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REVIEW

Whole cell microbial transformation in cloud point system

Zhilong Wang · Jian-He Xu · Daijie Chen

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Abstract Cloud point system, consisting of nonionic surfactant in an aqueous solution, has been developed as a novel medium for whole cell microbial transformation. The basic properties of cloud point system including phase separation and solubilization are introduced. The application of cloud point system for extractive microbial transformation is different from that of water-organic solvent two-phase partitioning system or aqueous two-phase system are discussed, which mainly focus on the biocompatibility of microorganism in a cloud point system and a downstream process of microbial transformation in cloud point system with oil-water-surfactant microemulsion liquid-liquid extraction for surfactant recovery and product separation. Finally, examples of whole cell microbial transformation in cloud point systems, especially in situ extraction of moderate polar substrate/product, are also presented.

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Z. Wang (✉)
School of Pharmacy, Shanghai Jiao Tong University,
200240 Shanghai, People's Republic of China
e-mail: zlwang@sjtu.edu.cn

J.-H. Xu
Laboratory of Biocatalysis and Bioprocessing,
State Key Laboratory of Bioreactor Engineering,
East China University of Science and Technology,
200237 Shanghai, People's Republic of China

D. Chen
Shanghai Institutes of Pharmacy Industry,
200040 Shanghai, People's Republic of China

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Introduction

Compared to isolated enzymes, application of whole cell as biocatalysts is usually more stable due to the surrounding of their natural environment and more economic due to the elimination of tedious protein purification processes. Furthermore, whole cell metabolism of cheap glucose is sufficient to drive the reaction in a bioprocesses involving cofactor regeneration [1]. Therefore, whole cell as a biocatalyst is more prevalent than that of isolated enzyme in a biosynthesis process [2]. The emerging biotechnological fields like the omics tool (genomics, transcriptomics, proteomics and metabolomics) will lead to more and more hyperproduction strains. However, besides structure, the biochemical system of a whole cell microorganism also possesses dynamic and regulatory properties. Such as for a whole cell microbial transformation in an aqueous solution, there are some obstacles including limited substrate accessibility to a microorganism due to the low aqueous solubility of most hydrophobic organic compounds, inhibition or toxicity of both substrate and product to the microorganism, and possibility of further degradation of products by the whole cell [3]. A similar process occurs in biodegradation [4–6]. Improvements in bioprocess techniques for dealing with the dynamic and regulatory properties should keep pace with the up-stream development.

A switch of microbial transformation medium from an aqueous solution to an organic solvent, especially the water-organic solvent two-phase partitioning system, has been studied extensively [7–10]. One of the main obstacles

for whole cell microbial transformation in an organic solvent is its biocompatibility, which has led to screening for organic solvent tolerant microorganism. The screening of organic solvent tolerant microorganisms and their tolerant mechanisms has been reviewed [11–14]. For examples, an organic solvent tolerant microorganism *Pseudomonas putida* has been shown to thrive in a high concentration of toluene [15]. Other examples includes a preparation of organic solvent-tolerant mutants from *E. coli* K-12 [16], utilization of a hydrophobic bacterium *Rhodococcus opacus* B-4 as a whole-cell catalyst in an anhydrous organic solvent [17]. Alternatively, selection of a biocompatible nonaqueous solvent other than an organic solvent, which is known as medium engineering, is also attracted attention in recently, such as aqueous two-phase system [18, 19] and room temperature ionic liquids [20, 21] etc. Cloud point system as a novel two-phase partitioning system has been applied in separation field and referred as cloud point extraction [22–26]. The cloud point system has also been successfully exploited for whole cell microbial transformations in our lab [27]. The potential application of cloud point system as a novel medium for biotransformation has also been reviewed [28]. In present work, the fundamentals about cloud point system including phase separation and solubilization are introduced. Then the bioprocess considerations for application of cloud point system for extractive microbial transformation, such as biocompatibility and downstream process, are discussed. Finally, examples of microbial transformation in cloud point systems, especially in situ extraction of moderate polar substrate/product, are also presented.

Cloud point system

Phase separation

Aqueous surfactant solution with a surfactant concentration above its critical micelle concentration (CMC) usually assembles themselves into many kinds of supramolecular assemblies, such as micelles, lamellar, vesicle, revere micelles, hexagonal and liquid crystalline etc. When an aqueous nonionic surfactant micelle solution is above a certain temperature or in the presence of certain additives, phase separation occurs to form a dilute phase and a surfactant-rich phase or a coacervate phase. The phase separation of a nonionic surfactant Triton X-100 aqueous solution is depended on temperature as shown in Fig. 1 [29]. The dilute phase is a surfactant micelle solution with a surfactant concentration above its critical micelle concentration. The surfactant in the coacervate phase may form a lamellar or vesicle structure, which depends on molecular structure of the surfactant and temperature. Such a two-phase partitioning

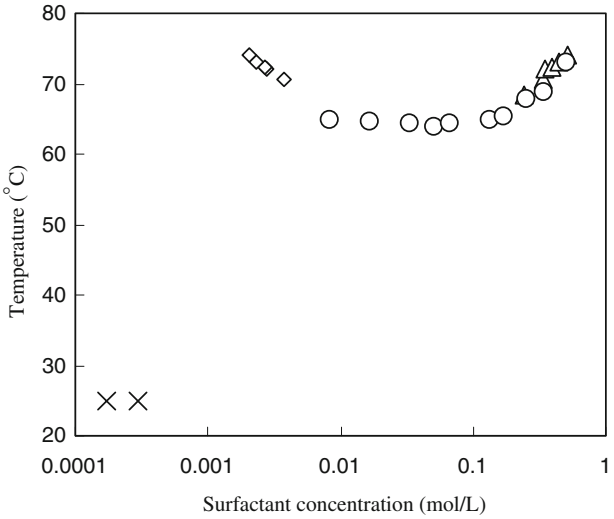

Fig. 1 Temperature–concentration phase diagram of Triton X-100 aqueous solution [29]. At the temperature blow the cloud point, the surfactant solution is a single isotropic solution phase region; at the temperature above cloud point, the solution separates into two coexisting isotropic phases. The surfactant-rich phase is known as coacervate phase, the other dilute phase. *Diamond* is surfactant concentration assayed after phase separation; Triangle is surfactant concentration calculated by conservation of mass after phase separation; *Circle* is determined from cloud point method and fork is CMC, which indicates that the nonionic surfactant concentration in the dilute phase of the cloud point system is higher than the CMC

system is called a cloud point system. The phase separation temperature is called cloud point. The cloud point is determined by the molecular structure of the employed nonionic surfactant [30] and is also affected by the surfactant concentration and additives. These are well detailed in some review articles [23, 24, 26].

To distinguish between a cloud point phenomenon and a surfactant dispersible in an aqueous solution should be emphasized. When an environmental temperature is at the Krafft point of a surfactant aqueous solution, the solubility of the surfactant is equal to its critical micelle concentration. Above Krafft point, the total solubility of the surfactant increases dramatically due to the formation of micelles. Below Krafft point, only surfactant monomers are presented and the solubility is drastically limited. Thus, a surfactant aqueous solution is cloudy at a temperature below its Krafft point, but becomes clear at a temperature above its Krafft point [31]. The phase change of 3% (v/v) Triton X-45 dispersible in water with temperature is shown in Fig. 2. The nonionic surfactant is dispersible in the aqueous solution and forms a white dispersible phase at room temperature. The cloudy dispersible phase becomes clear with an increase of temperature and becomes cloudy again with a further increase of temperature to its cloud point. However, the turbidity of surfactant dispersible in an aqueous solution at room temperature usually be regarded as the cloud point

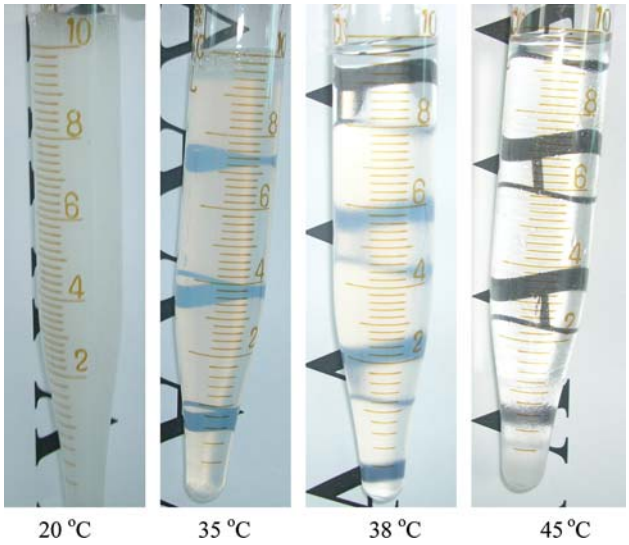

Fig. 2 Phase change of dispersible Triton X-45 in an aqueous solution with temperature [33]. At low temperatures, such as 20 °C, Triton X-45 is dispersible in the aqueous solution and forms an opaque, stable and white dispersible phase. As the temperature is increased to 35 °C, the dispersible phase turns into a clear, blue and transparent phase. The transparency can be detected from the background but the *blue* color that results from a light scatter of micelle particles can not be detected in the picture. This temperature is denoted as Krafft point. With the temperature increase to 38 °C, the surfactant in aqueous solution becomes cloudy again and separates into two clear phases. This temperature is denoted as cloud point. When the temperature is increased to 45 °C, the volume of surfactant-rich phase is further reduced

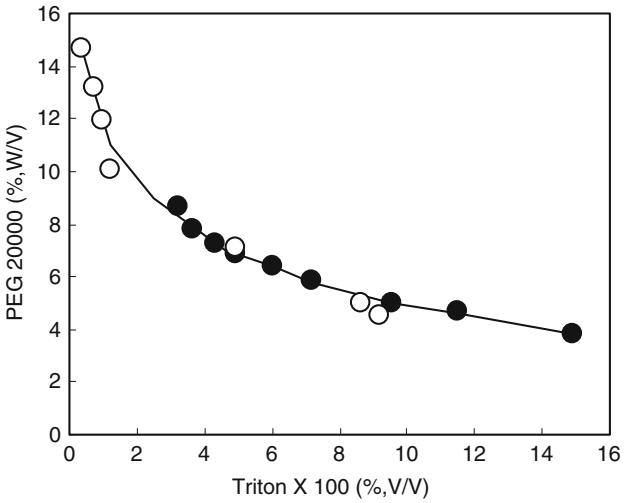

Fig. 3 PEG 20000 induced phase separation of Triton X-100 in an aqueous solution [38]. The phase diagram is designated by a binodal curve which separates the two-phase area from the single phase zone. The compositions represented by the points below the bimodal curve are homogeneous. Two phases are formed only by the compositions above the binodal curve. The phase diagram gives the exact compositions of the top phase and the bottom phase. The *circles* are obtained by titrated the cloudy PEG aqueous solution with an aqueous Triton X-100 solution. On the other hand, the *black dots* are obtained by titrated the cloudy Triton X-100 solution with an aqueous solution of PEG 20000

phenomenon and a below room temperature is taken as the cloud point of Triton X-45 [23]. There is a same phenomenon for nonionic surfactant Brij 30 and Span 20 etc [23, 32] in spite of the fact that a phase separation into two clear phases at a cloud point temperature do not occurred at a room temperature [33].

There are many kinds of commercial nonionic surfactant, however, the nonionic surfactants with a cloud point at room temperature (about 20–40 °C) for cloud point extraction are very limited. It has been demonstrated that the effect of polyethylene glycols (PEG) on the cloud point of a surfactant aqueous solution is related with the chain length of the polymer. A polymer with a short chain elevates the cloud point of a nonionic surfactant solution. On the other hand, a polymer with a long chain decreases the cloud point of a nonionic surfactant solution. A possible flocculation depletion mechanism about the polymer chains existing between two regular micelles has been suggested to explain the polymer and the surfactant interactions [34]. The effect of polyvinylpyrrolidone [35], β-cyclodextrin [36] and hydroxypropyl starch Reppal PES100 [37] to the cloud point of a nonionic surfactant aqueous solution have also been reported. A nonionic surfactant Triton X-100 aqueous solution with a cloud point 68 °C can be induced to form a cloud point system by an addition of a long chain PEG 20000 as shown in Fig. 3, in which, one is nonionic surfactant rich phase and the other is a polymer rich phase [38]. The phase diagram gives the exact composition of each phase, which is similar to that of conventional aqueous two-phase system. The PEG induced cloud point system makes it is possible for utilization of most of commercial nonionic surfactants for cloud point extraction at room temperature.

Solubilization

Solubilization is defined as "preparation of a thermodynamically stable isotropic solution of a substance normally insoluble or very slightly soluble in a given solvent by an introduction of an additional amphiphilic compound or components" [39]. The mechanism about solubilization of a solute in surfactant micelles has been studied comprehensively, such as hydrocarbon additive solubilized in the micelle hydrophobic core, a more polar material oriented in palisades region, polar additives solubilized in the region of the hydrated layer, and the additive directly associated with the electrical double layer [40] etc. Not only a hydrophobic solute but also a hydrophilic solute can also be solubilized into a surfactant micelle. Polar compounds, particularly those with hydrogen bond functional groups and aromatic hydrocarbon with C–H...π interaction, has also been found solubilization [41]. The solubilization of surfactant micelle

aqueous solution and the phase separation of nonionic surfactant aqueous solution make cloud point system is an important separation medium, which has been utilized in separation field known as cloud point extraction [23].

Cloud point extraction has been utilized in separation field for a long time and as an eco-friendly method has been attracted more and more attention in recent years [26]. However, the study of solubilization in the coacervate phase of a cloud point system is relatively few. The main obstacle is the difficulty to determine the free solute concentration, i.e., to distinguish solute between in the surfactant supramolecular assemblies and in aqueous solution. The different types of supramolecular assemblies of a nonionic surfactant in aqueous solution lead to its solubilization ability diversification. A rigorous experiment with a very low phenol concentration indicates that a solubilization capacity of a nonionic surfactant between in the dilute phase and in the coacervate phase is very small and the difference can be attributed to the experimental errors [42]. However, a remarkable change in the phenol solubilization in the coacervate phase of cloud point system with the increase of phenol concentration has been determined as shown in Fig. 4 [43]. The structure of supramolecular assemblies in the coacervate phase is altered by an addition of phenol to decrease cloud point of the nonionic surfactant aqueous solution, which is analogous to the supramolecular assemblies change from a lamellar to a vesicle structure with an increase of temperature [44]. The difference in solubilization capability can be contributed to the different structure of supramolecular assembly in the coacervate phase. A similar phenomenon has also been reported in cloud point system with a nonionic surfactant Brij 35, the solubilized phenol increases from 2.9 moles per mole of surfactant in the dilute phase to 7.5 moles per mole of surfactant in the coacervate phase [45]. The different solubilization capability in the dilute phase and the coacervate phase of the cloud point system further confirms that the solubilization is related to the structure of supramolecular assemblies of a nonionic surfactant.

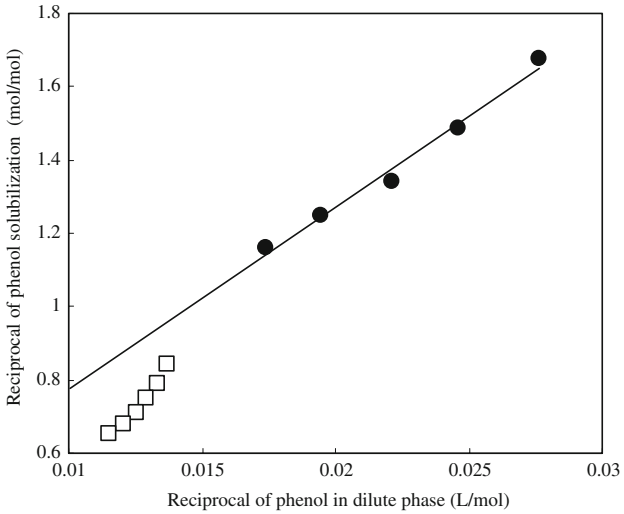

Fig. 4 A plot of Langmuir isotherm about phenol solubilization in the coacervate phase of a cloud point system [43]. With an increase of phenol concentration, aqueous micelle solution of the polyoxyethylene glycol monoether nonionic surfactant $C_{12}E_7$, in which 12 indicates the number of carbons in the alkyl chain and 7 indicates the average number of ethylene oxide units in the hydrophilic-moiety, changes from a single phase micelle solution to a two-phase system, then to a three phase system, and then to a two-phase system again. Symbol of *black circles* represent the region of the coacervate phase in the two-phase region with a relatively lower phenol concentration. In this phenol concentration region, the adsorbed phenol in the surfactant is fitted with Langmuir isotherm. Symbol of *blank squares* represent the region of the coacervate phase in the two-phase region with a relatively higher phenol concentration. In this phenol concentration region, the adsorbed phenol in the surfactant cannot be fitted with Langmuir isotherm and the solubilization increases with the increase of phenol concentration markedly

Bioprocess considerations

Water-organic solvent two phases partitioning system [8] and aqueous two-phase system [18, 19] have been used to extractive microbial transformation. However, the biocompatibility of water-organic solvent two phases partitioning system and the downstream process of aqueous two-phase system are limited for their industrial application. The biocompatibility and downstream process of application of cloud point system for whole cell microbial transformation are discussed in this section.

Biocompatibility

The biocompatibility of a nonaqueous medium is a prerequisite for its application for a whole cell microbial transformation. Some nonionic surfactants micelle solution with a relatively lower surfactant concentration has been applied to enhance the bioavailability of hydrophobic compounds [46–48]. However, the permeability of a nonionic surfactant to microbial cells makes the nonionic surfactant toxic to the microorganism [49].

A screening of *Mycobacterium* sp. in different kinds of nonionic surfactant aqueous solution finds that only Triton X-114, whose cloud point is below the culture temperature and phase separation occurs to form a dilute phase and a coacervate phase, is biocompatible. Nonionic surfactant Brij 30, which is dispersible in the aqueous solution as that of Triton X-45 as discussed in Fig. 2, along with the surfactant with a cloud point above the culture temperature, is toxic to the microorganism [27]. The relationship between the biocompatibility and the phase separation of a nonionic surfactant aqueous solution is further studied. The same

series of nonionic surfactants Triton X-100, Triton X-114 and Triton X-45, which have the same hydrophobic moiety but different numbers of ethylene oxide unit. Triton X-114 is biocompatible but Triton X-100 is toxic to microorganism *Mycobacterium* sp as shown in Fig. 5a. Although hydrophobic substrate can be solubilized with any ratio of Triton X-114 to Triton X-100, only above a certain volume ratio of Triton X-114 to Triton X-100, where the cloud point is below the culture temperature, the system is biocompatible [27]. The phase separation and biocompatibility of a series of mixture surfactants with different ratios of Triton X-114 to Triton X-45 are shown in Fig. 5b. A phase separation only occurs at a volume ratio of Triton X-45 to Triton 114 below 40%. Above those volume ratios, their Krafft points of the mixture nonionic surfactant aqueous solution are higher than the microorganism culture temperature, and then phase separation can only occur at an even higher temperature as shown in Fig. 2. The dispersible aqueous systems are toxic to the microorganism *Saccharomyces cerevisiae* and the biomass of *S. cerevisiae* growing in the series of nonionic surfactant aqueous systems is also consistent with the phase separation [50].

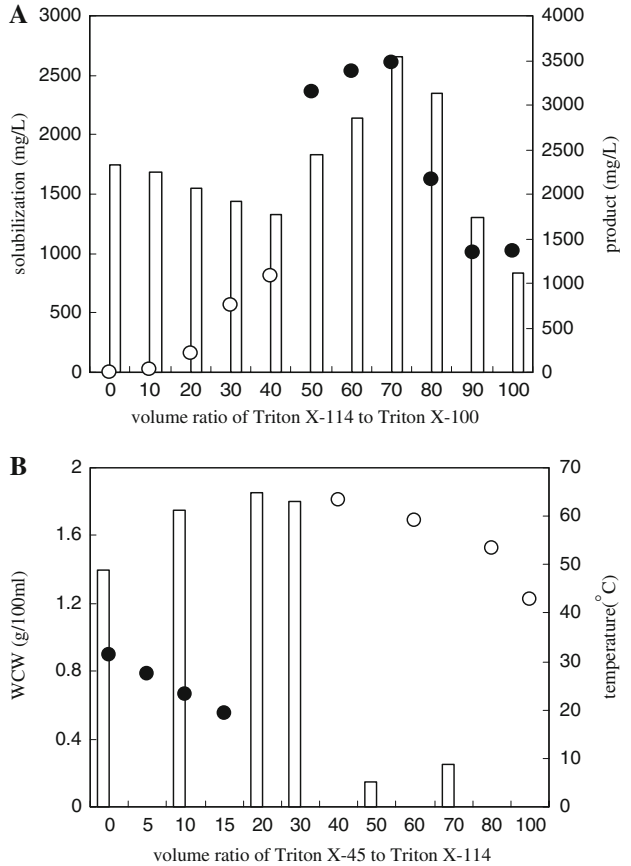

Fig. 5 Biocompatibility and the phase separation of a nonionic surfactant aqueous system. **a** Solubilization of product and the final product concentration of *Mycobacterium* sp. transformation of cholesterol into ADD in a surfactant-aqueous solution system with different volume ratios of Triton X-100 to Triton X-114. The product concentration is determined by growing cell whole cell microbial transformation after *Mycobacterium* sp growing in a culture medium containing 2% of nonionic surfactant for 7 days. The *blank bar* is the solubilization of product; the *open circle* is the product concentration in which the surfactant-aqueous system is a single phase; the *filled circle* is the product concentration in which the surfactant-aqueous system has separated into a two-phase system [27]. **b** Growth of *Saccharomyces cerevisiae* in surfactant-aqueous solution systems with different volume ratios of Triton X-114 to Triton X-45. The wet cell weight (WCW) is determined by *Saccharomyces cerevisiae* growing in a culture medium containing 10% of nonionic surfactant for 3 days. The *blank bar* is WCW; the *filled circle* is the cloud point and a phase separation occurred under culture temperature; the *open circle* is the cloud point above culture temperature and the surfactant is dispersible under the culture temperature [50]

Polyethylene glycol (PEG) induces a Triton X-100 aqueous solution to form a novel cloud point system is shown in Fig. 3. The biocompatibility of PEG induced cloud point system along with cloud point system is studied comparably with different water-organic solvent two-phase partitioning system as shown in Table 1. The accumulated biomass and the residual glucose are used to quantify the biocompatibility of a nonaqueous system. In an organic solvent mediated water-organic solvent two-phase partitioning system, the

Table 1 The cell growth and glucose metabolism of *Saccharomyces cerevisiae* in different two-phase partitioning systems [38]

Solvent system	Log *P*	Cell viability	
		Biomass (g)	Residual glucose (A_{540})
Control		0.52	0.137
Cloud point system		0.38	0.010
PEG induced cloud point system		0.28	0.049
Ethyl acetate	0.68	0.05	2.571
iso-butylalcohol	0.8	0.05	2.542
n-butyl acetate	1.7	0.06	2.571
Chloroform	2	0.05	2.571
n-octyl alcohol	2.81	0.10	2.542
Cyclohexane	3.2	0.18	0.965
Isooctane	4.27	0.61	0.183

The control is 20 ml of a nutrient medium, which consists of 90 g glucose, 10 g yeast extract, 10 g $(NH_4)_2SO_4$, 3 g KH_2PO_4, 2 g $Na_2HPO_4{\cdot}12H_2O$, 1 g $MgSO_4{\cdot}7H_2O$ and 0.05 g $CaCl_2{\cdot}2H_2O$ in per liter of tap water. The cloud point system is composed of 20 ml of the nutrient medium and 2 ml of a nonionic surfactant mixture of Triton X-114 and Triton X-45 with a volume ratio of 9:1. The PEG induced cloud point system is consisted of 20 ml of the nutrient medium with 8% (w/v) PEG 20,000 and 7% (v/v) Triton X-100. The water-organic solvent two-phase partitioning system is consisted of 20 ml of the nutrient medium with 2 ml of different organic solvents, respectively. Biomass is detected by wet cell weight after 2 days' whole cell growing culture in those systems and the residual glucose concentration is detected by measuring A_{540} of the aqueous phase with a standard 3, 5-dinitrosalicylic acid method after 2 days' whole cell growing culture

biocompatibility increases with the increase of log *P* (log *P* is defined as the partitioning coefficient between octanol and water, which is usually used to index the polarity of an organic solvent) of the organic solvent as predicted by log *P* criterion [8]. A nonionic surfactant cloud point system is more polar than that of water-organic solvent two-phase partitioning system, which has been discussed based on E_T (30) [28]. However, *S. cerevisiae* maintains its biocompatibility in the PEG induced cloud point system along with the cloud point system, which provides a biocompatible environment with a relatively higher polarity.

The toxic nonionic surfactant becomes biocompatible to a microorganism under a phase separation condition. The biocompatibility of cloud point system has a similar principle to that of water-organic solvent two-phase partitioning system [4, 14, 51, 52]. The different is the replacement of organic solvent auxiliary phase in a water-organic solvent two-phase partitioning system with the coacervate phase in a cloud point system. The microscopic structure of cloud point system [27] and a supposed mechanism for microbial transformation in a cloud point system are presented in Fig. 6.

It is well known that most of organic solvents are toxic to a microorganism. Monophase organic solvent system, in which only a limited concentration of organic solvent can be added to increase the solubility of some organic substrate, has been applied for microbial transformations [53]. The critical inhibition concentration of an organic solvent to a microorganism increases with the increase of an organic solvent polarity [8, 14]. However, the organic solvent toxicity to a microorganism in a water-organic solvent two-phase partitioning system is determined by its actual concentration in the aqueous environment other than the total organic solvent concentration. The well known log *P* criterion indicates that an organic solvent with a log *P* above 4 is biocompatible [7, 8, 54].

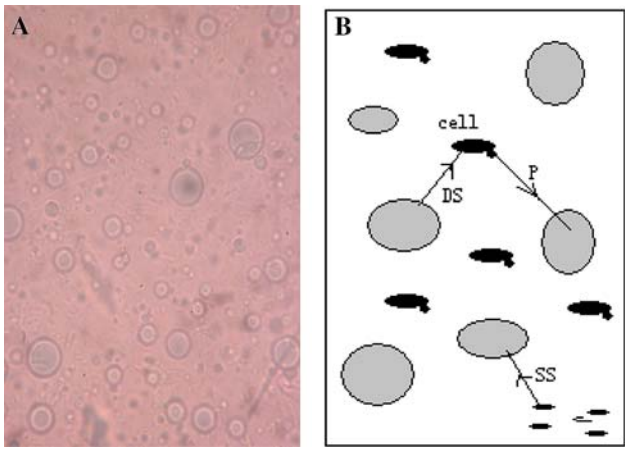

Fig. 6 Mechanism of whole cell microbial transformation in cloud point system. **a** Microscopic observation of oil-in-water emulsion (×400) with many surfactant vesicles (*grey dots*) in a cloud point system [27]. **b** Schematic representation of a whole cell microbial transformation in a cloud point system. The surfactant vesicle acts as a substrate reservoir, which enhances the dispersion and bioavailability for a water-insoluble solid substrate. At the same time, it controls the delivery of toxic substrate into the whole cell and eliminates the toxicity of substrate to the microorganism. The surfactant vesicle also acts as a product extractant. In situ product removal prevents the microorganism from the inhibition of the product and prevents the product from further degradation by the microorganism. At some times, it also shifts the equilibrium to further microbial transformation. *SS* is a solid substrate; *DS* is a dispersible substrate; *P* is a product. The *black circle* with a little bud represents the whole cell of a microorganism; the *grey circle* represents the surfactant vesicles; the *black circle* represents the solid substrate

Similar, the toxic nonionic surfactant is concentrated into the coacervate phase at a temperature above its cloud point and the surfactant concentration in the dilute phase is relatively lower. A very low nonionic surfactant micelle solution is biocompatible and has been applied in biotechnology [46–48]. Analogue to the log *P* is chosen as a parameter for an organic solvent, the cloud point of a nonionic surfactant may act as a criterion for a nonionic surfactant. However, the replacement of auxiliary phase with nonionic surfactant in the cloud point system possesses some advantages. For instance, the nonvolatilization and noninflammability of a nonionic surfactant fulfills the demands of green solvent and avoids the explosive risk existing in a water-organic solvent two-phase partitioning system under an aerobic condition [55]; a relatively higher polarity and a biocompatibility of nonionic surfactant in a cloud point system as discussed in Table 1 is potential for whole cell microbial transformation of a moderate polar substrate/product, where is inaccessible for a water-organic solvent two-phase partitioning system since the biocompatibility and extraction of product can not be fulfilled at the same time [56, 57].

Downstream process

Nonvolatile nonionic surfactant, which is an environmental benign solvent [26], makes it is impossible for the recovery product from the process of microbial transformation in cloud point system to copy the routine evaporation procedure as that of conventional water-organic solvent two-phase extraction. Exception of a tedious chromatographic separation [58], there are only a very few reports about the separation of nonionic surfactants from special target compounds in large-scale. For examples, the same as an aqueous two-phase system where only a neutral solute can be extracted [59], adjustment the pH of a cloud point system for back-extraction of a solute with an acidic or a basic moiety has been proposed [24]. Recovery of volatile organic compounds from surfactant solutions by pervaporation has also been documented in literature [60]. An addition of copolymer EOPO into the coacervate phase after the

cloud point extraction of protein forms a new two-phase system, in which the protein partitions to the water phase as its strong exclusion effect. This phenomenon has been used to separate the target protein from the nonionic surfactant [37]. Winsor II microemulsion has been successfully used for separation of hydrophilic cholesterol oxidase from a nonionic surfactant solution [61]. Development of a general strategy for product separation and nonionic surfactant recovery is indispensable for the industrial application of cloud point systems.

Microemulsion is composed of water, organic solvent and surfactant, occasionally with an alcohol as a co-surfactant. Two-phase systems, called Winsor I and Winsor II, correspond to oil in water microemulsion coexisting with an excess oil phase and water in oil microemulsion coexisting with an excess water phase, respectively. A Winsor III system corresponds to concentrate surfactant into a bicontinuous phase coexisting with excess oil and excess water. Sometimes, water, surfactant and organic solvent form a single phase, which is also called Winsor IV. The compositions, temperature and volume ratio of oil to water are important factors to affect the microemulsion type as shown in Table 2 [62]. The polarity of an organic solvent, such as microemulsion turns from Winsor II to Winsor I with the organic solvent change from *iso*-butyl alcohol, *n*-butyl acetate to ethyl ether as shown in No 1, 3 and 5 of Table 2, the hydrophile–lypophile balance (HLB) value of a nonionic surfactant, such as the microemulsion turns from Winsor III to Winsor I with the nonionic surfactant change from Triton X-45 to Triton X-114 and Triton X-100 as shown in No 10, 7 and 9 of Table 2, and the temperature, such as Winsor II microemulsion is formed with any one of the organic solvent at 25 °C as shown in No 2, 4 and 6 of Table 2, which are important factors to determine the microemulsion type. By selection of a hydrophile nonionic surfactant, a relatively polar organic solvent and under a relatively low temperature condition, a Winsor I microemulsion can be formed. If a microbial transformation product were solubilized in the organic solvent, the product and the nonionic surfactant should be separated in the downstream process of microbial transformation in a cloud point system.

By setting a microbial transformation of benzaldehyde into *L*-phenylacetylcarbinol by whole cell *S. cerevisiae* [63] in a cloud point system as an example, a general strategy with microemulsion extraction for product separation and a surfactant recovery has been established. As schematically shown in Fig. 7, the product and the nonionic surfactant in the microbial transformation broth is successfully separated with the application of a Winsor I microemulsion. Then the nonionic surfactant is recovered with a Winsor II microemulsion. In a single stage Winsor I microemulsion extraction process, the product recovery coefficient 76.9% and the nonionic surfactant recovery coefficient 66.5 % are achieved. A discrete countercurrent extraction operation improves the product to nearly complete extraction [62].

Microbial transformation

Some examples of whole cell microbial transformations as listed in Fig. 8 has been carried out in cloud point systems, which is detailed in Table 3.

Table 2 Effects of important factors on microemulsion type [62]

No	Surfactant	Organic solvent	Temperature (°C)	Concentration of surfactant in water (%, v/v)	Ratio of oil to water (ml:ml)	Fraction of surfactant in water (%)	Winsor
1	Triton X-114	*iso*-butyl alcohol	6	10	20:20	0.5	II
2	Triton X-114	*iso*-butyl alcohol	25	10	20:20	0.7	II
3	Triton X-114	*n*-butyl acetate	6	10	20:20	43.5	II
4	Triton X-114	*n*-butyl acetate	25	10	20:20	10.7	II
5	Triton X-114	Ethyl ether	6	10	20:20	81.7	I
6	Triton X-114	Ethyl ether	25	10	20:20	30.2	II
7	Triton X-114	Ethyl ether	6	10	40:20	58.5	I
8	Triton X-114	Ethyl ether	6	2.5	20:20	62.8	I
9	Triton X-100	Ethyl ether	6	10	20:20	91.7	I
10	Triton X-45	Ethyl ether	6	10	20:20	2.05	III

A certain concentration of nonionic surfactant aqueous solution is combined with an organic solvent with a certain volume ratio of oil phase to water phase. Then the combined microemulsion is put into a certain temperature environment for equilibrium. After the phase equilibrium, the nonionic surfactant concentration in the organic solvent phase is determined by evaporation. The microemulsion type can be determined by the fraction of a nonionic surfactant in the water phase or in the organic solvent phase

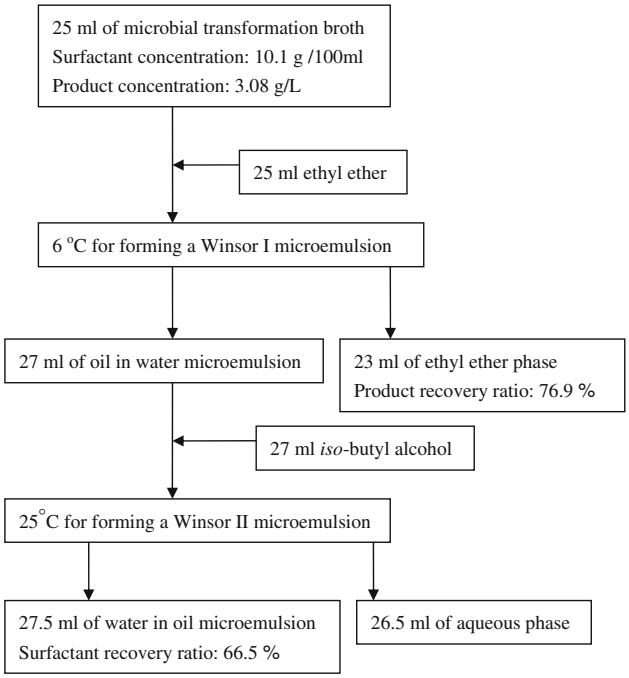

Fig. 7 A scheme of downstream process of microbial transformation in cloud point system [62]. Cloud point system for microbial transformation is composed of nonionic surfactant (Triton X-114 and Triton X-45 with a volume ratio of 9:1) and aqueous culture medium with a volume ratio of 1:10. By application of a Winsor I microemulsion liquid–liquid extraction, the product and the nonionic surfactant is separated. Then the product in the organic solvent phase is subjected to further purification. The nonionic surfactant in the W_m phase is extracted with a Winsor II microemulsion for removal of the hydrophilic impurity and recovery of nonionic surfactant to the organic solvent phase

Microbial transformation of sterols

The microbial transformation of sterol to produce steroid intermediate androst-1,4-diene-3,17-dione (ADD) as shown in Fig. 8a is a classic example of nonaqueous biotransformation, which involves a low solubility of substrate, both substrate and product inhibition, and further degradation of product by the microorganism [67–70]. Whole cell microbial transformation in water-organic solvent two-phase partitioning system [68] and aqueous two-phase system [67] has been carried out. However, the bioprocess is limited by the biocompatibility of organic solvent or solubilization of aqueous two-phase system. *Mycobacterium* sp maintains its biocompatibility in Triton X-114 or mixture of Triton X-114 and Triton X-100 cloud point system. The cloud point system enhances the substrate solubility and then bioavailability [27, 71]. A resting cell microbial transformation further confirms that the cloud point system eliminates the substrate and product inhibition, and prevents the product from further degradation [64]. All of those make the specific biocatalysis activity increases from 1 mg ADD per gram of cell per day in aqueous solution to 24 mg ADD per gram of cell per day in a cloud point system [64]. The final product concentration has been enhanced to above 10 g per liter by application of growing cell biotransformation [27] or 12 g per liter by application of resting cell biotransformation in the cloud point system [65].

Asymmetric biosynthesis of chiral 1-phenylethanol

Microbial transformation of prochiral acetophenone to produce chiral 1-phenylethanol by *S. cerevisiae* (Fig. 8b) is perhaps one of the most popular model reaction, which involves cofactor NAD(P)H regeneration with the viable cells [72–75]. However, the toxicity of the substrate/product to the microorganism [76] makes the utilization of whole cell microorganism, even with an immobilized cell technique [72–74], for a self-regeneration and a self-proliferation capability only possible at a very low substrate concentration [75]. The toxic substrate and product concentration at which the *S. cerevisiae* can grow is 0.3 and 0.4% in the aqueous solution, respectively. The cloud point system has been improved them to 0.6 and 0.7% (v/v) in the cloud point system, respectively. And reutilization of the whole cell microorganism as biocatalyst indicates that it maintains its biocatalytic activity at least four times [50].

Microbial transformation production of *L*-phenylacetylcarbinol

Biotransformation of benzaldehyde into *L*-phenylacetylcarbinol (PAC), an intermediate for the production of *L*-ephedrine and related pseudoephedrines, is fulfilled by pyruvate decarboxylase of *S. cerevisiae* through non-oxidative decarboxylation of pyruvic acid followed by carboligation to benzaldehyde. To make the process economic, pyruvic acid should be produced by a metabolism of glucose with the metabolizing cells, as shown in Fig. 8c [77, 78]. The whole cell microbial transformation can be considered as an interesting microbial transformation process for evaluation, as it includes both toxic substrate and end-product inhibition. Unfortunately, the product with a log *P* of 0.6 can only be effectively extracted by an organic solvent with relatively higher polarity, such as pentanol, octanol etc [79, 80]. It is difficult to maintain the biocompatibility in the relatively higher polarity organic solvent-water two-phase partitioning system [57]. Even an immobilized cell technique, in which the toxic substrate and/or product gradients are established within the immobilizing matrix and the toxic substrate and/or product are reduced by virtue of the diffusion limitations [81], is not very effective to this microbial transformation [63]. However, *S. cerevisiae* maintains its biocompatibility in the cloud point system or

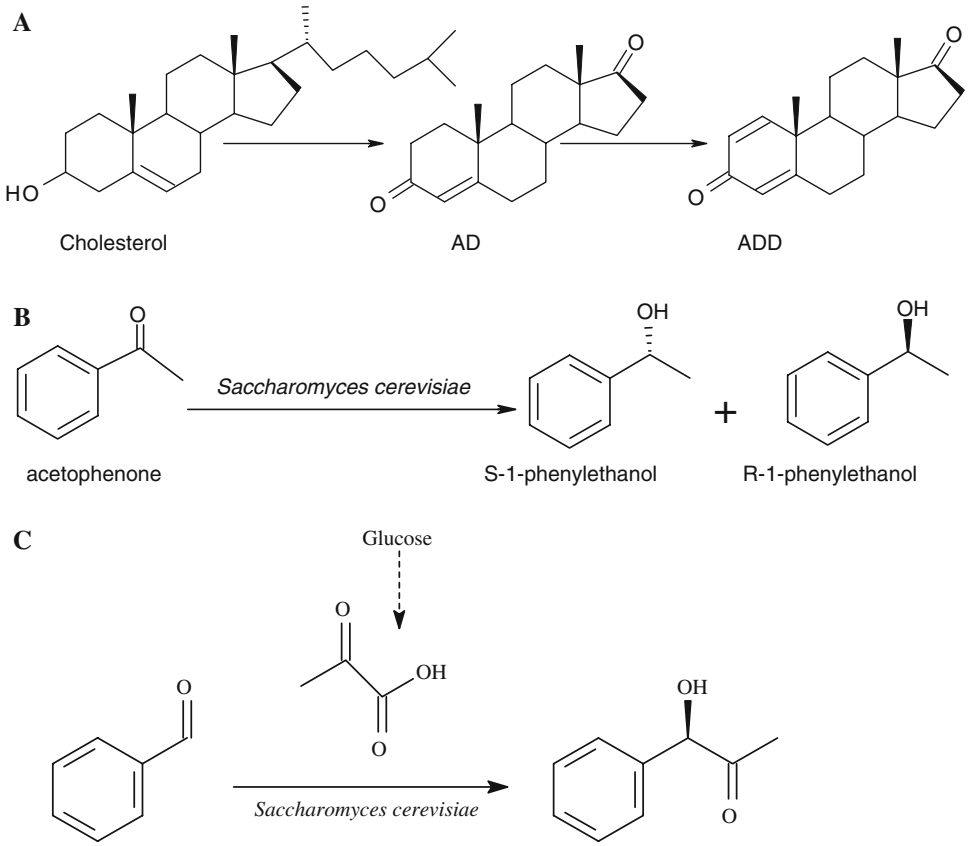

Fig. 8 Examples of whole cell microbial transformations in cloud point systems. **a** Biodegradation of insoluble sterol involving multiple enzymes of *Mycobacterium* sp [27]. **b** Bioreduction involving cofactor NAD (P) H regeneration with viable cells of *Saccharomyces cerevisiae* [50]. **c** Precursor fermentation involving glucose metabolism by *Saccharomyces cerevisiae* to produce pyruvic acid as one of its substrates [66]

Table 3 Examples of whole cell microbial transformation in cloud point system

Constitutes of cloud point system	Microorganism	Substrate	Product	References
Triton X-114 and Triton X-100	*Mycobacterium* sp (growing cell)	Cholesterol	ADD	[27]
Triton X-114 and Triton X-100	*Mycobacterium* sp (resting cell)	Cholesterol	ADD	[64]
Triton X-114 and Triton X-100	*Mycobacterium* sp (resting cell or growing cell)	Phytosterol	ADD	[65]
Triton X-45 and Triton X-114	*Saccharomyces cerevisiae* (growing cell and resting cell)	Acetophenone	1-phenylethanol	[50]
Triton X-100 and PEG 20000	*Saccharomyces cerevisiae* (resting cell)	Benzaldehyde	*L*-PAC	[66]

PEG induced cloud point system in spite of its relatively higher polarity as shown in Table 1. At the same time, the product phenylacetylcarbinol with a relatively higher polarity has also been extracted into the surfactant rich phase in the PEG induced cloud point system during the microbial transformation process [38]. The extraction of a relatively higher polarity product in the PEG induced cloud point system fills the gap of product polarity spectrum that left by application of water-organic solvent two-phase partitioning system for extractive microbial transformation of moderate polar substrate/product [56, 57]. At the same time, as the cloud point of a nonionic surfactant may act as a criterion for selection of a biocompatible nonionic surfactant, a decrease of the cloud point of a nonionic surfactant in aqueous solution by addition of PEG also extends the application of nonionic surfactants with relatively higher cloud points for whole cell microbial transformation at conventional microbial transformation temperatures. A further optimization of microbial transformation in the PEG induced cloud point system has led to the product concentration increase to about 8 g/L [66].

Future trends

Phase separation of a nonionic surfactant aqueous system is related to its biocompatibility and perhaps the cloud point of a nonionic surfactant can be utilized as a criterion for selection of a nonionic surfactant in whole cell microbial transformation. However, the cloud point system as a novel two-phase partitioning medium for whole cell microbial transformation is still in its infancy and the criterion should

be further validated by more model reactions. A deep insight into the mechanism behinds the phenomenon will be a major goal in the future research.

A mathematic model of biotransformation in a cloud point system with an immobilized enzyme as a biocatalyst has been established [82]. Although the physiology and application aspects about surfactant in microbiology and biotechnology has many studies [83, 84], a knowledge about viable cells including the cell physiology, cell growth and intrinsic kinetics etc in a cloud point system is still unclear. A similar simulation for whole cell microbial transformation is still far from maturity [71]. A search about the cell physiology in the future is important and possible.

Whole cell microbial transformation to produce a relatively higher polar product in a water-organic solvent two-phase partitioning system involves the conflict of biocompatibility and extractive ability of an organic solvent. The extraction of a relatively higher polar product of a microbial transformation in a cloud point system fills the gap. The relatively higher polar compounds, such as alcohol, ketone, organic acid and ester etc, are important "building blocks" for organic synthesis. The exploitation of these microbial transformations in cloud point systems should be extensively studied in the near future.

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