Production of Valuable Lipophilic Compounds by Using Three Types of Interface Bioprocesses: Solid–Liquid Interface Bioreactor, Liquid–Liquid Interface Bioreactor, and Extractive Liquid-Surface Immobilization System

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Abstract: Bioconversions such as enzymatic and microbial transformations are attractive alternatives to organic synthesis because of practical advantages such as resource conservation, energy efficiency, and environmentally harmonic properties. In addition, the production of secondary metabolites through microbial fermentation is also useful for manufacturing pharmaceuticals, agricultural chemicals, and aroma compounds. For microbial production of useful chemicals, the authors have developed three unique interfacial bioprocesses: a solid–liquid interface bioreactor (S/L-IBR), a liquid–liquid interface bioreactor (L/L-IBR), and an extractive liquid-surface immobilization (Ext-LSI) system. The S/L-IBR comprises a hydrophobic organic solvent (upper phase), a microbial film (middle phase), and a hydrophilic gel such as an agar plate (lower phase); the L/L-IBR and the Ext-LSI consist of a hydrophobic organic solvent (upper phase), a fungal mat with ballooned microspheres (middle phase), and a liquid medium (lower phase). All three systems have unique and practically important characteristics such as utilization of living cells, high concentration of lipophilic substrates/products in an organic phase, no requirement for aeration and agitation, efficient supply of oxygen, easy recovery of product, high regio- and stereoselectivity, and wide versatility. This paper reviews the principle, construction, characteristics, and application of these interfacial systems for producing lipophilic compounds such as useful aroma compounds, citronellol-related compounds, β-caryophyllene oxide, and 6-penty-α-pyrone.

Key words: interface bioreactor, liquid-surface immobilization, fungal bioconversion, terpenoid conversion, pyrone fermentation

1 Introduction

Biocatalysts such as enzymes, as well as whole cells of microorganisms, are known to catalyze many regio- and stereoselective reactions such as hydrolysis, esterification, and oxidoreduction¹ ². Microbial transformation is therefore widely applied in various industries, including pharmaceutical, cosmetic, food, and petrochemical industries, as a solution to critical problems such as environmental pollution and resource and energy conservation. However, two grave obstacles, namely, water-insolubility and biocidal activity of substrates and/or products, limit the industrial applications of these biocatalysts.

To overcome the first obstacle, many approaches such as addition of surfactants³ or water-miscible⁴ and immiscible organic solvents⁵, complexation with cyclodextrins⁶, use of fungal dry mycelia⁷, and immobilization of whole cells in organic solvents⁸ have been reported. In addition, solvent-tolerant bacteria such as Pseudomonas⁹, Bacillus¹⁰, Acinetobacter¹¹, and Staphylococcus¹², as well as some yeasts¹³ have been isolated and characterized. These sol-
vent-tolerant microorganisms are expected to be genetically superior sources of biocatalysts for non- or micro-aqueous bioconversion and producers of solvent-stable enzymes.

However, the efficacy of additives such as surfactants and water-miscible and immiscible solvents is limited by their toxicity towards many microorganisms. Addition of high concentrations of these additives and organic solvents can cause serious damages to microbial cells, including decomposition of cell membrane, inhibition of nutrient uptake, inhibition of membrane permeability, inhibition of oxygen uptake, inhibition of metabolism, inhibition of DNA synthesis, inhibition of respiration, denaturation of intercellular enzymes, inhibition of cell division, acidification of cells, and release of intercellular components such as K⁺, proteins, and DNA. Therefore, the surfactant or organic solvent must be carefully selected to minimize cell damage.

Complexation of water-insoluble substrates with cyclodextrins is another method to overcome the challenge of water-insolubility. However, removal of cyclodextrins from a broth is troublesome and costly, because chromatographic procedures are necessary in many cases. Furthermore, the use of free and immobilized cells in an organic phase is strictly restricted to coenzyme-independent bioconversions such as esterification and hydrolysis. Although some coenzyme-dependent reactions such as reduction and biodegradation with lyophilized and/or immobilized microbial cells have been reported in an organic phase, the organic solvent promptly inactivated the regeneration of enzymes in the microbial cells.

The second challenge is the biocidal activity of substrates and/or products. Organic–aqueous two-liquid phase systems are known to be effective in reducing the toxicity of substrates and/or products. Two-liquid phase systems have been used in many microbial transformations such as dehydrogenation, reduction, epoxidation, and hydroxylation. It was also reported that two-liquid phase systems are effective for side-chain cleavage of steroids and biodegradation of harmful substances. Organic–aqueous two-liquid phase systems, which are classified into dispersion and dispersion-free systems, are known to be effective for alleviating solvent toxicity. However, they have some disadvantages, including phase toxicity, transfer of nutrients from the aqueous phase into the organic phase, and emulsion formation. Depending on the agitation rate, phase toxicity can cause disruption of the cell membrane. Therefore, more practical and efficient microbial transformation devices need to be developed. In this paper, two unique interfacial biotransformation systems, S/L-IBR and L/L-IBR, and an interfacial fermentation system, Ext-LSI system, are introduced and reviewed.

2 Solid–liquid interface bioreactor (S/L-IBR)

2.1 Principle and characteristics of S/L-IBR

S/L-IBRs are interfacial microbial transformation systems that allow microbial cells to grow on an interface between a hydrophilic carrier and a hydrophobic organic solvent. Nutrient agar plates are often used as the hydrophilic carrier, and middle chain n-alkanes such as n-decane and n-dodecane or middle chain alkyl ethers such as di-n-hexyl ether and isoamyl ether are used as the hydrophobic organic solvent. These organic solvents can dissolve more oxygen than an aqueous phase. Thus, during cultivation with S/L-IBR, the culture is generally allowed to stand. Interestingly, the S/L-IBR can drastically reduce the toxicity of lipophilic compounds such as toluene and cholesterol, as the organic phase acts as a reservoir and a diluent of the toxic organic compounds. Thus, the concentration of the substrate and the accumulation of the product can be drastically enhanced in accordance with the toxicity alleviation effect on the solid–liquid interface.

Fig. 1 The principle of S/L-IBR. A microbial film formed on an interface between an agar plate and a hydrophobic organic solvent efficiently catalyzes various microbial transformations of lipophilic substrates. The concentration of substrate and the accumulation of product can be drastically enhanced in accordance with the toxicity alleviation effect on the solid–liquid interface.

- Alleviation of substrate and product toxicities
- Solubilization of substrate and product
- Easy recovery of product
- Efficient oxygen supply
- Wide application
- Accumulation of harmful by-product in a carrier
- Difficulty of pH control in a carrier
- Difficulty of nutrient supplementation

☆ Advantage  ★ Disadvantage
The S/L-IBR has been applied in many microbial transformations. Almost all bacteria, actinomycetes, yeasts, and fungi can actively grow on the carrier/solvent interface and efficiently catalyze various microbial reactions such as hydrolysis, esterification (Fig. 2), oxidation (Fig. 3), reduction, and biodegradation (Fig. 4).

The S/L-IBR has many characteristics of practical importance, such as high concentrations of substrates and products in the organic phase, solubilization of water-insoluble substrates and products, efficient supply of oxygen, easy recovery of products, and wide application (Fig. 1). The protocol for using S/L-IBR is generally easy, as aeration, agitation, and solvent-extraction are unnecessary.

The S/L-IBR also has some practical disadvantages. For example, harmful polar by-products such as organic acids may accumulate in the culture. In addition, it is difficult to control the pH in a carrier, and nutrient supplementation is difficult. These problems stem from the fact that the aqueous phase in a carrier cannot be modified (Fig. 1).

2.2 Application of S/L-IBR to hydrolysis, esterification, and transacetylation

Enzymatic or microbial hydrolysis\(^{36}\), esterification\(^{37}\), and transesterification\(^{38}\) have been frequently used for the optical resolution of various enantiomers. The S/L-IBR has been used for the hydrolysis of 2-ethylhexyl acetate into 2-ethyl-1-hexanol by *Candida cylindracea* ATCC 14830, which was growing on an interface between a synthetic polymer gel (polyvinyl alcohol–glutaraldehyde/alginate–Ca\(^{2+}\)) and neat 2-ethylhexyl acetate. Although the enantioselectivity of the reaction was very low, 280 g/L of 2-ethyl-1-hexanol was obtained in 10 days (Fig. 2A)\(^{39}\). Additionally, the enantiofacially selective hydrolysis of 2-benzycyclohexane enol ester into (R)-2-benzycyclohexaneone by *Pichia farinosa* IAM 4682 was also achieved with the S/L-IBR. The enantiomeric excess (ee) and the molar yield of (R)-2-benzycyclohexaneone reached 80% and 50%, respectively (Fig. 2B)\(^{40}\).

For the esterification of cholesterol by octanoic acid (Fig. 2C)\(^{33}\), a unique microbial transformation system for producing acetate esters, the double coupling system, was de-
veloped. In this system, yeasts such as Hansenula and Pichia could produce various aliphatic and aromatic acetate esters through transacetylation of the corresponding primary alcohols by acetyl coenzyme A, which was produced via the metabolism of glucose and free fatty acids. This reaction is catalyzed by alcohol acetyltransferase, which is known to catalyze the acetylation of isoamyl alcohol by acetyl-CoA in sake production in Saccharomyces. The authors have firstly applied the enzyme to organic synthesis.

The AATFase of Pichia kluyveri IFO 1165 could catalyze the enantioselective acetylation of RS-citronellol by acetyl-CoA to produce (S)-citronellyl acetate and (R)-citronellol at an E value of 40. The AATFase of Hansenula saturnus IFO 0809, and the enantioselective oxidation of (RS)-citronellol to (S)-citronelic acid by Hansenula saturnus IFO 0809, and the enantioselective oxidation of (RS)-citronellol to (R)-citronellal by Rhodococcus equi IFO 3730.

2.3 Application of S/L-IBR in oxidative reactions

While alcohol dehydrogenase (ADH) is useful for producing aldehydes or ketones from alcohols, aldehyde dehydrogenase (ALDH) is useful for preparing carboxylic acids from aldehydes. The S/L-IBR was used for the ADH-dependent oxidation of methylcyclohexanols by Rhodococcus equi IFO 3730. 2-Methylcyclohexanone was regioselectively synthesized from 2-methylcyclohexanol with a high accumulation rate of 11 g/L, despite their high biotoxicities.

The superior ability of S/L-IBR to alleviate toxicity was also observed during the oxidative synthesis of decanoic acid, an antiseptic, from 1-decanol by Issatchenkia scutulata var. scutulata IFO 10070. Middle chain 1-alkanols (C₆–C₁₀) are known to have high molecular toxicity, and decanoic acid is more biotoxic against Gram-positive bacteria, yeast, and fungi. In these reports, the minimal inhibitory concentration (MIC) of decanoic acid was found to be less than 1 mM (0.172 g/L); however, with S/L-IBR, accumulation of high amounts of decanoic acid (32.5 g/L) was observed.

While many yeasts such as Hansenula and Candida catalyze (S)-selective oxidation of citronellol to produce (S)-citronelic acid and (R)-citronellal, some acti-
nomycetes such as *Rhodococcus equi* catalyze *(R)*-selective oxidation of citronellol to yield *(R)*-citronellal and *(S)*-citronellol (Fig. 3D)\(^{55,56}\). Using *Geotrichum candidum* as a biocatalyst, 65 g/L of citronelic acid was produced in 13 days, despite the high biotoxicity of the substrate and product. Thus, by combining enantioselective transacylation and oxidation by select microorganisms, *(R)*-citronellol, *(R)*-citronellal, *(R)*-citronelic acid, *(S)*-citronellol, and *(S)*-citronelic acid were synthesized with high values of ee and yield\(^{27}\).

### 2.4 Application of S/L-IBR in reductive reactions

The reductase enzyme of microorganisms is a versatile and useful enzyme for preparing chiral alcohols by asymmetric reduction\(^{28}\). S/L-IBR has been used for reducing ketones, as microbial asymmetric reduction is important for preparing chiral alcohols in industries. Sugai *et al.* had applied S/L-IBR in asymmetric reduction of 6-methyl-5-heptene-2-one to *(R)*-sulcatol, a useful chiral synthon, by *Pichia farinosa* IAM 4682 (Fig. 4A)\(^{50}\). The ee and conversion yield of *(R)*-sulcatol were 90% and 51%, respectively.

Ethyl *(R)*-2-hydroxy-4-phenylbutanoate (EOPB) is an important intermediate in the synthesis of angiotensin-converting enzyme (ACE) inhibitors such as enalapril and Lisinopril\(^{50}\). S/L-IBR has been used for producing *(R)*-EOPB through microbial asymmetric reduction of a prochiral precursor, ethyl 2-oxo-4-phenylbutanoate (EOPB) (Fig. 4B)\(^{51}\). An yeast, *Candida holmii* KPY 12402, could *(R)*-preferentially reduce EOPB to *(R)*-EOPB in an n-decane layer. The overall yield, chemical purity, and ee of the *(R)*-EOPB were 58%, 99%, and 90%, respectively\(^{51}\).
Furthermore, methyl ursodeoxycholate (Me-UDCA), which is widely used as a cholesterol gallstone-dissolving agent\(^{39}\), was synthesized by asymmetrically reducing methyl 7-ketolithocholate (Me-7KLC) with an anaerobic bacterium, *Eubacterium aerofaciens* JCM 7790 (Fig. 4C)\(^{63-65}\). Anaerobic conditions in the S/L-IBR were regulated using a set of GasPak\textsuperscript{TM} catalyst and an oxygen indicator. The productivity of Me-UDCA was enhanced by a combination of glucose and mannitol as the carbon and hydride sources and by using phosphate buffer as the aqueous phase in the gel\(^{65}\).

2.5 Application of S/L-IBR in biodegradation

S/L-IBR has been applied in the synthesis of (S)-ibuprofen, a pharmacologically active anti-inflammatory drug\(^{46}\), via enantioselective biodegradation of racemic ibuprofen by a yeast, *Trichosporon cutaneum* KFY 30802 (Fig. 4D)\(^{67}\). The addition of hydroglobe (10 mM) into a hydrophilic carrier effectively increased the ee and repressed excess degradation of (S)-ibuprofen (E value: 9.3).

S/L-IBR has also been used for the biodesulfurization of dibenzothiophene into 2-hydroxybiphenyl by *Rhodococcus erythropolis* ATCC 53968 (Fig. 4E)\(^{68}\). The development of a practical process for the biodesulfurization of organo-sulfur compounds such as dibenzothiophene in fossil fuels was considered necessary, as the sulfur dioxide formed during the combustion of fossil fuels caused acid rains\(^{80}\). It was observed that a biofilm of the *R. erythropolis* ATCC 53968 strain peeled off from the surface of the agar plate to form an organic phase (n-dodecane or n-tetradecane). To overcome this challenge, screening was performed for a UV mutant that could strongly adhere onto the carrier surface. The selected UV mutant (UM-021) could effectively catalyze the biodesulfurization, and the biofilm did not peel off from the carrier surface. The final removal rate of dibenzothiophene (2 mM) by the mutant was 95%, after still cultivation for 5 days\(^{68}\).

2.6 Practical disadvantages of S/L-IBR

As described in the previous sections, S/L-IBR generates high yield and ee of products, in addition to many other merits, as shown in Fig. 1. However, S/L-IBR also has some fatal disadvantages. These disadvantages result from the fact that the aqueous phase in the hydrophilic carrier of the S/L-IBR cannot be modified. For instance, harmful by-products such as organic\(^{39}, \)\(^{63}\) and inorganic acids\(^{68}\) could accumulate in the hydrophilic carrier, the carrier pH could decrease, and the nutrients may get depleted\(^{46}\). Therefore, the development of a new type of interface bioreactor with a modifiable aqueous phase is necessary. Thus, the next generation of interface bioreactors, the L/L-IBR has an aqueous phase in the hydrophilic carrier that can be modified or exchanged.

3 Liquid–liquid interface bioreactor (L/L-IBR)

3.1 Principle and characteristics of liquid-surface immobilization (LSI) systems

Balloon microspheres (MS), used as fillers in composite materials such as paints and putty, have micro-scale diameters and very low density\(^{70}\). MS were used as key materials for generating fungal mats on the surface of an aqueous phase (liquid medium). Fungal cells, mycelia, and spores could float on liquid surfaces with a crowd of MS, as shown in Fig. 5A. Many fungi were found to form thick fungus–MS mats with many aerial hyphae and spores, after only 3 days of stationary cultivation (Figs. 5B, 5C). This interfacial cultivation system for fungi was called the liquid-surface immobilization (LSI) system (Fig. 6A). The fungus–MS mat was physically strong enough to maintain its form, even if the vessel was overturned (Fig. 5D)\(^{71}\). Fungus–MS mats were also formed on wider liquid surfaces in stainless steel trays (30×30 cm).

Matsumoto Yushi-Seiyaku, Co., Ltd., Osaka produces different types of MS, including MFL-80GCA (CaCO\(_3\)-coated; mean diameter, 21 μm; density, 0.20)\(^{72}\), MFL-80GTA (talc-coated; mean diameter, 37 μm; density, 0.20), and MMF-DE-1 (non-coated; mean diameter, 30 μm; density, 0.06). MS are made of polyacrylonitrile; they are synthesized through polymerization of emulsions or suspensions, followed by thermal expansion\(^{70}\). A recently developed porous microparticle, Advacend HB-2051 (polymethylmethacrylate; diameter, 20 μm; Sekisui Chemical Co., Ltd., Tokyo) is currently being used in our laboratory.

The fungus–MS mat has been used for the production of two useful enzymes, xylanase\(^{72}\) and β-glucosidase\(^{73}\), by a transformant of *Aspergillus oryzae* RIB40 (Fig. 6A). Both the enzymes were produced efficiently, compared to the conventional cultivation system, submerged cultivation (SmC). Similar to solid-state cultivation, the digestion of the enzymes by inherent proteases was alleviated in the LSI system\(^{74}\).

3.2 Principle and characteristics of L/L-IBR

As mentioned in the previous section, the LSI system is useful for producing enzymes and water-soluble metabolites\(^{71-73}\). A unique organic–aqueous interfacial cultivation system can be constructed by adding a harmless hydrophobic organic solvent onto a fungus–MS mat. In this system, the aqueous phase under the fungus–MS mat can be easily controlled or exchanged, unlike in the S/L-IBR. However, the organic phase above the fungus–MS mat acts as a reaction solvent, similar to the S/L-IBR. This interfacial microbrial transformation system was called the liquid–liquid interface bioreactor (L/L-IBR; Fig. 6B)\(^{71}\), and has been used in many types of fungal bioconversions.

The L/L-IBR has shown excellent product accumulation and regio- and stereo-selectivity in many cases; the protocol for L/L-IBR, similar to that for S/L-IBR, is easy, as aeration,
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Fig. 5 Formation of a fungal mat together with ballooned microspheres. Numerous number of polyacrylonitrile microspheres (MS; diameter, 20–100 μm; density, 0.03–0.20) suspended in an aqueous phase rapidly come to the liquid-surface together with fungal cells (A). The fungal cells immobilized into the MS layer grow and differentiate to vegetative and aerial hyphae, sporangia, and spores during appropriate cultivation period to form a physically strong fungal cells–MS mat on the liquid-surface (B, C). The fungus–MS mat did not collapse even by rolling of the vessel (D).

Fig. 6 A genealogy of three types of interfacial cultivation/application systems using ballooned microsphere (MS). In the LSI system (A), fungal cells produce hydrophilic metabolites and/or enzymes into an aqueous phase. The L/L-IBR system (B) affords hydrophobic products into an organic phase via interfacial biotransformation of lipophilic substrate dissolved in the organic solvent. In the Ext-LSI system (C), hydrophobic metabolites are produced via interfacial fermentation to accumulate into an organic phase.
agitation, and solvent extraction are not required (Fig. 7). The concentration of the product and the residual substrate in the organic phase can be directly assessed using HPLC and GLC, without extraction and condensation.

3.3 Application of L/L-IBR in hydrolytic reactions

L/L-IBR was first used for the hydrolysis of 2-ethylhexyl acetate into 2-ethyl-1-hexanol by *Absidia coerulea* NBRC 442371. The type culture fungus was screened from approximately 400 strains using the S/L-IBR, to yield 51.1 g/L of 2-ethyl-1-hexanol from a 50% (w/v) solution of 2-ethylhexyl acetate in *n*-decane in 5 days. The hydrolytic reaction was performed using submerged cultivation (SmC), organic–aqueous two-liquid phase system (TLP), S/L-IBR, and L/L-IBR (MS, CaCO3-coated MFL-80GCA). As shown in Fig. 8, the L/L-IBR showed the most efficient performance among the four studied systems; it yielded 107 g/L of 2-ethyl-1-hexanol after 8 days of cultivation71. Later, the performance of L/L-IBR was attributed to the activating lipase of *A. coerulea*, which used Ca2+ from the CaCO3-coated MFL-80GCA MS75, similar to the other lipases76.

3.4 Application of L/L-IBR in reductive reactions

Next, L/L-IBR was used in the asymmetric reduction of benzil to (S)-benzoin by *Penicillium claviforme* IAM 7294 (Fig. 9)77. Although (S)-benzoin, a useful chiral auxiliary, has been produced through asymmetric reduction by fungi and bacteria, the low solubility of benzil and the toxicity of benzil and benzoin have limited the practical applications78. Therefore, we tried to achieve a high concentration and ee of (S)-benzoin using L/L-IBR. Among the 25 fungal strains tested with S/L-IBR, *Penicillium claviforme* IAM 7294 was the most effective; it efficiently reduced 3% benzil to yield 12.9 g/L (S)-benzoin (95.2% ee) in di-*n*-hexyl ether in 3 days77.

Reduction was also compared among four cultivation systems, SmC, TLP, S/L-IBR, and L/L-IBR (MS, MFL-80GCA) systems. As shown in Fig. 9, the L/L-IBR showed the highest accumulation and ee of (S)-benzoin, and many needle-like crystals of excess (S)-benzoin were found on the fungus-MS mat. Thus, the efficacy of the L/L-IBR for asymmetric microbial reduction was also confirmed.
subterminal hydroxylation of \( n \)-alkanes. Regio- and stereoselective subterminal hydroxylation of \( n \)-groups hydroxylates the terminal methyl and C-2 methylene -alkanes, \( n \)-droxylation of \( n \)-hexadecanols isms and/or subterminal hydroxylation by many microorganisms are known to be decomposed by terminal sites that occur at activated positions, generally benzyl and aryl groups. The cytochrome P450 system is known to preferentially hydroxylate a terminal methyl group of \( n \)-alkanes, \( n \)-Cladosporium resinae sp. and \( n \)-Staphylococcus epidermidis. There have been no reports of fungal hydroxylation of \( n \)-alkanes such as \( n \)-decane and \( n \)-hexadecane. It was found that the hydroxylation activity of the strain was significantly affected by hydrophobicity and charge on the surface of the synthetic polymer microparticles mixed into the polycrylonitrile MS layer.

Further investigations were carried out for the regio- and stereoselective subterminal hydroxylation of \( n \)-decane by \( n \)-Monilliera sp. NAP 00702, in the L/L-IBR. It was found that the hydroxylation activity of the strain was significantly affected by hydrophobicity and charge on the surface of the synthetic polymer microparticles mixed into the polycrylonitrile MS layer.

Many reports have been published on the relationship between surface or interface hydrophobicity and microbial cells. For example, the adsorption of \( n \)-Escherichia coli cells onto droplets of \( n \)-alkanes such as \( n \)-decane and \( n \)-hexadecane was reported to cause an increase in respiration and a decrease in glucose uptake. The same phenomenon was also confirmed in cells that adhered onto the surface of hydrophobic synthetic polymers such as styrene–divinylbenzene copolymer and polytetrafluoroethylene. It was reported that the growth rate of \( n \)-Staphylococcus epidermidis and \( n \)-Pseudomonas aeruginosa on the cell surface hydrophobicity of \( n \)-Fusarium solani, and the morphology of \( n \)-Cylindrocarpon and \( n \)-Acremonium cells were significantly affected by the adhesion onto hydrophobic solid surfaces.

In addition to the phenomena described previously, researchers have found that the subterminal hydroxylation activity of \( n \)-Monilliera sp. NAP 00702 is significantly affected by contact with hydrophobic polymer microparticles mixed into an MS layer in the L/L-IBR system. Higher the hydrophobicity of the polymer particles mixed, higher the \( n \)-4-decanol accumulation concentration observed. The coefficient of determination \( (R^2) \) between the polymer hydrophobicity and the \( n \)-4-decanol accumulation was 0.747. Thus, the contact between fungal cells and the hydrophobic solid surface is moderately effective in improving the microbial hydroxylation activity.

It has also been reported that the charge (cationic or anionic) on the solid-surface significantly affected the microbial cells. For instance, the adhesion of microbial cells onto an anionic resin significantly affected their growth rate, the optimum pH for the growth of \( n \)-Escherichia coli, the spore formation of \( n \)-Bacillus subtilis, and the oxidative activity of \( n \)-Nocardia coralline.

Apart from these observations, researchers have observed a unique phenomenon: the adhesion of anionic resin microparticles onto an MS layer significantly enhances the hydroxylation activity of \( n \)-Monilliera sp. NAP 00702 in the L/L-IBR system. While the addition of cation-exchange...
and the chelating resin microparticles onto an MS layer caused an inhibition of fungal growth and hydroxylation activity, the addition of anion-exchange resin microparticles with moderate total capacity \( \leq 1.00 \text{ meq/g} \) promoted the production of \((-\)-4-decanol in the L/L-IBR system. The positive effect of the anion-exchange resin microparticles was also observed during the fermentative production of a fungicidal secondary metabolite, \( \beta \)-pentyl-\( \alpha \)-pyrone (6PP), by \textit{Trichoderma atroviride}, using a novel interfacial fermentation system called the extractive liquid-surface immobilization (Ext-LSI) system, described in detail later (Fig. 6C)\(^{105, 106} \). In this case, spore formation by \textit{T. atroviride} was repressed by the addition of anion-exchange resins.

3.6 Application of L/L-IBR in epoxidation

In general, enzymatic epoxidation selectively yields optically active regioisomers\(^{107} \). Epoxy groups in many terpenes are known to enhance various biological activities, including antibacterial\(^{108} \), antifungal\(^{112} \), anti-inflammatory\(^{114} \), insecticidal\(^{115} \), and cytotoxic activities\(^{116} \).

From approximately 400 strains tested, a basidiomycete, \textit{Nemania aenea} SF 10099-1, was selected as the best strain for producing \((-\)-\( \beta \)-caryophyllene oxide. After optimizing the culture conditions, 35.4 g/L of the epoxide was obtained in an organic phase (dimethyl silicon oil; KF-96L-1CS), despite the biotoxicity of the epoxide (MIC, 66–200 mg/L)\(^{112, 113} \). Despite the high biotoxicity of the sesquiterpene, the optimal initial substrate concentration was 30 w/v (Fig. 11).

3.7 Construction of multistory L/L-IBR system

A unique interfacial bioreactor, a multistory L/L-IBR, was constructed and used for producing \((-\)-\( \beta \)-caryophyllene oxide. In this system, five L/L-IBR units were stacked and connected by overflow and circulation lines. A 10% solution of \( \beta \)-caryophyllene in low-density dimethylsilicone oil (KF-96L-1CS) naturally overflowed from the upper unit into the lower ones; it was then directed from a reservoir.
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4 Extractive liquid-surface immobilization (Ext-LSI) system

A unique interfacial fermentation system, called the Ext-LSI system, is stably constructed by adding a hydrophobic organic solvent onto a fungal cell-MS layer in the LSI system (Fig. 6C). In this system, lipophilic secondary metabolites are spontaneously extracted from fungal cells into an organic phase, thus inhibiting feedback and/or end-product inhibition by the metabolites. The Ext-LSI has been used for producing a fungicidal coconut-like aroma compound, 6-pentyl-α-pyrone (6PP).

Fungicidal secondary metabolites have been produced by some Trichoderma spp. in various fermentation systems, including submerged, aqueous two-liquid phase, organic-aqueous two-liquid phase, liquid-surface, and solid-state cultivation systems. However, only low levels of 6PP were accumulated, because of its strong anti-fungal activity. Indeed, the maximum reported levels of accumulation of 6PP in submerged, liquid-surface, and solid-state fermentations were 474 mg/L, 455 mg/L, and 3 g/kg, respectively. In contrast, the accumulation of 6PP in the Ext-LSI system reached 7.1 g/L in a dimethylsilicon oil layer, despite its strong fungicidal activity (MICs against some fungi, 0.1–0.5 g/L) (Fig. 13).

5 Future prospects

Bioprocess such as enzymatic and microbial transformations are expected to replace organic synthesis because of their advantages such as resource conservation, energy efficiency, and environmentally harmonic properties. However, the substrate concentration, product accumulation, reaction rate, biocatalyst stability, and productivity in bioprocesses are generally inferior to those in organic synth-
theses. The insolubility of synthetic substrates in the aqueous phase also limits the practical use of bioprocesses.

Despite these shortcomings of traditional bioprocesses, the S/L-IBR and the L/L-IBR have some practically important advantages, compared to SmC and TLP. For example, high concentrations of substrates and products are found in the organic phase, aeration and agitation are not required in the reactor, oxygen supply is efficient, product recovery is easy, and the microbial reactions have high regio- and stereoselectivity as well as wide versatility.

Recently, it was found that it might be possible to use fungal spores as "tough" biocatalysts in moderately polar organic solvents\(^{125}\). This possibility is considered very important to the pharmaceutical industry, because the intermediates of many drugs are insoluble in both water and hydrophobic organic solvents. The unique non-aqueous microbial transformation systems, S/L-IBR, L/L-IBR, Ext-LSI, and fungal spore biotransformation systems in organic solvents, might be practically useful for the production of industrially useful chemicals.

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