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Bioconversion of Hydrocortisone to Prednisolone by Immobilized Bacterial Cells in a Two-Liquid-Phase System

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Abstract: Bioconversion of hydrocortisone to prednisolone by free, immobilized and reused immobilized cells of three bacterial strains (*Bacillus sphaericus* ATCC 13805, *Bacillus sphaericus* SRP III and *Arthrobacter simplex* 6946) in an aqueous and a two-liquid-phase system using different organic solvents was investigated. The experiments were carried out in a 125 cm^3 shake flask at 27 $\pm$ 2°C, 220 rpm for 96 h. The contents of prednisolone and hydrocortisone in samples taken at 0, 3, 6, 24, 48, 72, 96 and 144 h were determined by HPLC analysis. The immobilized bacterial cells showed higher prednisolone yield than the free form in an aqueous system. In the two-phase systems, the butyl acetate to aqueous media ratio of 1 : 30 for all three bacterial strains in immobilized forms gave the highest prednisolone yields, at an incubation time of 144 h, of 87·6, 70·6 and 88·3% respectively. For an *n*-decane to aqueous ratio of 1 : 6, moderate prednisolone yields of 81·8, 47·9 and 71·4% were obtained with shorter incubation times of 72, 96 and 6 h respectively. For cyclohexane and other alcohols, the organisms produced low yields of prednisolone (0–30%). Single reuse of all three immobilized bacterial cells gave a 3–20% lower yield of prednisolone than the non-reused cells. The increase in hydrocortisone concentration decreased the prednisolone production whereas increasing the *n*-decane to aqueous ratio from 1 : 6 to 1 : 3 caused no significant change in the productivity. © 1998 Society of Chemical Industry

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Key words: bioconversion; hydrocortisone; prednisolone; *Bacillus sphaericus*; *Arthrobacter simplex*

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1 INTRODUCTION

One of the main problems in steroidal drug production by biotechnology is the solubility of precursors in the reaction mixtures. Many approaches have been developed in order to solve this problem. The use of water-immiscible organic solvents in the two-phase system can offer several potential advantages in biocatalysis by increasing the solubility of the substrates, thereby making it possible to operate at high substrate and product concentrations. Hocknull and Lilly have investigated various organic solvents which are not toxic to Gram-positive bacteria and can stabilize the Δ^1-dehydrogenase enzyme.[1] PVP (polyvinylpyrrolidone) has been used to increase the solubility of hydrocortisone in the liquid medium but the Δ^1-dehydrogenase activity in *Arthrobacter simplex* is also decreased.[2] It has been found that methanol (5–10% in aqueous media) decreased the activity of *Arthrobacter simplex*[3] especially at the water–organic solvent interface.[1,4] Methanol and ethanol can inhibit the enzymatic function of Δ^1-dehydrogenase.[5] However, this inhibition effect can be masked by immobilization of the cells. Free bacterial cells have been shown to tolerate methanol up to 0·05% whereas a tolerance of up to 10% in the immobilized forms has been observed.[5] Immobilization of *A. simplex* in calcium alginate can prolong the half-life of Δ^1-dehydrogenase activity from 17 to 24 h since immobilization of *A. simplex* in calcium alginate is a mild reaction with no problem of nutrient penetration into the beads.[1] Immobilized bacteria have been reused up to 69 times.[6] Other carriers which have been used to immobilize the bacteria are polyacrylamide hydrazide beads (PAAH),[7,8] polyacrylamide gel,[9,10] collagen,[11] thermally reversible hydrogel[12] and liposomal medium.[13]

The present study compared the effects on prednisolone yield of free and immobilized as well as the reused immobilized cells of *B. sphaericus* ATCC 13805 and *B. sphaericus* SRP III with *A. simplex* ATCC 6946 in an aqueous and a two-liquid-phase system using various organic solvents.

2 MATERIALS AND METHODS

2.1 Maintenance of microorganisms

Three strains of bacteria, *Bacillus sphaericus* ATCC 13805, *Bacillus sphaericus* SRP III (screened from soils in Chiang Mai, Thailand[14]) and *Arthrobacter simplex* ATCC 6946, which kept in mineral oil layered agar slants, were subcultured in TSA (Tryptic Soy Agar, Difco, USA) for 2 days. Each strain was grown in culture medium in shake flasks at 27 ± 2°C, 200 rpm for 48 h. The cultivation medium had the following composition (g dm^{-3}): yeast extract, 5; $(NH_4)_2SO_4$, 3·0; and $MgCl_2$, 2·0 in 50 mmol dm^{-3} Tris–HCl buffer, pH 7·8.

2.2 Immobilized cultures

A 5·0 cm^3 portion of the bacterial cell suspension was mixed with 0·020 g of sodium alginate. The bacteria–alginate mixture was then dispersed in 0·2 mol dm^{-3} $CaCl_2$ solution in 50 mmol dm^{-3} Tris–HCl buffer, pH 7·8. The resulting mixture was pumped through a needle (No. 18) into 0·2 mol dm^{-3} $CaCl_2$ in 50 mmol dm^{-3} Tris–HCl buffer, pH 7·8. The beads were collected and allowed to harden for 1–2 h at 27 ± 2°C prior to use.

The optimum bacterial cell concentration for immobilization was previously primarily determined at different time intervals by the plate count method. The bead samples were dissolved by incubating in 10 cm^3 of 0·20 mol dm^{-3} dipotassium hydrogen phosphate in 50 mmol dm^{-3} Tris–HCl buffer, pH 7·8 for 1 h.

2.3 Bioconversions

For aqueous systems, 30 mg of $CaCl_2$ was added to 29·0 cm^3 of the culture medium in a 125 cm^3 Erlenmeyer flask and left to equilibrate for 10 min at 27 ± 2°C with stirring (200 rpm). Hydrocortisone solution (15 mg in 1·0 cm^3 of 95% ethanol) was added, mixed well and equilibrated for 10 min. The free or immobilized bacterial cell suspension (5·0 cm^3) was added into the mixture and samples (0·5 cm^3) withdrawn at 0, 3, 6, 24, 48, 72, 96 and 144 h.

For a two-phase system, the cultivation reaction and conditions were the same as the aqueous system except that varying amounts of hydrocortisone were dissolved in different organic solvents before being added to the reaction mixture.

In the experiments with reused immobilized bacterial cells, the used immobilized bacterial beads were rinsed aseptically and used again in the reaction mixture; the procedure was the same as the two-phase system described above.

2.4 HPLC analysis

Samples (0·5 cm^3) taken from the cultivation reaction were extracted with 2·0 cm^3 of chloroform by vortex mixing for 30 s. A portion (0·5 cm^3) of the chloroform phase was transferred to a sampling vial. Hydrocortisone and prednisolone levels were determined by high performance liquid chromatography (HPLC, Thermo Separation Products Inc., California, USA) at 254 nm. Each sample (5 mm^3) was analysed on a 250 × 4·6 mm Spherisorb 5 Sil column employing chloroform/methanol/acetic acid (90 : 10 : 0·5 by volume) as the

mobile phase at a flow rate of 0·70 cm^3 min^{-1}. The standard steroid compounds used were of reference grade (Sigma Co., USA) and all other chemicals were of reagent grade. Hydrocortisone and prednisolone gave separations with retention times of 8·3 and 8·8 min respectively. No other compounds in the media samples were detected.

3 RESULTS AND DISCUSSION

3.1 Optimum amount of bacterial cells

The optimum concentrations of bacteria producing the highest yield of prednisolone are shown in Table 1. The starting bacterial concentrations that gave maximum prednisolone yield were 2×10^5, 18×10^5 and 4×10^6 cell cm^{-3} for *B. sphaericus* ATCC 13805. *B. sphaericus* SRP III and *A. simplex* ATCC 6946 respectively. *B. sphaericus* ATCC 13805 (2×10^5 cell cm^{-3}) gave the highest prednisolone yield of 92·1% at 48 h. These concentrations were used for the further experiments.

3.2 Bioconversions

Tables 2 and 3 compare the prednisolone yield of free and immobilized bacteria between the two aqueous systems. Method 1 involved the concomitant addition of hydrocortisone with a 2-day cultivation of immobilized or free bacterial cells. Method 2 involved the sequential addition of hydrocortisone after a 2-day cultivation of immobilized or free bacterial cells. Method 1 appeared to be the preferable procedure since it gave higher yields of prednisolone in both free and immobilized cells. Therefore, this method was used for two-phase experiments. *B. sphaericus* ATCC 13805 and *B. sphaericus* SRP III in immobilized forms give yields of prednisolone about 5–15% higher than the free bacteria whereas *A. simplex* ATCC 6946 showed no significant difference between free and immobilized bacteria.

For two-phase systems, the organic solvents used were cyclohexane, amyl alcohol, butyl acetate, lauryl alcohol, *n*-decane and *n*-decyl alcohol at various aqueous ratios. The effects of organic solvent to aqueous ratios on the maximum yield of prednisolone in the two-phase system using *B. sphaericus* ATCC 13805, *B. sphaericus* SRP III and *A. simplex* ATCC 6946 are presented in Tables 4, 5 and 6 respectively. Immobilized bacteria gave a higher prednisolone yield than the free form in solvent/aqueous ratios of butyl acetate/aqueous = 1 : 30 and of *n*-decane/aqueous = 1 : 6 for *B. sphaericus* ATCC 13805 and of *n*-decane/aqueous = 1 : 6 for *B. sphaericus* SRP III. Prednisolone production appeared to depend on the types and ratios of the organic solvents. It was observed that an *n*-decane to aqueous ratio of 1 : 6 for the immobilized *B. sphaericus* ATCC 13805 cells and the free *A. simplex* 6946 cells gave the maximum prednisolone yields of 81·80 and 84·40% at 48 and 6 h respectively. For butyl acetate at an organic solvent to aqueous ratio of 1 : 30, immobilized *B. sphaericus* ATCC 13805, *B. sphaericus* SRP III and *A. simplex* 6946 showed maximum prednisolone yields of 87·60, 70·60 and 88·30% respectively, but with longer incubation times than the *n*-decane solvent of 144, 120 and 144 h respectively. The *n*-decane solvent system seemed to be the best system because of the shorter incubation period at which maximum yield was observed. The *n*-decane solvent is one of the alkanes group which has a log *P* value (the logarithm of water to *n*-decane partition coefficient) of equal to or more than 3·6, which will not be toxic to the activity of the bacterial enzyme Δ^1-dehydrogenase,[15] especially for the Gram-positive bacteria.[1] The solubility of *n*-decane in water is quite low. Therefore, the activity of the enzyme is destroyed only at the solvent–water interface.

In cyclohexane or other alcohol systems, prednisolone production was quite low, 0–30%. Cyclohexane

TABLE 1
Comparison of Optimum Immobilized Bacterial Cell Concentrations Producing the Highest Prednisolone Yield in an Aqueous Phase System in Time Courses Studied at 0, 4, 24, 48, 72 and 96 h

Bacterial strains	*Starting bacterial cell concentration studied* (*cell* cm^{-3})	*Conditions at maximum prednisolone yield* (%)		
		Bacterial cell concentration (*cell* cm^{-3})	*Time* (*h*)	*Maximum prednisolone yield observed* (% ± *SD*)
Bacillus sphaericus ATCC 13805	2, 4, 6 and 8×10^5	2×10^5	48	92·1 ± 2·2
Bacillus sphaericus SRP III	6, 12, 18 and 24×10^5	18×10^5	96	83·5 ± 1·3
Arthrobacter simplex ATCC 6946	4, 8, 12 and 16×10^6	4×10^6	72	78·8 ± 1·9

$$\text{Prednisolone yield} = \frac{\text{prednisolone conc. in media at time '}t\text{'} \times 100}{\text{initial hydrocortisone conc.}}$$

TABLE 2

Time Courses of Bioconversion of Hydrocortisone to Prednisolone of *Method 1* by Free and Immobilized Bacterial Cells in the Aqueous System at which the Starting Cell Concentrations gave the Highest Yield (% ± SD) of Prednisolone

Time (*h*)	B. sphaericus *ATCC 13805* (% ± *SD at* 2×10^5 *cell* cm^{-3})				B. sphaericus *SRP III* (% ± *SD at* 14×10^5 *cell* cm^{-3})				A. simplex *ATCC 6946* (% ± *SD at* 2×10^6 *cell* cm^{-3})			
	Free		*Immobilized*		*Free*		*Immobilized*		*Free*		*Immobilized*	
	H	*P*	*H*	*P*	*H*	*P*	*H*	*P*	*H*	*P*	*H*	*P*
0	95·8 ± 1·1	0·0	95·9 ± 2·0	0·0	95·9 ± 2·5	0·0	88·1 ± 2·7	0·0	88·3 ± 2·0	0	92·6 ± 1·7	0·0
4	94·0 ± 2·0	0·0	94·6 ± 1·1	0·0	89·2 ± 1·6	0·0	82·9 ± 1·7	0·0	83·3 ± 2·8	5·8 ± 2·2	86·2 ± 1·24	5·9 ± 1·6
24	41·0 ± 1·4	57·1 ± 1·0	75·6 ± 1·4	34·5 ± 1·6	89·1 ± 2·6	0·0	80·0 ± 2·5	0·0	77·8 ± 1·0	11·5 ± 1·45	82·4 ± 1·0	9·8 ± 1·2
48	6·2 ± 1·4	92·1 ± 1·2	5·3 ± 1·8	96·3 ± 1·5	76·2 ± 1·0	17·5 ± 1·3	76·6 ± 1·3	13·6 ± 1·2	12·3 ± 1·6	83·6 ± 1·80	44·6 ± 1·9	41·6 ± 1·1
72	4·8 ± 1·3	83·4 ± 1·0	4·1 ± 2·0	94·2 ± 1·6	45·1 ± 1·1	44·6 ± 1·6	56·1 ± 1·7	34·0 ± 1·5	0·0	65·3 ± 1·5	0·0	67·7 ± 1·2
96	6·4 ± 1·6	68·9 ± 1·2	3·7 ± 1·2	72·4 ± 1·3	5·5 ± 1·27	78·9 ± 1·9	38 ± 1·7	89·6 ± 1·8	0·0	33·3 ± 1·4	0·0	75·3 ± 1·7

Method 1 involved adding hydrocortisone and the bacterial cells concomitantly.

$$\% \text{ yield of prednisolone or hydrocortisone} = \frac{\text{prednisolone or hydrocortisone conc. in media at time '}t\text{'} \times 100}{\text{initial hydrocortisone conc.}}$$

H, Hydrocortisone; P, prednisolone.

TABLE 3

Time Courses of Bioconversion of Hydrocortisone to Prednisolone of *Method 2* by Free and Immobilized Bacterial Cells in the Aqueous System at which the Starting Cells Concentrations gave the Highest Yield (% ± SD) of Prednisolone

Time (*h*)	B. sphaericus *ATCC 13805* (% ± *SD at* 6×10^5 *cell* cm^{-3})				B. sphaericus *SRP III* (% ± *SD at* 26×10^5 *cell* cm^{-3})				A. simplex *ATCC 6946* (% ± *SD at* 4×10^6 *cell* cm^{-3})			
	Free		*Immobilized*		*Free*		*Immobilized*		*Free*		*Immobilized*	
	H	*P*	*H*	*P*	*H*	*P*	*H*	*P*	*H*	*P*	*H*	*P*
0	95·6 ± 1·9	0·0	92·3 ± 1·7	0·0	93·2 ± 1·4	0·0	91·1 ± 1·5	0·0	95·7 ± 1·6	0·0	92·3 ± 1·5	0·0
2	78·6 ± 1·5	0·0	81·0 ± 2·0	0·0	84·0 ± 2·1	0·0	86·3 ± 1·9	0·0	33·9 ± 1·2	52·8 ± 2·0	72·6 ± 1·7	40·2 ± 2·0
6	43·8 ± 1·5	12·5 ± 1·3	67·9 ± 1·9	21·1 ± 1·7	80·5 ± 1·8	13·2 ± 2·2	80·7 ± 2·1	5·7 ± 1·6	4·4 ± 1·7	74·2 ± 1·3	53·3 ± 1·6	75·2 ± 1·5
24	50·9 ± 1·3	34·0 ± 1·3	54·4 ± 1·7	43·3 ± 2·1	58·7 ± 1·7	40·6 ± 1·9	62·9 ± 2·2	21·5 ± 1·9	4·4 ± 1·3	48·5 ± 1·3	8·2 ± 2·0	40·8 ± 1·7
48	19·5 ± 1·7	72·3 ± 2·0	9·6 ± 1·2	91·8 ± 1·4	45·6 ± 1·4	65·8 ± 1·4	39·9 ± 1·5	51·3 ± 1·6	3·5 ± 1·8	14·9 ± 1·4	5·3 ± 2·1	3·5 ± 1·3
72	5·7 ± 1·9	84·7 ± 1·6	4·7 ± 1·7	86·9 ± 1·6	22·4 ± 2·0	55·5 ± 1·2	15·2 ± 2·0	80·1 ± 2·1	3·1 ± 1·6	0·0	0·0	0·0
96	5·3 ± 1·9	63·7 ± 1·9	3·2 ± 2·0	68·7 ± 1·7	9·8 ± 2·1	49·1 ± 2·0	7·7 ± 1·3	68·4 ± 1·8	0·0	0·0	0·0	0·0

Method 2 involved culturing the free or immobilized cells for 2 days before the addition of hydrocortisone.
H, hydrocortisone; P, prednisolone.

TABLE 4
Effects of Organic Solvents/Aqueous Ratios on the Maximum Yield of Prednisolone Using Free and Immobilized *B. sphaericus* ATCC 13805 Bacteria in the Two-Phase System

Solvents	*Ratio (organic solvent/aqueous)*	*Maximum yield of prednisolone*			
		Free bacteria		*Immobilized bacteria*	
		Time (h)	%	*Time (h)*	%
Water	—	72	84·7	48	91·8
Cyclohexane	1 : 30	—	0·0	—	0·0
	1 : 6	—	0·0	—	0·0
Amyl alcohol	1 : 6	144	4·7	—	0·0
Butyl acetate	1 : 30	144	86·8	144	87·6
	1 : 6	—	—	6	3·4
Lauryl alcohol	1 : 6	4	4·0	—	0·0
n-Decane	1 : 6	72	71·9	48	81·8
n-Decyl alcohol	1 : 6	4	7·3	—	0·0

TABLE 5
Effects of Organic Solvents/Aqueous Ratios on the Maximum Yield of Prednisolone Using Free and Immobilized *B. sphaericus* SRP III Cells in the Two-Phase System

Solvents	*Ratio (organic solvent/aqueous)*	*Maximum yield of prednisolone*			
		Free bacteria		*Immobilized bacteria*	
		Time (h)	%	*Time (h)*	%
Water	—	48	65·8	48	80·1
Cyclohexane	1 : 30	—	0·0	—	0·0
	1 : 6	—	0·0	—	0·0
Amyl alcohol	1 : 6	—	0·0	—	0·0
Butyl acetate	1 : 30	144	94·6	120	70·6
	1 : 6	—	—	72	4·1
Lauryl alcohol	1 : 6	—	0·0	—	0·0
n-Decane	1 : 6	96	19·2	96	47·9
n-Decyl alcohol	1 : 6	—	0·0	—	0·0

TABLE 6
Effects of Organic Solvents/Aqueous Ratios on the Maximum Yield of Prednisolone Using Free and Immobilized *A. simplex* ATCC 6946 in the Two-Phase System

Solvents	*Ratio (organic solvent/aqueous)*	*Maximum yield of prednisolone*			
		Free bacteria		*Immobilized bacteria*	
		Time (h)	%	*Time (h)*	%
Water	–	6	74·2	6	75·2
Cyclohexane	1 : 30	—	0·0	—	0·0
	1 : 6	—	0·0	—	0·0
Amyl alcohol	1 : 6	168	6·7	—	0·0
Butyl acetate	1 : 30	144	90·2	144	88·3
	1 : 6	—	—	72	3·4
Lauryl alcohol	1 : 6	72	4·2	24	26·0
n-Decane	1 : 6	6	84·4	6	71·4
n-Decyl alcohol	1 : 6	—	0·0	—	0·0

and other alcohol systems are more polar solvents than *n*-decane and their log *P* values are expected to be equal to or less than 3·4. When these solvents, especially alcohols, are soluble in water, they can be toxic to bacteria by limiting the bacterial nutrients. Thus, the effect of bacterial activity in prednisolone productivity is related to the log *P* value of these solvents. It has been previously reported that the log *P* value which gives the highest yield of dehydrogenation products is 4·0.[1]

The organic solvent to aqueous ratios of *n*-decane (1 : 6) and butyl acetate (1 : 30) were further used for studies on the reuse of immobilized bacteria. Table 7 compares effects of one reuse on prednisolone yield in the aqueous system and the two-phase system when *n*-decane and butyl acetate were used. The results show that a lower yield of prednisolone (3–20%) was obtained when the immobilized bacterial beads were reused. The doubling of substrate concentration (i.e. hydrocortisone) from 15 mg to 30 mg lowered prednisolone production by a factor of about 2 since using a higher hydrocortisone concentration than the optimum may inhibit the propagation of the bacterial cells, thereby decreasing the prednisolone production. Changing the *n*-decane to aqueous ratio from 1 : 6 to 1 : 3 gave no significant changes for *B. sphaericus* ATCC 13805 and *B. sphaericus* SRP III but gave a decreased yield of about 19% for *A. simplex* ATCC 6946, as shown in Table 8.

4 CONCLUSION

In comparing the selected three bacterial strains (both free and immobilized forms) in an aqueous and a two-phase system, it was found that the starting bacterial concentrations in the aqueous and the two-phase system affected the production of prednisolone differently. Immobilization of the bacterial cells using calcium alginate may not be able to prevent the leakage of the cells, hence the active bacterial cells were both inside and outside the beads. In comparing the three immobilized bacterial cells, the *B. sphaericus* ATCC 13805 and *B. sphaericus* SRP III gave about a 5–30% higher prednisolone yield in the aqueous phase than in the two-phase system, whereas *A. simplex* ATCC 6946

TABLE 7
Comparison of the Reuse of Immobilized Bacterial Cells on Maximum Yield of Prednisolone by the Three Bacterial Strains in the Aqueous Phase and the Two-Phase System

System	*Numbers of reuses*	*Maximum yield of prednisolone*					
		B. sphaericus *ATCC 13805*		B. sphaericus *SRP III*		A. simplex *ATCC 6946*	
		Time (*days*)	%	*Time* (*days*)	%	*Time* (*days*)	%
Aqueous	0	2	91·8	3	80·1	0·25	75·2
	1	3	75·9	3	71·1	0·2	70·1
Two-phase							
Butyl acetate/H_2O = 1 : 30	0	6	87·6	5	70·6	6	88·3
	1	6	86·6	6	78·0	6	75·8
n-Decane/H_2O = 1 : 6	0	2	81·8	4	47·9	0·25	71·4
	1	2	95·0	3	44·2	1	58·1

TABLE 8
Effects of Hydrocortisone Concentrations and the *n*-Decane to Aqueous Ratios on Maximum Yield of Prednisolone

Hydrocortisone (*mg*)	*Ratio* (*by volume*)	*Maximum yield of prednisolone*		
		B. sphaericus *ATCC 13805*	B. sphaericus *SRP III*	A. simplex *ATCC 6946*
30·0	1 : 3	41·9	26·9	47·8
30·0	1 : 6	46·1	21·7	67·1
15·0	1 : 6	81·8	47·9	71·4
7·5	1 : 6	96·8	77·1	65·9

gave lower activity in the aqueous phase than in the two-phase system. The best solvent system in the two-step phase for all immobilized bacterial cells was an *n*-decane to aqueous ratio of 1 : 6 since this system produced moderate prednisolone yield with a short incubation time. A butyl acetate to aqueous ratio of 1 : 30 gave the highest yield but took too long to reach maximum prednisolone production.

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