bacterium used in this study (*Nocardia sp.*) was deposited under the number 92-344 at the DSM (FRG) and identified as an isolate of Rhodococcus sp Sallam *et al.,* (1995).

Chemicals

Cortical (hydrocortisone), prednisolone (authentic), were obtained from Sigma Chemicals Company. All chemicals used in the present investigation were of fine analytical grade obtained from Merck.

Cultivation

Rhodococcus was cultivated in 500 ml shaking flask containing 200 ml of growth medium with 200 rpm orbital shaking at 30°C. The growth medium contained 3 gL^{-1} beef extract, 5 gL^{-1} peptone, 10 gL^{-1} glucose in distilled water (Sallam *et al.,* 1995). Growth of the culture proceeded for 48 h, the cells were harvested by centrifugation and washed with four times their volume of reaction medium, used directly as a wet cell (2g /100 ml). Reaction medium was composed of 0.25 % dextrose in 50 mM phosphate buffer, pH 7.4.

Dehydrogenation in the presence of organic solvents.

Cell cake (standard inoculum of wet cell) was resuspended in reaction medium in a 250 ml stoppered flask, organic solvents containing cortisol 0.1 gL^{-1} (unless otherwise stated) was added to the flask, agitation was at 200 rpm in an orbital shaking incubator at 30°C, samples of both phases were removed for assay.

Assessment of the effect of organic solvents

Aliquots (25 ml) from previously prepared culture were contacted by shaking with an equal volume of organic solvent for 0, 2, 5, 10 and 15 min. After the appropriate time the flasks contents were harvested and washed. The wet cell obtained was resuspended in 25 ml of reaction medium containing 2.5 mg cortisol slurried in 1 ml 0.01 % Tween 80. The flasks were agitated for 18 h and assayed for product levels. The ability of solvent treated cells to transform the particulate steroid was compared with an aqueous control which had not been contacted with the solvent.

Analysis of steroid conversion

At the end of the transformation period, aqueous samples were extracted and treated to give a semisolid residue "test material" (Naim *et al.*, 2002; Sallam *et al.*, 1995). Organic solvent samples were removed and filtered through 0.45 µm filters before evaporation, (test material).

The test materials were identified using high-pressure liquid chromatography (HPLC) (Kloosterman and Lilly, 1984), in which 1 ml of the previously extracted sample containing the steroids was mixed with 1 ml methylene chloride, vortexed for 1 min., and the steroid allowed to separate. A 10 µl sample of the methylene chloride phase was injected into an HPLC, using a lichrosorb Si-605- µm column, methylene chloride as a mobile phase containing 5% methanol and 0.55 % acetic acid, 1 ml/min flow rate and UV detector at 254 nm.

Table 1 Effect of various water–immiscible organic solvents on the conversion to prednisolone in aqueous /organic solvent two-phase system.

Organic solvent	Conversion (%)[a]	LogP value[b]
Ethyl acetate	33.3	0.68
Butan-1-ol	16.0	0.8
Petroleum ether	19.2	ND[c]
1,2-dichloroethane	18.0	ND
Chloroform	39.4	0.2
Toluene	46.0	2.5
Carbon tetrachloride	19.0	3.0
n-hexane	48.0	3.5

a. bioconversion of hydrocortisone to prednisolone by *Rhodococcus* cells was carried out in a 30 ml aqueous solvent (2: 1 v/v) two-phase system with 250 ml Erlenmeyer flask (200 rpm) at 30°C for 12h. The hydrocortisone concentration was 0.1 g l-1
b. Cited from reference (Laane *et al.*, 1987).
c. Not determined

Experimental results

Comparison of different reaction systems of Δ^1-dehydrogenation of hydrocortisone

Effect of various water immiscible organic solvents on the conversion of hydrocortisone to prednisolone by *Rhodococcus sp.* DSM 92-344 was tested in as aqueous /organic solvent two-phase system (2:1, v/v). As shown in Table 1, it was found that solvents with high log P values

such as hexane, toluene gave much higher conversions than those solvents with low log P values, such as ethyl acetate and butan-1-ol. Laane *et al.*, (1987) have proposed that solvent of log P value in the range of 2-4 may be used in an aqueous /organic solvent two –phase system. The productivity of prednisolone in an aqueous /organic solvent two-phase system was superior to that in aqueous or cosolvent systems (Table 2).

Table 2 Comparison of the conversion to prednisolone by *Rhodococcus* sp. DSM 92-341 in various reaction systems.

Reaction system	Reaction time	Prednisolone accumulated (mgL^{-1})	Productivity ($mg\ L^{-1}\ h^{-1}$)
Aqueous	12	24	2
Cosolvent			
Methanol	12	36	3
Ethanol	12	23	1.9
Acetone	12	20	1.6
Two phase			
n-hexane	12	48	4
Toluene	12	46	3.8

The bioconversion conditions were the same a in Table 1.

Assessment of the effect of organic solvents on Rhodococcus sp

To assess the effect of four different solvents (including n.hexane and toluene) alone on the cells, the solvent pretreated cells in the absence of steroid, described in the experiment procedure was used. From Figure 1 it is evidenct that the loss of Δ^1- dehydrogenation ability of Rhodococcus sp. is not significant in using n-hexane and toluene but the loss of Δ^1-dehydrogenation ability is rapid using both acetone and ethanol (after 10 and 15 min respectively), it follows a trend to the solvents of greater solubility in water having the more rapid disrupting effect on the cells, this is in accord with Brink and Tramper (1985).

The results show the suitable nature of n-hexane and toluene for use with *Rhodococcus sp.*, therefore n-hexane and toluene were chosen for all further work.

Optimization of aqueous/toluene or aqueous /n-hexane two phase system for Δ^1-dehydrogenation of hydrocortisone.

Effect of reaction time on the conversion of hydrocortisone to prednisolone in an aqueous n-hexane and aqueous /toluene (3:1, v/v) two-phase system were investigated. As shown in Fig.2, it was found that fifty percent of hydrocortisone could be converted to prednisolone within 18 h using aqueous /n-hexane two- phase system and within 48 h using aqueous/toluene two-phase system. After 6 h the productivity of hydrocortisone to prednisolone in an aqueous/n-hexane two –phase system was 7.96 mg $l^{-1}h^{-i}$ and 6.68 mg $l^{-1}h^{-i}$ in aqueous /toluene two –phase system. Generally, the productivity of prednisolone in an aqueous –n-hexane two phases system is higher than that in aqueous /toluene two-phase system after 6, 12 and 18 h reaction time. On the

other hand after 24 h and 48 h the productivity in an aqueous /toluene two-phase system (2.06, 1.03 mg $l^{-1}h^{-1}$) is higher than in aqueous /n-hexane two-phase system (2.03-0.96 mg $l^{-1}h^{-1}$).

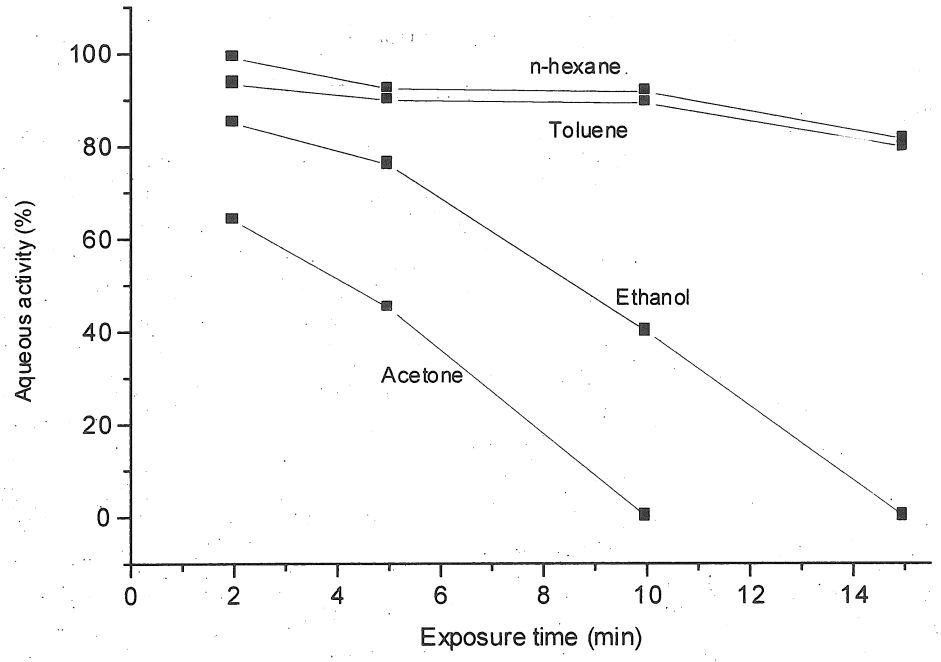

Fig (1) Percentage of reaction aqueous phase activity with time of exposure *Rhodococcus* cells to organic solvents

The decrease of prednisolone yield in using aqueous /n. hexane two –phase system after 24 and 48 h may be explained by the reversibility of prednisolone to cortisol which was demonstrated by some investigators (Martin, 1984, Naim *et al.*, 2003and Adham *et al.*, 2003). Furthermore the increase in prednisolone yield in using aqueous/toluene two phase system after 48 h reaction time not significant comparative with that of 24h.

In order to improve the productivity of prednisolone the ration of aqueous /organic solvent in the two-phase system must be changed. Increase in the solvent conc from 10% to 20% was favorable for an increase in productivity which resulted from an increase in the accumulation of prednisolone in both n-hexane (2.75-3.22 mg $l^{-1}h^{-1}$) and toluene (2.10-2.24 mg $l^{-1}h^{-1}$) (Table 3 and Fig 3), then the productivity decrease in parallel with an decreased in the ratio of aqueous/ organic phase.

The loss of Δ^1- dehydrogenation activity of *Rhodococcus* by decrease in the ratio of aqueous organic phase may be due to solvent action on the dehydrogenase enzyme per-cells or due to solvent action on the cofactor regeneration step of the reaction. Hocknull and Lilly (1988) also Pinheiro *et al.*, (1993) studied the Δ^1-dehydrogenation system of *Arthrobacter simplex* in organic solvent-aqueous two –liquid phase, they recorded that the primary target of organic solvent was the cofactor regeneration step of the reaction rather than the Δ^1- dehydrogenase.

Some workers (Bar, 1987; Cho and Schuler, 1966) have suggested a number of mechanisms by which the organic phase may be toxic to a microorganisms including partitioning of nutrients, limited access of nutrients of the cells, and disrupstion of the outer layer of the cell structure,

the first two explanation may be proposed. In addition, there was no evidence of cell lysis or of cellular material free in the reaction media.

Table 3. Effect of organic / aqueous solvent two-phase ratio on Δ^1-dehydrogenation activity of *Rhodococcus* sp

Solvent	Two-phase	Reaction time	Prednisolone accumulated %
n-hexane	45/5	18	49.5
	40/10	18	58
	35/15	18	50
	30/20	18	47.9
	25/25	18	46
Toluene	45/5	24	50.5
	40/10	24	53.9
	35/15	24	49.6
	30/20	24	44
	25/25	24	38.2

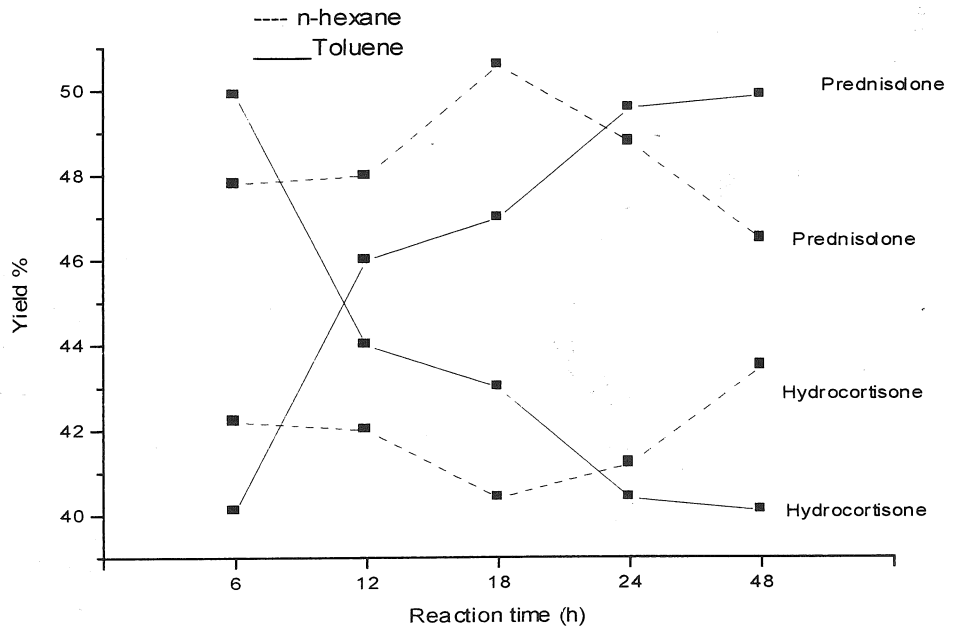

Fig 2. Effect of time on Δ^1- dehydrogenase activity in an aqueous /organic two-phase system

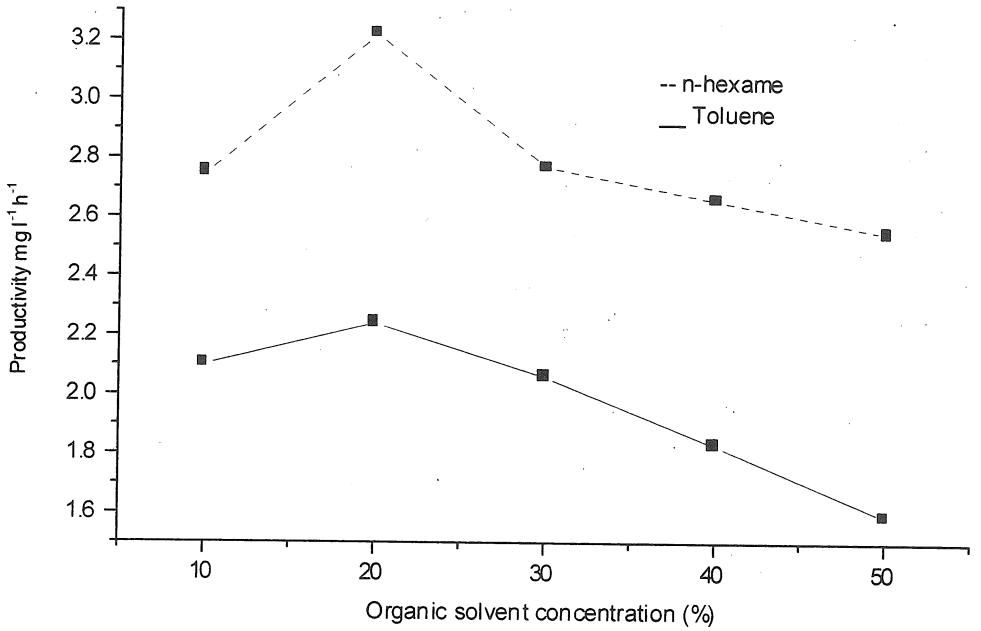

Fig 3. The effect solvent concentration on the productivity of prednisolone in organic-aqueous two-phase system in optimum reaction time.

Schneider (1968) suggested that the side of solvent action in cells was either the lipid layers of the membrane or else involved, a protein that undergoes conformational changes in response to organic solvents, other workers (Gordon *et al.*, 1980) have proposed that solvents act by displacing the annular lipids of integral membrane proteins, such a mechanism would account for loss of enzyme activity. Also Hocknull and Lilly (1988) recorded that the loss of cofactors regeneration function could result either from more solvent entering the membrane or from a redistribution of solvent within the membrane.

Possibility of increasing concentration of poorly water soluble substrate and or products are the most important reason to justify the use of organic media instead of aqueous solutions. Three sets of experiments were carried out to evaluate the effect of substrate concentrations on the prednisolone productivity, the hydrocortisone concentration was varied 10-80 % in an aqueous/n-hexane (4:1 v/v) two phase system in the optimum reaction time (18h); an aqueous / toluene (4:1 v/v) two phase system in the optimum reaction time (24h) and an aqueous system in the optimum reaction time (24h- data not shown), as shown in Table 4 and Fig 4, the productivity of prednisolone in an aqueous toluene, two phase system (11 mg/L/h) was higher than that in an aqueous /n-hexane system under high hydrocortisone concentration 80 mg/100 ml. The reason may be due to the fact that the partition coefficient of prednisolone in the toluene phase is for better than that in the n-hexane phase. From the data shown in Table 4 the best prednisolone yields (58%) achieved by using 20 mg hydrocortisone in both n-hexane and toluene. However, the best prednisolone yield were obtained in an aqueous /toluene two phase system using 30,40,80 mg hydrocortisone. The Δ^1-dehydrogenase system of *Rhodococcus sp.* shows a good stability by using substrate concentration up to 0.2 mg/ml and the bacterium could tolerate a substrate concentration up 0.8 mg/ml.

Table 4. Effect of hydrocortisone concentration on the bioconversion to prednisolone in an aqueous /organic two phase system (4:1)

Hydrocortisone conc. Mg/100 ml	Prednisone conversion %		
	No solvent*	n-hexane	Toluene
10	24.0	58.0	53.9
20	23.3	58.0	58.0
30	19.3	46.8	50.0
40	14.4	35.0	40.0
80	13.0	20.0	33.0

-The bioconversion conditions were the same as in Table 3
- The optimum reaction times were used from both n-hexane and Toluene (18 and 24 h respectively).

*The bioconversion was carried out in 50 ml reaction media for 24 h (the optimum reaction time).

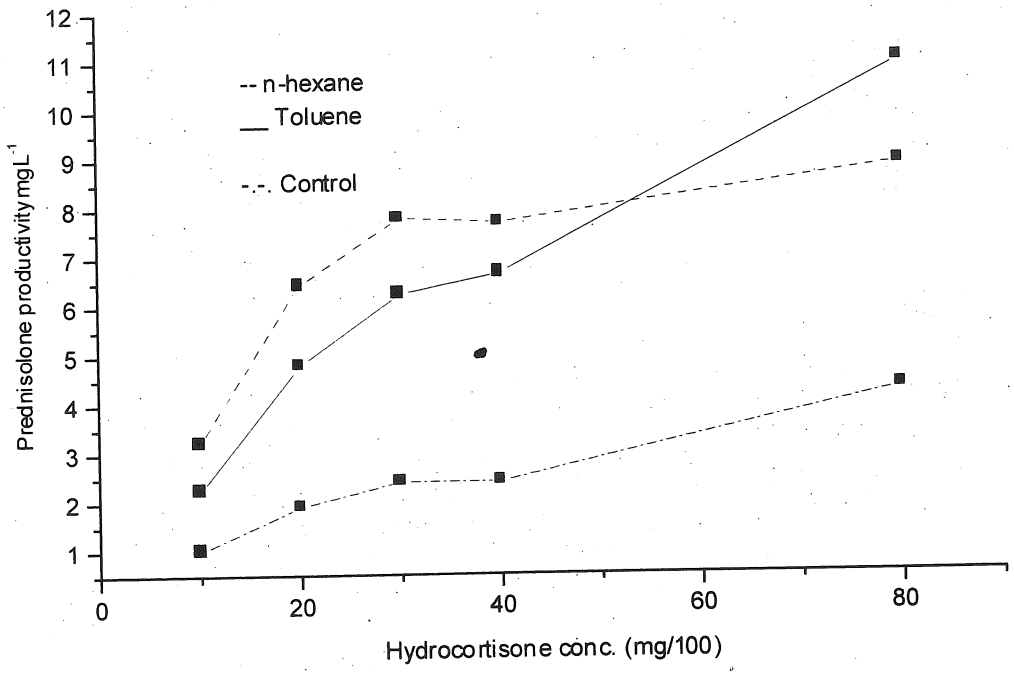

Fig 4. Effect of hydrocortisone concentration on the productivity of prednisolone in an aqueous organic two phase system (4:1)

All bioconversion conditions were the same as in Table 4.

The Δ^1- dehydrogenation of hydrocortisone by various wet cells was carried out in a 50 ml aqueous/toluene (40:10) two-phae system for 24h the hydrocortisone concentration was 80 mg/100 ml.

In the above experiments the maximum productivity value was recorded by using high substrate concentration (80 mg/ml) dissolved in toluene as organic phase, the influence of wet mass on the maximum productivity was investigated . As shown in Table 5, the increase of wet cell mass in aqueous phase gave an increase in the productivity, about 56 % of hydrocortisone added could be converted to prednisolone within 24 h.The maximum productivity was 21.7 mg L^{-1} h^{-1}.

Table 5. Effect of cell mass on the bioconversion of hydrocortisone in aqueous /toluene two phase system.

Cell mass (g)	Prednisolone Yied %	Productivity
1	33	11.0
2	52	17.3
3	65	21.7

Conclusion

Biocatalyst in water /organic solvent two phase is a promising branch in the broad spectrum of biotechnology conversion, the organic phase must be highly insoluble in water to reduce the detrimental effects on the microbial cells. However, cell activity predictions based solely on log P value of the solvent may not be totally accurate.

A two –liquid-liquid phase system composed of 80% v/v phosphate buffer 7.4 and 20 % v/v toluene or n-hexane can be used for Δ^1- dehydrogenation of hydrocortisone .

Use of a two liquid phase system composed of 80 % v/v phosphate buffer pH 7.4 and 20 % v/v toluene will have the possibility of higher substrate and /or product concentration.

References

Adham, N.Z., Abd El –Hady, A. and Naim, N. (2003). Biochemical studies on the microbial Δ^1-dehydrogenation of cortisol by *Pseudomonas fluorescens*. Process Biochemistry 38: 897-902.

Angelova, B., Schmauder, H. P., (1999). Lipophilic compounds in biotechnology-interaction with cells and technological problems. *J. Biotechnol.;* 67: 13-32.

Bar, R. (1987). In bioctalysis in organic Media (Laane, C Tramper, J and Lilly M.D., eds) Elsevier, Amsterdam, 147-153.

Berrie, J. R., Williams, R.A.D., Smith, K.E. (1999). Microbial transformation of steroids -XI- progesterone transformation by *Streptomyces roseochromogenes*- purifications and characterization of the cytochrome P450 progesterone 16α-hydoxylase system. *J. steroid Biochem. Mol. Biol.* 71: 153-165.

Ceen, E.G., Dunnill, P. and Herrmann, J.P.R. (1988). Two –liquid phase reactor studies of 11α-hydroxylation of progesterone by *Aspergillus ochraceus* . *Biotechnol. Bioeng.* 31: 743-746.

Cho, T. and Schler, M.C. (1968). Multimembrane bioreactor for extractive fermentation. *Biotechnol. Prog.* 2: 53-60

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

Gordon, L. M., Sauerheber, R.D., Esgate, J. A., Dipple, I., Marchmont, R. J., Hoslay, M.D. (1980) The increase in bilayer fluidity of rat liver plasma membranes achieved by the local anesthetic benzyl alcohol effects the activity of intrinsic membrane enzymes. *J. Biol. Chem.* 255: 4519-4527.

Gruz, A., Fermandes, P., Cabral, J.M.S., Pinheiro, H. M. (2001). Whole –cell bioconversion of β-sitosterol in aqueous –organic two systems. *J. Mol Catal. Enzymatic* 11: 579-585.

Hocknull, M. D., Lilly, M.D., (1988) Stability of the steroid Δ^1-hdehydrogenation system of *Arthrobacter simplex* in organic solvent- two water –liquid phase environments. *Enzyme Microb. Technol.* 10: 669-674.

Hocknull, M. D., Lilly, M.D., (1987). The Δ-dehydrogenation of hydrocotisone by *Arthrobacter simplex* cells in organic aqueous two-liquid phase environments. In: Biocatalysis in Organic Media (Laane, C., Tramper, J. and Lilly, M.D.eds.) Elsevier, Amsterdam, 393-398.

Hocknull, M. D., Lilly, M.D., (1990). The use of free and immobilized *Arthrobacter simplex* in organic solvent/aqueous two liquid-phase reactors. *Appl. Microbiol. Biotechnol.* 33: 148-153.

Kloosterman, J. I. V., 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.

Kutney, J., Milanova, R. K., Vassiliev, C. D., Stefanov, S S, Nedelcheva N.V. (1999). Process for the microbial conversion of phytosterols to androstenedione and androstadienedione. WO Patent 9949075 .

Laane, C., Boeren, S. and Vos K. (1985). On optimizing organic solvents in mutli-fluid-liquid-phase biocatalysis. *Trend in Biotechnology* 3: 251-252

Laane, C., Boeren, S. and Vos K. (1987). Rules for optimization of biocatalysis in organic solvents *Biotechnol. Bioeng.* 30: 81-87.

Leon, R., Fermandes, P., Pinheiro, H. M., Cabral, J.M.S. (1998). Whole-cell biocatalysis in organic media. *Enzyme Microb. Technol.* 23: 483-500.

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

Mahato, S. B., Mukherjee, A. (1984). Steroid transformation by microorganisms, *Phytochemistry* 23: 2131-2154.

Martin, C.K.A., (1984) Biotechnology, Vol.6a, H.J. Rehm and G. Reed, Chapter 3, pp.79.

Naim, N., Adham, N.Z., Abd El-Rehim, H., El-hady, A. (2003) Prednisolone production using *Pseudomonas fluorescens* cells immobilized with polymer carrier produced by radiation polymerization, *Process biochemistry* 38: 1083-1089.

Naim, N., Adham, N. Z., El-Hady, A. (2002) Microbial dehydrogenation of cortisol. Bull. NRC, Egypt, 27, No., 4, pp:425-445.

Oda, S., Sugai, T., Ohta, H. (2001). Synthesis of methyl urodeoxycholate via microbial reduction of 7-ketolithocholate with *Eubacterium aerofaciens* JCM 7790 grown on two kinds of carbon and hydride source, glucose and mannitol. *J. Biosci. Bioeng.* 91: 178-183.

Pinheiro, H. M., Cabral, J. M. S., Adlercreutz, P. (1993). Quinones as external electron acceptors in steroid Δ^1-dehydrogenation with entrapped cells in organic medium. *Biocataysis* 7:83-96.

Ramelmeier, R. A. and Blanch, H. W. (1989). Mass transfer and cholesterol oxidase kinetics in a liquid two-phase system. *Biocatalysis* 2: 97-120.

Redikulstev, Y. V., Koshcheyenko, K.A., Skryabin, G. K., Livinenko, L. A., Borman, E. A., Sukhodolbskaya, G. V., (1995). Method of preparing prednisolone. Patent 1471561.

Riva, S. (1991). Enzymatic modification of steroids. In: applied biocatalysis (Blanch, H. W. and Clark, D.S., eds.) Marcel Dekker, Inc., Ny, 179-221.

Sallam, L.A.R, El-Abyad, M. S., El-Refai, A. H., EL-Menofi, H. A., Adham, N. Z. (1995). Bioconversion of 19 nortesrosterone by *Rhodococcus sp* DSM 92-344: Optimization of transformation paramteres. *Process Biochem.* 30(1): 25-34.

Schneider, H. (1968). Inhramembrane location of alcohol anesthetics *Biochim. Biophys. Acta*, 163:451-458 .

Srivastava, A., and Patil, S. (1995). Dependence of steroid 1 (2) –dehydrogenation on the C-17 side chain during cholesterol metabolism by immobilized *Mycobacterum fortuitum. World J. Microbiol Biotechnol.* 11: 284-286.

Van Sonsbeck, H.M., Beeftink, H.H., and Tramper, J.(1993). Two liquid-phase biorectors. *Enzyme Microb.Technol.* 15:723-729.

Received : 04.01.2005
Accepted : 28.02.2005