J. Biol. Macromol., **13**(1), 1-11 (2013)

Review

Morphological, physiological, and biochemical properties of fungal cells located on a liquid-surface and an organic–aqueous interface

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In general, morphological, physiological, and biochemical properties of fungal cells are drastically different among solid-state (SSC), liquid-surface (LSC), and submerged cultivation (SmC) systems. In addition to these traditional cultivation systems, a unique liquid-surface cultivation [liquid-surface immobilization (LSI)] system and interface cultivation systems [extractive liquid-surface immobilization (Ext-LSI) system; liquid–liquid interface bioreactor (L–L IBR)] bring some interesting fungal morphological, physiological, and biochemical characteristics. The unique properties of fungal cells located on a liquid-surface and an organic–aqueous interface are very important to application of fungi to the production of enzymes, metabolites, and bioconversion products. In this review, the unique and important characteristics of fungal cells immobilized in the LSI, L–L IBR, and Ext-LSI systems are explained in detail. Moreover, some application of the cultivation systems to fermentation, enzyme production, and microbial transformation are introduced.

Received April 1, 2013; Accepted June 5, 2013

Key words: filamentous fungi, interface bioreactor, liquid-surface cultivation, secondary metabolite, microbial transformation

Introduction

Filamentous fungi have been applied to various industries, such as traditional food and brewery industries, the production of metabolites, enzymes, cosmetic materials, and pharmaceutical intermediates. In many cases, fungal cells are cultivated in submerged cultivation (SmC) system to form pelleted, filamentous, or clumped forms as morphological pattern. In general, the pelleted form measuring 1–2 mm in diameter is the ideal morphology for fungi in the SmC because this form can maintain a low viscous broth and enables efficient oxygen supply[(1)]. However, a large number of factors, such as type and size of inoculum, composition and pH of medium,

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agitation rate, incubation temperature, and a number of additives (surfactant, polymer, and chelator) must be optimized for the formation of fungal pellets[2]. Thus, although the control of the fungal morphology is practically very important, this process is troublesome to implement in general.

On the other hand, in a traditional cultivation system named solid-state cultivation (SSC), fungal cells growing on solid substrate or inert support particles such as polyurethane foam naturally differentiate to form much aerial and vegetative hyphae, sporangia and spores[3]. The SSC has some practical advantages, such as higher productivity, lower risk of contamination and lower energy consumption, and lower cost of equipment compared with the SmC[4]. Moreover, derepression of catabolite repression and restraint of digestion of targeted proteins by inherent protease are favorable for the production of metabolites and enzymes in the SSC[5,6]. However, although the SSC has various advantages abovementioned, some serious disadvantages, such as poor heat dissipation, low water activity, and slow diffusion rates of nutrients, products and oxygen in a packed bed have been observed[4]. Therefore, it is fervently desired to develop a higher performance of cultivation system for fungi.

Recently, we have developed three types of unique fungal cultivation systems, a liquid-surface immobilization (LSI)[7,8], an extractive LSI (Ext-LSI)[9,10], and a liquid–liquid interface bioreactor (L–L IBR) systems (Fig. 1)[11,12]. While the LSI system consists of a fungus–polyacrylonitrile (PAN) ballooned microsphere (MS) mat (a top phase) and liquid medium (a bottom phase), the Ext-LSI and L–L IBR systems consist of hydrophobic organic

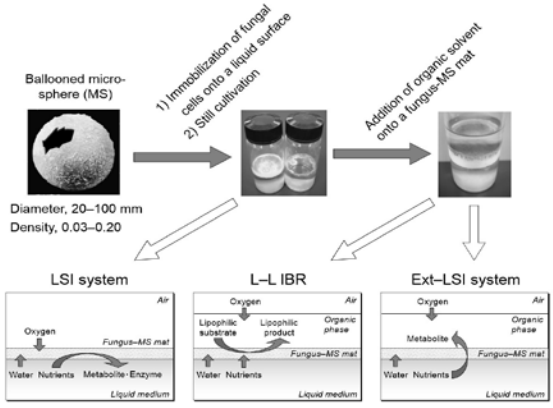

Fig. 1. Principles of three types of cultivation/application devices for fungal cells. LSI, liquid-surface immobilization; L–L IBR, liquid–liquid interface bioreactor; Ext-LSI, extractive liquid-surface immobilization. In all systems, fungal cells are trapped on a ballooned polyacrylonitrile microsphere (MS; diameter, 20–100 μm; density, 0.03–0.20) layer formed on a liquid-surface of medium. The fungal cells grow and differentiate to vegetative and aerial hyphae, sporangia, and spores during appropriate precultivation period to form a physically strong fungus-MS mat on the liquid-surface. The fungus-MS mat is used as a biocatalyst to produce hydrophilic metabolites and enzymes (LSI system). In the case of overlaying the fungus-MS mat with a harmless hydrophobic organic solvent, such as middle-chain alkanes, esters, ethers, and low viscous dimethylsilicone oils, two types of unique cultivation/applica-tion devices can be constructed. The L–L IBR is an interfacial microbial transformation device of hydrophobic substrates dissolved in the organic phase. The Ext-LSI system is an interfacial fermentation device of hydrophobic metabolites by using nutrients dissolved in the liquid medium. In all cases, targeted products are remarkably accumulated into a liquid medium (LSI system) or an organic phase (Ext-LSI and L–L IBR systems) because of the alleviation of some unfavorable phenomena, such as catabolite repression, feed-back inhibition, substrate/product inhibition, and digestion of products by inherent proteases. In both the L–L IBR and the Ext-LSI system, the organic phase plays as a reservoir, an extractant, and a solvent of water-insoluble substrates and products.

solvent (a top phase), fungus–MS mat (a middle phase), and liquid medium (a bottom phase). In all systems, almost all fungal cells suspended in a liquid medium are floated together with numerous PAN-MS onto the surface of the liquid medium to form a physically strong fungus–MS mat onto the medium.

In this article, the principles, characteristics, and applications of the LSI, L–L IBR, and Ext-LSI systems are reviewed in detail. Furthermore, the relationship between the physicochemical properties (electric charge and hydrophobicity) of an organic–aqueous interface and the morphological, physiological and biochemical properties of fungal cells is discussed.

Liquid-surface immobilization (LSI) system

In many cases, the SmC causes strong carbon catabolite repression in the presence of excess easily metabolizable carbon sources, such as glucose, fructose, and sucrose[13]. The unfavorable phenomenon, which is brought about by some repressor proteins such as CreA[14], induces a decrease in enzyme and metabolite productivities. Furthermore, targeted enzymes produced by fungi are generally digested by inherent proteases produced simultaneously in the SmC[15]. Both phenomena are serious problems for many microbial industries.

On the other hand, it has been reported that the unfavorable phenomena above-mentioned can be avoided by adopting other traditional cultivation system, the SSC[16,17]. Indeed, the SSC generally enables superior productivities of enzymes and metabolites compared with the SmC[16,17]. The morphology control of fungal cells in SSC is generally easier compared with the SmC. While fungal cells form unnatural morphologies such as filamentous, clump, or pelleted forms in the SmC, the SSC generally leads fungal cells to natural differentiation to vegetative and aerial hyphae, sporangia, and spores. Thus, the SSC brings about the superior productivities of targeted enzymes and metabolite.

Recently, we have observed that the productivities of lipase by *Absidia coerulea* IFO 4423[7], and of xylanase[18] and β-glucosidase[8] by *Aspergillus oryzae* RIB40 in LSI system are significantly higher than those in the SmC. It is easily assumed that the production of inherent proteases is repressed in the LSI system likely to the SSC[6]. Moreover, fungal cells trapped in an MS layer differentiate to natural forms, i.e., vegetative and aerial hyphae, sporangia, and spores likely to the SSC. The natural differentiation in the LSI system must be favorable to display the inherent abilities.

Extractive liquid-surface immobilization (Ext-LSI) system

Fungal cells can grow and differentiate on an interface between a liquid medium and a harmless hydrophobic organic solvent such as *n*-alkanes, middle-chain fatty esters and ethers, and lowly viscous dimethyl silicone oil by the aid of the MS. Firstly, the fungal cells located on the organic–aqueous interface were applied to the production of a fungicidal secondary metabolite, 6-pentyl-α-pyrone (6PP)[9] which was useful for coconut-aroma. 6PP was produced by some *Trichoderma* spp. in the SmC[19], aqueous two–liquid–phase [20], organic–aqueous two–liquid–phase[21], the LSC[22], and the SSC systems[23]. However, the accumulation of 6PP was generally limited to very low levels because of its strong fungicidal

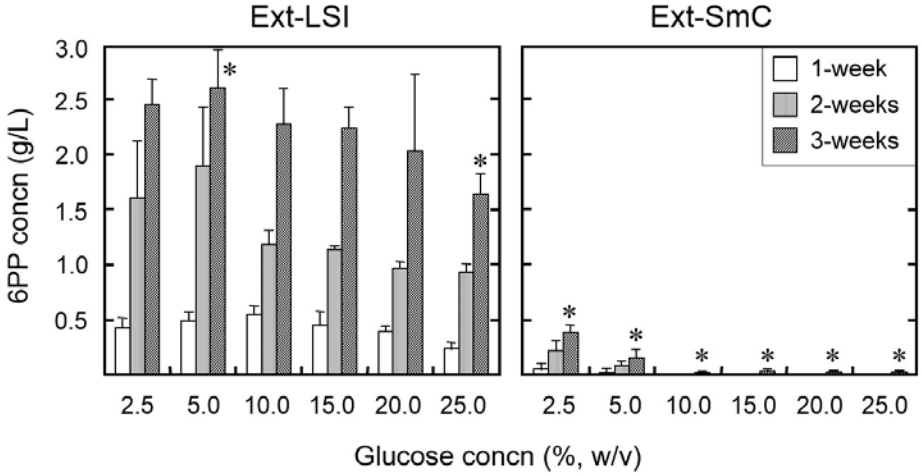

Fig. 2. Derepression of carbon catabolite repression in Ext-LSI system. The incubation was done at 25 °C without shaking. While strong carbon catabolite repression by excess glucose appeared in the Ext-SmC system, Ext-LSI system could alleviate the unfavorable phenomenone.

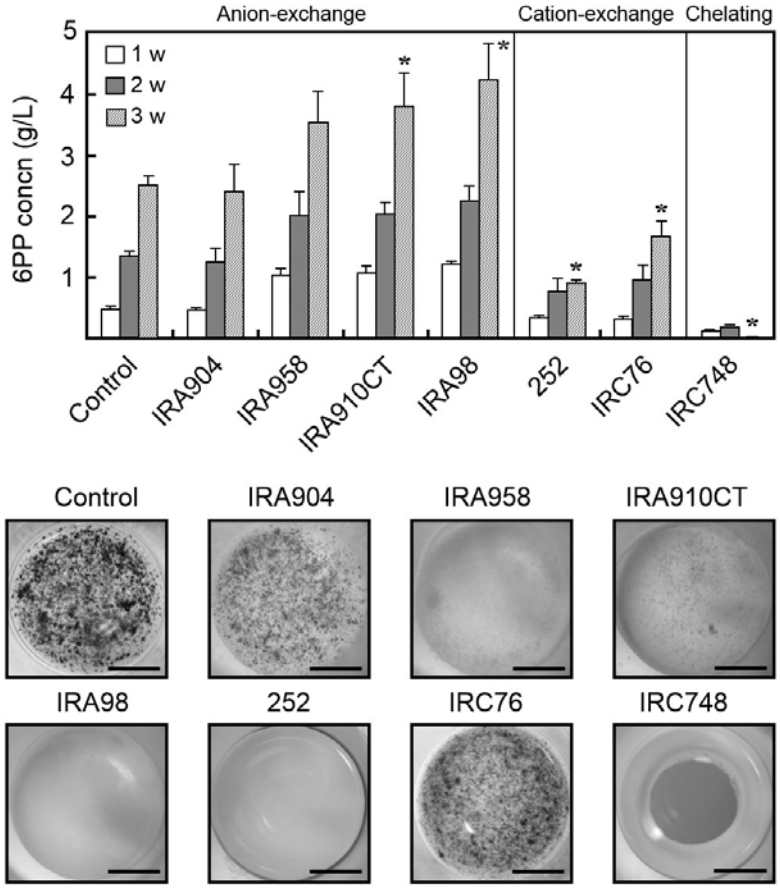

Fig. 3. Effect of mixing of ion-exchange resin microparticles into a MS layer on the production of 6-pentyl-α-pyrone (6PP) and the formation of spores of *Trichoderma atroviride* AG2755-5NM398. Control: PAN only; anion-exchange resins: IRA904 (total capacity, ≥ 0.65 meq/g), IRA958 (≥ 0.80), IRA910CT (≥ 1.00), IRA98 (≥ 1.35); cation-exchange resins: 252 (≥ 1.80), IRC76 (≥ 3.90); chelating resin: IRC748 (≥ 1.35). All ion-exchange resins (50 μm pass) were mixed in an MS layer at the ratio of 5-fold weight on PAN-MS. Thus, thickness of the fungus–MS-resin layer was approximately 3 mm in all cases. The incubation was performed at 25 °C without shaking.

activity (MICs = 0.2–0.5 g/L)[19,24]. Indeed, the maximum accumulations of 6PP produced in the SmC and the SSC are only 474 mg/L and 3 g/kg, respectively[25,26].

To the contrary, the accumulation of 6PP in an organic phase (dimethyl silicone oil) in the Ext-LSI system reached to 7.1 g/L because the organic phase played as a reservoir and an extractant of toxic 6PP[9]. Recently, it was observed that fungal cells located on the organic–aqueous interface in the Ext-LSI system are liberated from carbon catabolite repression to produce much 6PP compared with traditional extractive fermentation (Fig. 2)[10]. It is concluded that the Ext-LSI system may be liberate fungal cells from product toxicity, feed-back inhibition, and catabolite repression to afford large quantities of lipophilic secondary metabolites.

Ballooned PAN-MS (MMF-DE-1, Matsumoto Yushi Seiyaku, Co., Ltd., Osaka) is electrically neutral[27] and has relatively hydrophilic surface (contact angle, 50 °). The electric charge of an MS layer can be modified by mixing ion-exchange resin microparticles. All ion-exchange resins (50 μm pass) tested were mixed in the MS layer at the ratio of 5-fold weight on the PAN-MS. Thus, thickness of the fungus–MS-resin layer was approximately 3 mm in all cases. In the case of mixing cation-exchange (252 and IRC76; Organo Co., Ltd., Tokyo) and chelating resin (IRC748) microparticles into the PAN-MS layer, fungal morphology and fermentative activity drastically changed as shown in Fig. 3[28].

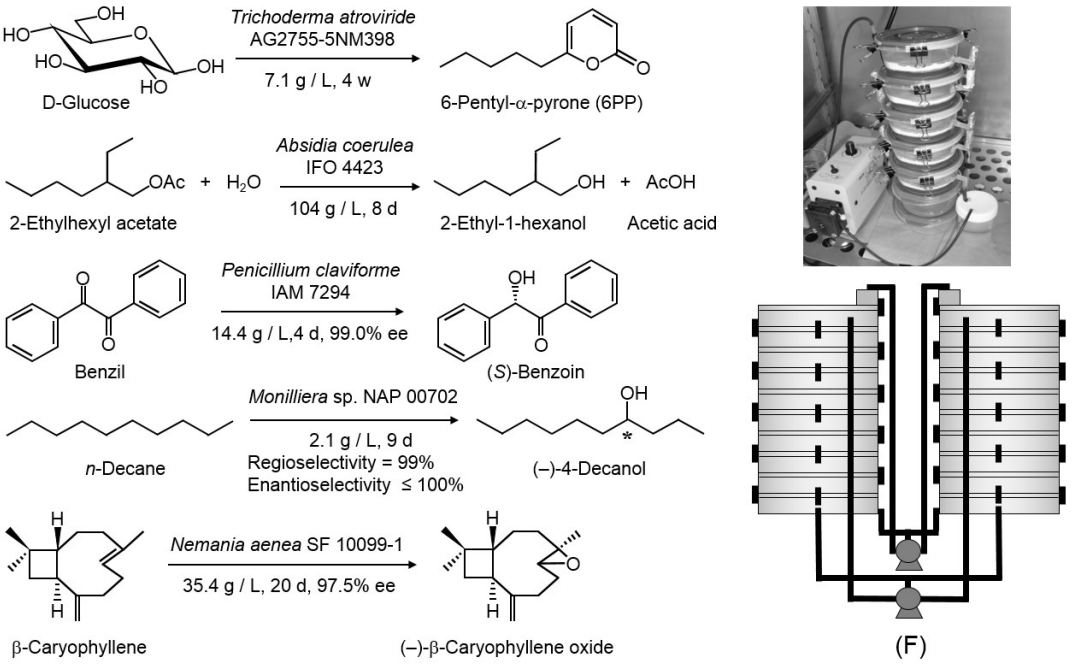

Fig. 4. Application of L–L IBR to various fungal bioconversions and a photograph and a schematic diagram of a multi-story L–L IBR system. The system consisted of stacked reactor units, diaphragm-pump, and solvent-circulation lines to connect an organic phase.

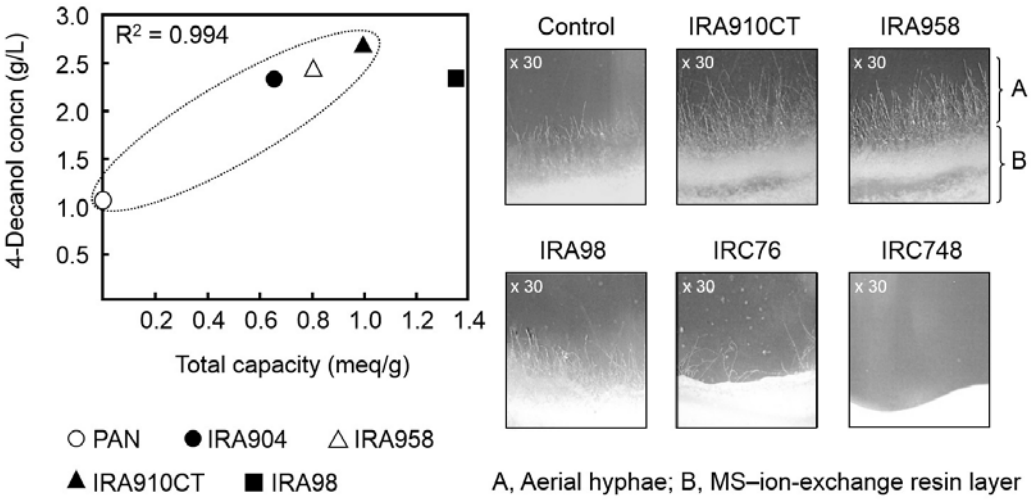

Fig. 5. Effect of mixing of ion-exchange resin microparticles into a MS layer on production of (–)-4-decanol and the formation of aerial mycelia of *Monilliera* sp. NAP 00702. All ion-exchange resins (50 μm pass) were mixed in an MS layer at the ratio of 5-fold weight on PAN-MS to form approximately 3 mm-thickness of fungus–MS-resin layer. The correlation coefficient (R^2) was calculated among PAN, IRA904, IRA958, and IRA910CT. The vertical sections of MS-layers were photographed with a stereomicroscope. The incubation was carried out at 25 °C without shaking for 14 days.

For the addition of anion-exchange resin microparticles (IRA904, 958, 910CT, and 98) into the PAN-MS layer, the higher the total capacity of anion-exchange resin, the higher the 6PP production and the lower the spore formation were observed. The correlation coefficient (R^2) between the total capacity and the 6PP production reached 0.724. On the other hand, the addition of cation-exchange (252 and IRC76) and chelating (IRC748) resin microparticles decreased the 6PP production, and IRC748 in particular exhibited the strongest inhibitory activity on fungal growth. Thus, it is concluded that the quaternary ammonium ion of the anion-exchange resin microparticles enhanced the production of 6PP[28].

As for the effects of anion-exchange resin on microbial cells, some reports have been published so far. It has been reported that some morphological, physiological, and biochemical properties of microorganism, such as oxidation activity[29], optimum pH for the bacterial growth[30], spore formation[31], and growth rate[30,32], drastically change in the presence of anion-exchange resin. Although the exact mechanisms of the phenomena caused by anion-exchange resin are not clear at the present time, it is assumed that electric signal generated by adhesion onto the charged resin surface causes abovementioned interesting phenomena.

Liquid–liquid interface bioreactor (L–L IBR)

The L–L IBR is defined as a bioreactor using living fungal cells located in an MS layer (a middle phase) formed on an interface between an aqueous phase (a bottom phase; liquid medium) and an organic phase (a top phase; hydrophobic organic solvent) likely to the

Ext-LSI system. In the L–L IBR, lipophilic substrates were dissolved in the organic phase and converted to lipophilic products (accumulated in the organic phase) or to hydrophilic products (accumulated in the aqueous phase). The reactor exhibited various merit for the fungal bioconversion, such as the alleviation of toxicity of substrates and products and much accumulation of poisonous products in the organic phase, high dissolution of lipophilic substrates and products into the organic phase, high oxygen supply in stationary cultivation, excellent regio- and stereoselectivities, and very wide applications. The bioreactor has been applied to various fungal bioconversions, such as hydrolysis[7,33], reduction[11], hydroxylation[34-36], and epoxidation[12] and has exhibited the excellent productivity of various useful chemicals compared with the SmC and an organic–aqueous two–liquid–phase system (Fig. 4).

Among these applications of the L–L IBR to fungal bioconversion, regio- and stereoselective hydroxylation of *n*-decane to (–)-4-decanol by *Monilliera* sp. NAP 00702 is very interesting. The regio- and stereoselectivities of the reaction reached 99% and almost 100% ee, respectively[34]. The discovery firstly overturned the established theory insisting on the shortage of regio- and stereoselectivities of microbial subterminal hydroxylation[37–39].

As for the hydroxylation of *n*-decane by *Monilliera* sp. NAP 00702, recently, it was reported that interfacial charge in the L–L IBR drastically affected the growth and the hydroxylation activity of the fungal cells[35]. Although the addition of cation-exchange (IRC76; Organo Co., Ltd., Tokyo) and chelating resins (IRC748) strongly inhibited the fungal

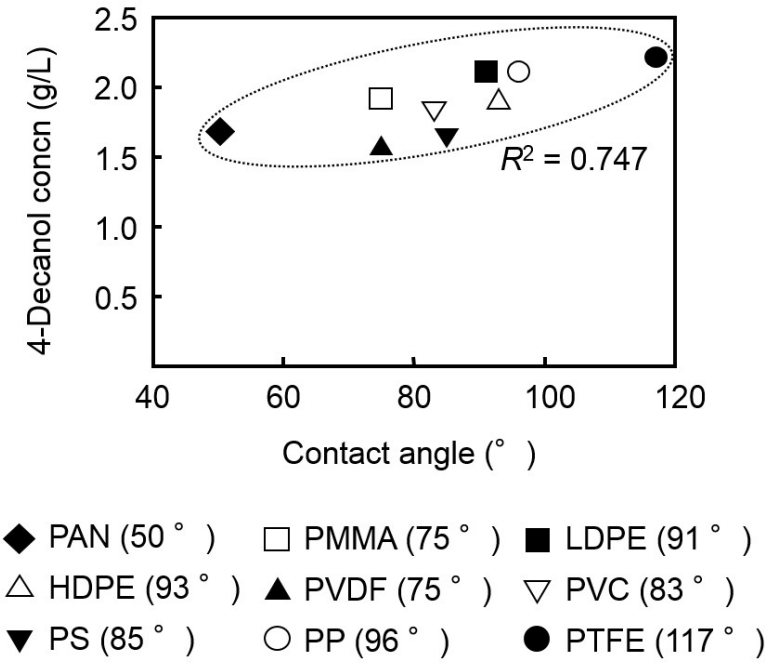

Fig. 6. Relationship between hydrophobicity of resin added and (–)-4-decanol production. All hydrophobic resins (50 μm pass) were mixed in an MS layer at the ratio of 5-fold weight on PAN-MS to form approximately 3 mm-thickness of fungus–MS-resin layer. The incubation was done at 25 °C without shaking for 14 days

growth, the addition of anion-exchange resin (IRA98, 910CT, 958, and 904) led the significant increase of hydroxylation activity with the enhancement of hyphal growth (Fig. 5). However, the effect of the strongest anion-exchange resin, IRA98 (total capacity ≥ 1.35 meq/g), declined the hydroxylation activity to some extent. The correlation coefficient (R^2) between the 4-decanol production and the total anion-exchange capacity of PAN only, PAN+IRA904 (1:5), PAN+IRA958 (1:5), and PAN+IRA910CT (1:5) was 0.994[35]. It is concluded that the moderate anion-exchange capacity (total capacity = 1.0 meq/g) in an MS layer enhanced the growth and hydroxylation activity of fungal cells in addition to fermentative activity, while the cation-exchange and chelating activities in the MS layer are unfavorable to fungal growth, hydroxylation and fermentative activities.

Recently, it was reported that the interfacial hydrophobicity in an MS layer of the L–L IBR system also affected the subterminal hydroxylation of *n*-decane to (–)-4-decanol by *Monilliera* sp. NAP00702. As shown in Fig. 6, the mixing of hydrophobic resin particles such as polypropylene (PP) and polytetrafluoroethylene (PTFE) in the PAN-MS enhanced the hydroxylation activity. The positive correlation between the contact angle of hydrophobic resin and (–)-4-decanol production was observed (R^2 = 0.747). However, the mixing of hydrophobic resin microparticles did not accelerate hyphal growth.

Concerning the effect of hydrophobic resin on microbial cells, some interesting phenomena have been reported. The addition of hydrophobic resin into microbial cell suspension leads some unique morphological, physiological, and biochemical characteristics of fungal cells, such as increase of growth rate[40] and respiration activity[41], decrease of the rate of glucose uptake[41], initiation of the formation of spores[42] and extracellular matrix[43]. It is also known that hydrophobic organic solvents, such as *n*-dodecane and *n*-tetradecane, lead the increase of respiration activity and the decrease of the rate of glucose uptake[44].

Although the exact mechanisms of the phenomena caused by hydrophobic resin are not clear at this time, it is assumed that the physicochemical properties of cell surface change by adhering onto surface of hydrophobic substrata. Indeed, it was reported that the surface hydrophobicity of fungal cells was remarkably increased by the adaptation to hydrophobic circumstance[45]. It is expected that mechanisms of the change of morphological, physicochemical, and biochemical characteristics of microbial cells containing fungal cells are clarified and reflected to the industrial application of microorganisms.

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Communicated by Ueno Hiroshi