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Microbial transformation of hydrophobic compound in cloud point system

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Abstract

Microbial transformations of hydrophobic compounds are often constrained by practical difficulties, such as substrate solubility and product inhibition. A novel approach, microbial transformation in cloud point system (CPS), was developed. The system could provide microbial cells with aqueous environment and could dissolve substrate and products in surfactant phase. A microbial transformation of cholesterol to androst-1,4-diene-3,17-dione (ADD) and androst-4-ene-3,17-dione (4-AD) has been carried out in the system consisting of nonionic surfactants Triton X-100 and Triton X-114, which is more effective than the microbial transformation in conventional media. The biocompatibility and bioavailability in the cloud point system were investigated by determination of solubilization of surfactant phase and observation of dilute phase and coacervate phase under microscopy. The system parameters of microbial transformation were optimized. It indicated that CPS has the potential capability to be utilized as an effective microbial transformation medium.
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Keywords: Microbial transformation; Hydrophobic compound; Nonionic surfactant; Cloud point system; Solubilization; Parameters optimization

1. Introduction

Microbial transformations of hydrophobic compounds are often hindered by two obstacles [1]: limited substrate accessibility to microorganism because of the low aqueous solubility of most organics, and inhibition or toxicity of both substrate and product exerted upon the microorganism. This also applies to removal of hazardous pollutants via biodegradation [2]. Medium engineering is an attempt to alleviate or overcome these problems by adding different kinds of inherently biocompatible and non-biodegradable ingredients into the essentially aqueous medium to form various types of microbial transformation medium. Many medium systems, such as aqueous organic two-phase system [3], aqueous two-phase polymer system [4], liposome medium [5], direct micelle system [6], water-in-oil microemulsion or reverse micelle system [7,8], have been reported. Although aqueous organic two-phase and aqueous two-phase polymer systems have been studied extensively, examples of application on an industrial scale are still scarce [9,10].

When an aqueous micelle solution of a nonionic surfactant is at a temperature above its cloud point (CP) or in the presence of certain additives, phase separation occurs to form a surfactant diluted phase and a surfactant-rich phase or coacervate phase. Such a system is called a cloud point system (CPS) and has been used in separation technology as cloud point extraction (CPE) [11], but has not been previously applied in microbial transformations. The system is attractive because it provides a separation method which is easy to manipulate, reliable to scale up, simple and effective to operate. Especially, it provides a mild environment so that cells or proteins will not be damaged and will partition in the phases depending on the properties of the phase system and the partitioned substance [12,13]. In microbial transformation in a CPS, the potential toxicity or inhibition effects may be reduced and the biocompatibility may be increased. Moreover, CPS offers the possibility for replacing the tedious mechanical separation of cells from products with an extraction process. Surfactants are known to increase the apparent aqueous solubility of hydrophobic compounds (often known as solubilization) and may be used to enhance the bioavailability and stimulate microbial transformation [14,15].

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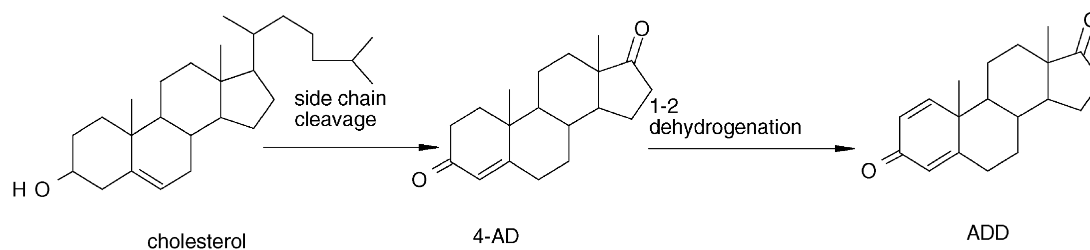

Fig. 1. The pathway of microbial transformation of cholesterol to ADD and 4-AD.

The pathway of side-chain cleavage of cholesterol to androst-1,4-diene-3,17-dione (ADD) and androst-4-ene-3,17-dione (4-AD) by *Mycobacterium* sp. is shown in Fig. 1. The mole ratio of ADD to 4-AD is about 10 [16]. Microbial transformations of steroids are severely limited by two obstacles. First, the aqueous solubility of steroids mostly ranges from 0.01 to 0.1 mM while that of sterols, such as cholesterol, is often below 1 μM [17]. Second, the substrate and product are toxic to the microorganism [18].

In this paper, a novel approach, microbial transformation of cholesterol to ADD by *mycobacterium* sp. in CPS, was developed. The parameters of the microbial transformation were optimized. The biocompatibility and bioavailability in this novel system are discussed.

2. Experiment

2.1. Materials

Fourteen kinds of nonionic surfactants, belonging to three main classes, were chosen for this study. The basic properties of them are shown in Table 1. Polyoxyethylene alcohols (Brij 30, Brij 35, Fluka; Brij 56, $C_{12}E_7$, Shanghai Surfactant Factory) were used because Brij 35 has facilitating effects on microbial transformation of sterol to ADD in aqueous two-phase system by *Mycobacterium* sp. [16]. Polyoxyethylene sorbitan fatty acid esters (Span 20, Span 40, Span 60, Span 80, Tween 20, Tween 40, Tween 60, Tween 80, Shanghai Agent Co. Ltd.) were chosen because Tween 80 has been applied widely in microbial transformation studies [19]. Alkylphenol ethoxylates (Triton X-100, Shanghai Agent Co. Ltd.; Triton X-114, Fluka) were chosen because they were reported with good effect on sediment biodegradation study [15] and are usually used in biotechnology [20].

2.2. Microorganism culture and microbial transformation

Microorganism strain *Mycobacterium* sp. NRRL B-3683 was used in our study. The stock culture was on agar slants consisting of 0.5 g yeast extract, 1.2 g agar powder, 1 g glycerol, 0.05 g K_2HPO_4, 0.1 g NH_4Cl, and 0.05 g $MgSO_4{\cdot}7H_2O$ in 100 ml of water. The activation culture medium consisted of 0.5 g $(NH_4)_2SO_4$, 0.45 g Na_2HPO_4, 0.34 g KH_2PO_4, 0.05 g $MgSO_4{\cdot}7H_2O$, 1.0 g glycerol,

Table 1
Basic properties of some nonionic surfactants

Nonionic surfactant	General structure*	Hydrophobic group	CMC (mM)	HLB	CP (°C)
Polyoxyethylene alcohols					
Brij 30	$C_{12}E_4$	Dodecanol	0.02–0.06	9.5	4
–	$C_{12}E_7$	Dodecanol	0.07	12.5	65
Brij 35	$C_{12}E_{23}$	Dodecanol	0.09	16.9	>100
Brij 56	$C_{16}E_{10}$	Blubber		12.9	64–69
Polyoxyethylene sorbitan fatty acid esters					
Span 20	$C_{12}S_6$	Lauric acid		8.6	
Span 40	$C_{16}S_6$	Palmitic acid		6.7	
Span 60	$C_{18}S_6$	Stearic acid		4.7	
Span 80	$C_{18}S_6$	Oleic acid		4.3	
Tween 20	$C_{12}S_6E_{20}$	Lauric acid	0.04–0.06	16.7	
Tween 40	$C_{16}S_6E_{20}$	Palmitic acid	29[a]	15.6	
Tween 60	$C_{18}S_6E_{20}$	Stearic acid	27[a]	14.9	
Tween 80	$C_{18}S_6E_{20}$	Oleic acid	0.01–0.02	15	
Alkylphenol ethoxylates					
Triton X-100	$C_8\Phi E_{9-10}$	Octylphenol	0.2	13.5	64
Triton X-114	$C_8\Phi E_{7-8}$	Octylphenol	0.3	12.8	22

E_n: number of ethylene oxide group; S_6: sorbitan ring; Φ: phenol ring.
* C_n: number of carbons in alkyl chain.
[a] mg/l.

0.2 g cholesterol, and 0.2 g Triton X-100 in 100 ml of water. The transformation culture medium consisted of 1.0 g $(NH_4)_2SO_4$, 0.45 g Na_2HPO_4, 0.34 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.5 g cholesterol, and a certain amount of nonionic surfactant in 100 ml of water.

The microorganism was grown aerobically at 28 °C in two steps, i.e. activation culture for 3 days and transformation culture for 7 days. After activation culture, 2 ml of culture liquid was withdrawn and added to 20 ml of transformation culture medium in a 250 ml Erlenmeyer flask, which was then shaken at 220 r/min for transformation. Some of the well-mixed transformation culture broth was withdrawn for analysis. The microorganism growth and cholesterol transformation were fulfilled during transformation culture simultaneously.

2.3. Analytical methods

One milliliter of sample was withdrawn from culture broth and extracted by 4 ml of methanol for 2 h. After centrifugation, 0.8 ml of supernatant was taken for HPLC analysis. Steroids were determined by HPLC on Hypersil C_{18} column using methanol:water (80:20) as mobile phase and 0.7 ml/min flow rate, detected at 254 nm. Retention times of ADD and 4-AD were 4.8 and 6.2 min, respectively.

TLC analysis was performed to determine the distribution of substrate and products in CPS. Samples were spotted on Silica gel 60 F_{254} (Merck) high-performance thin layer chromatography plates, run in chloroform:ether (1:3) solvent system and stained by phosphomolybdic acid solution (1% (w/v)). The R_f values of cholesterol, 4-AD and ADD were 0.62, 0.47, and 0.42, respectively.

2.4. Cloud point determination

A glass tube containing sample was placed in a thermo-regulated device. The temperature of sample solution was raised in small increments until the solution became cloudy. Then, the temperature was lowered by small step until the cloud disappeared. The cloud point was determined as the average of abovementioned two temperature values [13].

2.5. Solubilization determination

A series of surfactant solutions were prepared with excessive ADD and different weight ratio of Triton X-100 to Triton X-114. Each 10 ml of solution was placed in a vial and was shaken at 220 r/min and 28 °C for 72 h. Then, the treated solutions were filtrated with a filter (20 μm pore-size) and analyzed by HPLC.

3. Results and discussion

3.1. Screening of nonionic surfactant

For selecting appropriate medium of microbial transformation, many factors, such as biocompatibility, solubilization and non-biodegradability, should be considered [2]. The biocompatibility or potential toxicity of surfactant to microorganism is most important. It can be demonstrated by measuring the product (ADD) accumulation in surfactant-amended transformation culture [21]. Fig. 2 shows the final ADD concentration after 7-day microbial transformation culture with different kinds and concentrations of surfactants. When the transformation culture medium did not contain surfactant, the final ADD concentration was very low (20–80 mg/l), which was not shown in Fig. 2. When surfactant concentration was high, most surfactants were toxic to the microorganism. Only Triton X-114 was biocompatible with the microorganism under the condition of microbial transformation culture. Although

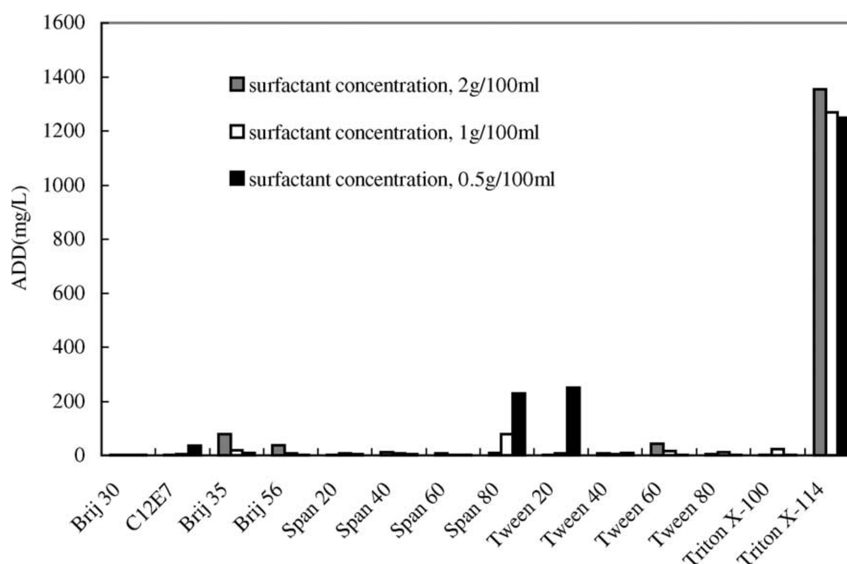

Fig. 2. ADD production in surfactant-amended transformation culture.

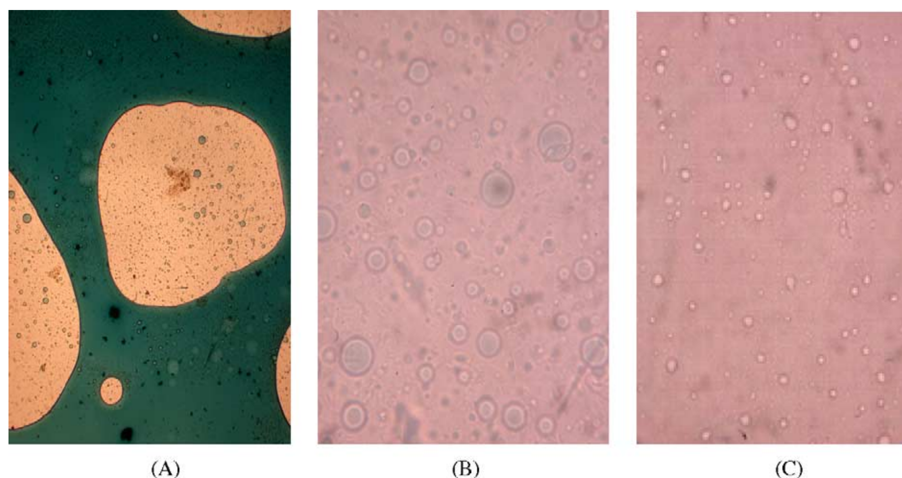

Fig. 3. Microscopic observation of dilute phase and coacervate phase. A: coacervate phase, water-in-oil emulsion (40×); B: large water vesicle in coacervate phase, oil-in-water emulsion (400×); C: dilute phase, oil-in-water emulsion (400×).

Triton X-100 is in the same class with Triton X-114, it was incompatible with the microorganism. Brij 30 was also incompatible to the microorganism. This surfactant may be incorporated into membrane lipids, which affects microbial surface structure, composition, properties, and functions [22]. On the other hand, the membrane lipids can be extracted from cells to form lipid-surfactant complexes, affecting the surfactant properties. Thus, the interaction between surfactants and microorganism becomes complicated.

3.2. Characteristics of CPS

After phase separation, CPS can be divided into dilute phase and coacervate phase. The two phases may be observed under microscopy after staining with oil-soluble dye Sudan black B [23]. The photographs are shown in Fig. 3. In the dilute phase, an oil-in-water microemulsion was formed. The small surfactant drops or micelles were visualized as dark spots (Fig. 3C). In the coacervate phase, water-in-oil microemulsion was formed. The dark background showed the continuous surfactant phase, which contained most substrate, ADD and 4-AD, acted as a substrate reservoir and product extractant to eliminate transformation inhibition by products and protect products from degradation (Fig. 3A). The large water vesicles in the coacervate phase acted as microreactors and retained most cells, where they could be sheltered from detrimental effects of surfactant. In large water vesicles, oil-in-water microemulsion could be visualized, which was similar to that in dilute phase (Fig. 3B). Mass transfer took place between two surfactant oil drops, two large water vesicles, or between the surfactant phase and the aqueous phase. Upon collision of two drops, their coalescence occurred through a transient dimer, which permitted a rapid mass exchange [24]. Thereby the bioavailibility of hydrophobic substrate was enhanced.

The distribution of substrate and products in CPS was demonstrated by TLC as shown in Fig. 4. Both substrate and products were concentrated in the coacervate phase. The partition coefficient of microorganism cell between coacervate phase and dilute phase was about 10, which was consistent with the report that *Mycobacterium* is a hydrophobic microorganism [22]. The substrate and cells tended to distribute in the coacervate phase, which enhanced the bioavailability. The microorganism growing in the two-phase organic system had a strong affinity for the immiscible liquid. The further colonization of microorganism on the surface of droplets had also been reported [25]. The microbial transformation can take place not only in the aqueous phase where substrate dissolves or diffuses, but also on the interface between surfactant and water, where the microorganism cells grow as a bio-membrane covering the interface of aqueous phase droplets, consuming the substrate from the surfactant phase reservoir [2].

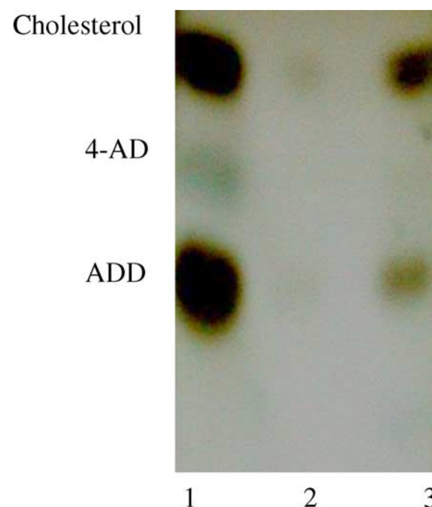

Fig. 4. TLC of substrate and products in CPS. 1: standard sample; 2: dilute phase; 3: coacervate phase.

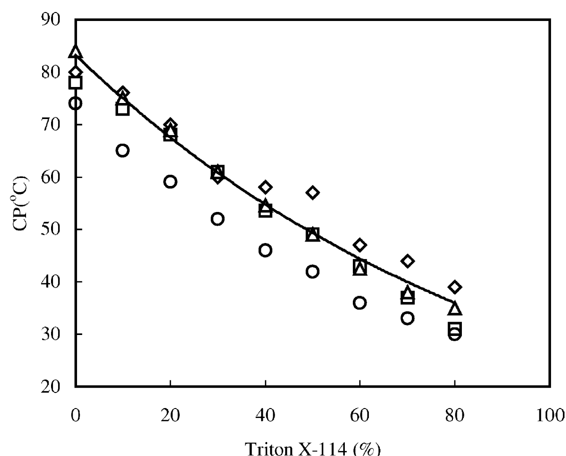

Fig. 5. CP of mixture system. The 2.0 g nonionic surfactant in 100 ml of water (□); 1.0 g $(NH_4)_2SO_4$, 0.45 g Na_2HPO_4, 0.34 g KH_2PO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 2.0 g nonionic surfactant in 100 ml of water (◇); 1.0 g$(NH_4)_2SO_4$, 0.45 g Na_2HPO_4, 0.34 g KH_2PO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.1 g cholesterol, 2.0 g nonionic surfactant in 100 ml of water (△); 1.0 g $(NH_4)_2SO_4$, 0.45 g Na_2HPO_4, 0.34 g KH_2PO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.1 g ADD, and 2.0 g nonionic surfactant in 100 ml of water (O).

3.3. Parameters optimization

3.3.1. Enhancement of solubilization in CPS

As shown in Fig. 2, only Triton X-114 aqueous solution, with CP below the temperature of microbial transformation culture, was biocompatible with the microorganism. The CP and the solubilization of Triton X-114 solution can be adjusted by addition of another surfactant to form a mixed surfactant solution. In order to enhance the solubilization of CPS, Triton X-100, which is in the same series as Triton X-114, was chosen to form a mixed system. The CP of this system with a weight fraction of Triton X-114 below 80% was higher than the temperature of the microbial transformation culture (28 °C), as shown in Fig. 5. Salts and cholesterol had little influence on the CP. However, ADD can decrease the CP.

The solubilization of some mixed surfactant solutions with different weight ratios of Triton X-114 to Triton X-100 is shown in Fig. 6. The total surfactant concentration in each solution was 2 g/100 ml. The solubilization of Triton X-100 alone is higher than that of Triton X-114. The solubilization in mixed surfactant micelle solution decreased with increasing weight ratio of Triton X-114 until the Triton X-114 content was 40%, then increased and reached the maximum value at 70% Triton X-114. The solubilization of surfactant micelle solution is strongly affected by its CP. At the temperature near the CP of the surfactant solution, the solubilization of surfactant solution increased drastically [26]. The amount of solute associated with a mole of surfactant in coacervate phase was much higher than that in dilute phase [27]. At the Triton X-114 weight ratio below 50%, no phase separation occurred and the final ADD concentration was lower than that in pure Triton X-114 system. When the Triton X-114 weight ratio was higher than 50%, phase separation appeared and the final ADD concentration was higher, the change trend of which was consistent with the change of solubilization. These results indicated that two-phase CPS was the prerequisite for microbial transformation in high concentration surfactant solutions.

3.3.2. Time course of microbial transformation reaction

The time course of microbial transformation was studied at substrate concentration of 1.5 g/100 ml and surfactant concentration of 4 g/100 ml. The weight ratio of Triton X-114 to Triton X-100 was 1:1 in all subsequent studies. Fig. 7 indicates that the final product concentration reached a maximum on the Day 7. When the transformation time was prolonged, the final product concentration decreased. The reason may be the degradation of product by the microorganism.

3.3.3. Evaluation of substrate/surfactant concentration

The effect of mixed surfactant concentration was investigated at mixture surfactant concentrations ranging from 0.5

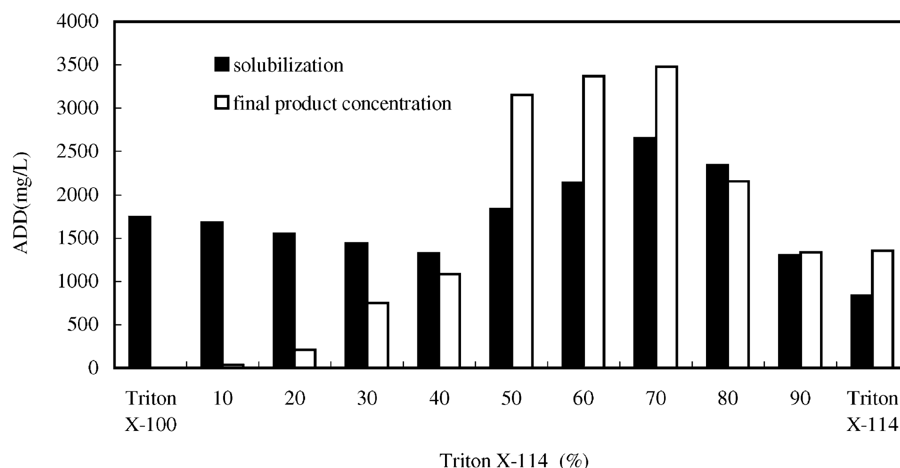

Fig. 6. Change of solubilization and final product concentration with fraction ratio of mixed surfactant system.

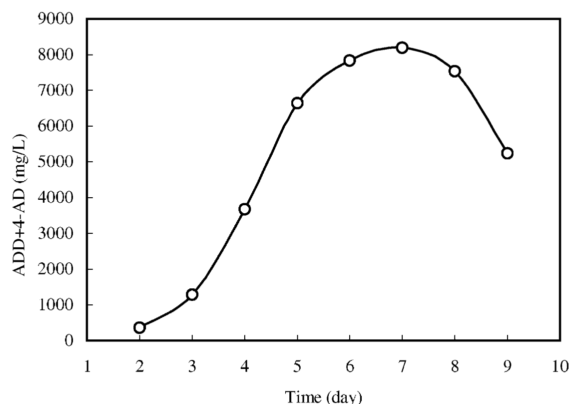

Fig. 7. Time course of microbial transformation of cholesterol to ADD and 4-AD.

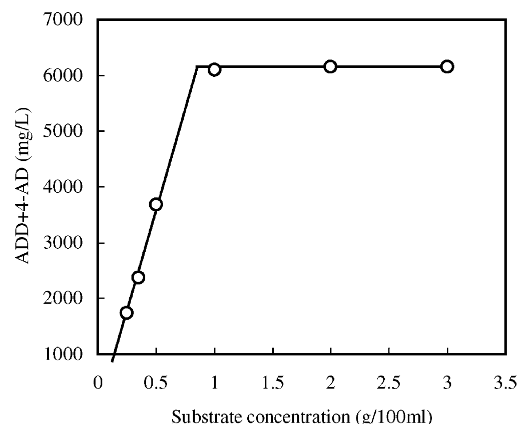

Fig. 9. Change of final product concentration with substrate concentration.

to 4.0 g/100 ml with substrate concentration of 0.5 g/100 ml. The results are shown in Fig. 8. At low surfactant concentration, the final product concentration steadily increased with increasing surfactant concentration, which may be a result of solubilization of surfactant. However, at high surfactant concentration, the final product concentration decreased with increasing surfactant concentration. Only in the proceeding of microbial transformation, ADD would induce the CP of surfactant micelle solution below the temperature of microbial transformation culture. Because the CP of surfactant micelle solution increased with the increase of surfactant concentration, the inhibitory effect of high surfactant concentration could be attributed to its CP above the temperature of microbial transformation culture. There was an optimum surfactant concentration for certain substrate concentration.

The effect of substrate concentration was investigated in the range (0.025–3) g/100 ml with a mixed surfactant concentration of 2 g/100 ml. As shown in Fig. 9, the final product concentration increased steadily and linearly with increasing substrate concentration up to a maximum value at a substrate concentration of 0.85 g/100 ml. Further increase in substrate concentration did not result in a higher final product concentration. These results could be attributed to the solubilization of surfactant micelle. For a certain surfactant concentration there is a critical substrate concentration at which the highest conversion efficiency is achieved.

The final product concentration and the conversion efficiency are important factors affecting the economy of the microbial transformation process. Substrate/surfactant concentration was evaluated at substrate concentrations ranging from 1 to 2.2 g/100 ml with different mixed surfactant concentrations. The result (including the data in Fig. 9) is shown in Fig. 10. When the surfactant concentration was increased from 2 to 8 g/100 ml, the maximum value of final product concentration also increased. However, at high surfactant concentration, the increasing trend was retarded. The maximum final product concentration at the surfactant concentration of 10 g/100 ml was nearly the same as that at the surfactant concentration of 8 g/100 ml.

The critical substrate concentration at a surfactant concentration of 8 g/100 ml was approximately 1.45 g/100 ml (see Fig. 10), corresponding to a final product concentration of 10 g/l. A complete microbial transformation of 1 g cholesterol can produce 0.74 g of ADD and 4-AD (ADD:4-AD = 10:1) [15]. The conversion yield is defined as the mole ratio of produced ADD and 4-AD to original cholesterol.

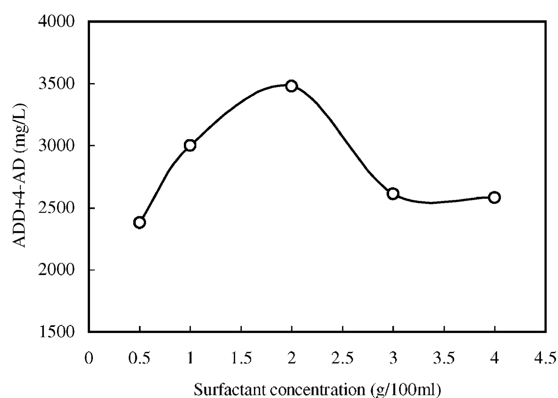

Fig. 8. Change of final product concentration with surfactant concentration.

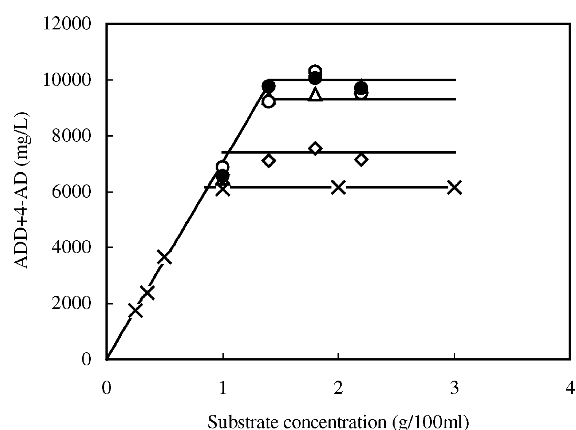

Fig. 10. Evaluation of substrate/surfactant concentration. Surfactant concentration (g/100 ml): (×) (2); (◇) (4); (△) (6); (○) (8); (●) (10).

Table 2
Basic parameters of microbial transformation system

Substrate	Product	Medium system	Microorganism	Substrate concentration (g/l)	Conversion yield (%)	Reference
Cholesterol	ADD, 4-AD	Two-phase aqueous system	*Mycobacterium*	1	80	[15]
Sitosterol	4-AD	Organic media	Immobilization *Mycobacterium*	5.28	89	[28]
Phytosterol	4-AD	PPG solvent	*Mycobacterium*	5–30	90	[29]
Sitosterol	4-AD	Water-immiscible organic solvent	Immobilization *Mycobacterium*	5	70	[16]
Cholesterol	ADD, 4-AD	Cloud point system	*Mycobacterium*	14.5	93	This work

The conversion yields of some transformation systems are calculated and shown in Table 2. Compared with previously reported results, the conversion yield and final product concentration have been improved markedly with the CPS system. We conclude that microbial transformations of hydrophobic substrates in cloud point systems have practical potential.

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