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Modified liquid–liquid interface cultivation system with floating microspheres and binder micro-pieces for slow-growing or unicellular microorganisms: Application to interfacial bioconversions with an actinomycete and yeasts

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ABSTRACT

Liquid–liquid interface bioreactor (L–L IBR) is a unique non-aqueous bioconversion system which comprises a hydrophobic organic solvent (upper phase), a fungal cells–floating microspheres (MS) layer (middle phase), and a liquid medium (lower phase). In this study, a modified L–L IBR with actinomycetes and yeasts was developed by using binder micro-pieces (BM) and estimated its availability through some bioconversions. This modified interface cultivation system was named a tacky liquid–liquid interface bioreactor (L–L IBR_{tac}). After the detailed estimation of its characteristics, the system was applied to oxidation of citronellol to citronellal, 2-methylcyclohexanol to 2-methylcyclohexanone, and 2-octanol to 2-octanone with *Rhodococcus hoagii* NBRC 3730, oxidation of citronellal to citronellic acid with *Candida viswanathii* NBRC 10321, and transacetylation of citronellol by acetyl coenzyme A (acetyl-CoA) produced from glucose by *Pichia kluyveri* NBRC 1165. The accumulation of citronellal, 2-methylcyclohexanone, and 2-octanone reached 3.1 (16 days), 2.3 (12 days), and 32.9 g/l (12 days) in spite of strong biotoxicities of the substrates/products without collapse of a cells–MS–BM layer. On the other hand, 6.1 g/l of citronellic acid and 2.8 g/l of citronellyl acetate were produced from 5% citronellal and 10% citronellol for 12 days, respectively.

1. Introduction

In recent years, microbial transformation of water-insoluble substrates is expected as an alternative manufacturing procedure of useful chemicals such as pharmaceutical intermediates to organic synthesis. However, its practical realization is considerably difficult because of the appearance of substrate and/or product inhibition and water-insolubility of substrate [1,2]. To overcome these problems, many trials have been done, such as addition of surfactant [3,4], cyclodextrin [5,6], water-miscible [7,8] and water-immiscible organic solvent (organic–aqueous two-liquid-phase system) [7,9], usages of dry mycelia [10,11] and immobilized resting cells [12,13] in an organic solvent. However, these procedures often decreased the productivity due to the toxicity of added surfactant and solvent.

Concerning the microbial transformation of water-insoluble substrates, the authors have developed a solid–liquid interface bioreactor (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) [14–16]. This interface cultivation system was applied to many microbial transformations with living bacteria, actinomycetes, and yeasts, such as hydrolysis [17], oxidation [18], reduction [19], decomposition [20], and transacetylation [21]. In all cases, the bioconversion efficiently proceeded because of toxicity alleviation effect and solubilization of hydrophobic substrates and products [14,22]. However, the S–L IBR has some severe disadvantages, such as impossibility of pH control and nutrient supplementation, and poisonous metabolite accumulation in the carrier [17,23,24].

In order to overcome the abovementioned disadvantages in the S–L IBR, two kinds of liquid–liquid cultivation systems, liquid–liquid interface bioreactor (L–L IBR) and extractive liquid-surface immobilization (Ext-LSI) systems, were developed. The L–L IBR comprises a hydrophobic organic solvent (upper phase), a fungal cells–floating microspheres (MS) layer (middle phase), and a liquid medium (lower phase) [16,25].

MS is classified to two types of micro-particles, ballooned and porous ones. The former MS is prepared from polyacrylonitrile (PAN)

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and the later one is made from polymethylmethacrylate (PMMA). The both types of MS micro-particles densely float onto a liquid-surface together with fungal cells because of their low density (0.06–0.50) [16,25–29]. After the formation of a fungal mat on the surface of the liquid medium together with numerous MS micro-particles during pre-cultivation, the fungal mat is overlaid by a hydrophobic organic solvent such as low viscous dimethylsilicone oil (L–L IBR). So far the L–L IBR has been applied to various bioconversions, such as hydrolysis [25,26], reduction [27], hydroxylation [28], and epoxidation [29], and in many cases, the L–L IBR gave very high concentrations of products with higher selectivity compared with submerged and water–aqueous two-liquid-phase systems.

The Ext-LSI system having structure similar to the L–L IBR has been applied to secondary metabolite production by fungal cells [16,30] and screening of antibiotic-producing fungi [31]. However, the liquid–liquid cultivation systems also have a common practical disadvantage. Namely, it is difficult to apply to the cultivation of slow-growing fungi and actinomycetes, unicellular microorganisms such as bacteria and yeasts because the microbial cells–MS layer easily breaks down by addition of hydrophobic organic solvent.

In this study, an improved L–L IBR system applicable to the slow-growing and unicellular microorganisms was constructed with binder micro-pieces (BM) prepared from water-insoluble carboxymethylcellulose (Fig. 1). The novel bioreactor was tentatively named a tacky liquid–liquid interface bioreactor (L–L IBR_{tac}) and its availability was confirmed through the application to five kinds of coenzyme-dependent microbial transformations adopted in our previous studies (Fig. 2).

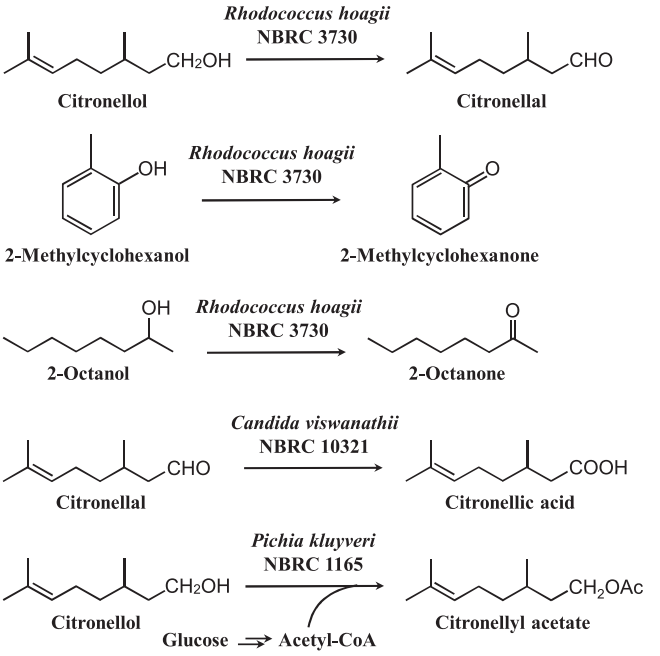

Fig. 2. Application of L–L IBR_{tac} to microbial oxidation and transacetylation by an actinomycete and yeasts.

2. Materials and methods

2.1. Microorganisms, media, and chemicals

As an actinomycete, *Rhodococcus hoagii* (former name, *R. equi*) NBRC 3730 was used for the oxidation of citronellol to citronellal [18], 2-methylcyclohexanol to 2-methylcyclohexanone [14], and 2-octanol to 2-octanone [14] (Fig. 2). As yeasts, *Candida viswanathii* NBRC 10,321 and *Pichia kluyveri* NBRC 1165 were used for the oxidation of citronellal to citronellic acid [32] and the transacetylation of citronellol to citronellyl acetate with acetyl coenzyme A (acetyl-CoA) produced via glucose metabolism [21,23,32], respectively (Fig. 2).

R. hoagii NBRC 3730 was cultivated in B-medium consisted of 10.0 g of polypeptone, 2.0 g of yeast extract, 1.0 g of $MgSO_4{\cdot}7H_2O$, and 1.0 l of reverse osmosis water (pH 7.0) at 30 °C. *C. viswanathii* NBRC 10,321 was cultivated in a modified YM-medium consisted of 10.0 g of glucose, 5.0 g of peptone, 3.0 g of yeast extract, 3.0 g of malt extract, 1.0 g of $MgSO_4{\cdot}7H_2O$, and 1.0 l of reverse osmosis water (pH 6.0) at 30 °C. *P. kluyveri* NBRC 1165 was cultivated in a CA-medium consisted of 200.0 g

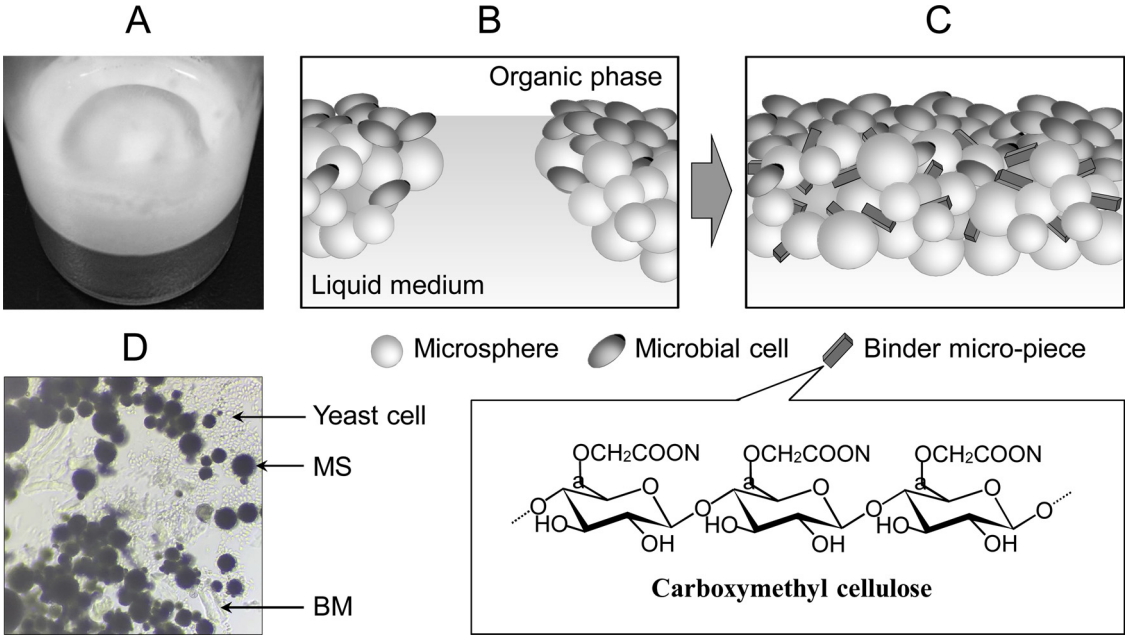

Fig. 1. A principle for tacky liquid–liquid interface bioreactor (L–L IBR_{tac}). A microbial cells–floating microspheres (MS) layer of a traditional L–L IBR with low-growing and/or unicellular microorganisms easily collapsed by overlaying with a hydrophobic organic solvent (A and B). In the L–L IBR_{tac}, a microbial cells–MS layer becomes physically strong by adhering the MS to each other via binder micro-pieces. The cells–MS-BM layer was not collapsed by overlaying a hydrophobic organic solvent (C and D).

Table 1
Physiochemical properties of carboxymethylcellulose binder micro-pieces[a].

	Sunrose SLD-F1	Sunrose SLD-FM
Color	white	⟵
Dry mean diameter (μm)	50	20
Wet mean diameter (μm)	104.7	57.9
pH	6.0–8.0	⟵
ca. Molecular weight	2.3×10^4	⟵
Adsorbed moisture (g/g)	17.5	19.0
1% Viscosity (mPa·s)	50–150	⟵
Ether content (mol%)	20–30	⟵
Purity (%)	> 99.0	⟵
Water solubility	insoluble	⟵
Supplier	Nippon Paper Industries	

[a] http://www.nipponpapergroup.com/english/products/chemical/sunrose/.

of glucose, 5.0 g of peptone, 3.0 g of yeast extract, 3.0 g of malt extract, 1.0 g of $MgSO_4{\cdot}7H_2O$, and 1.0 l of reverse osmosis water (pH 7.0) at 30 °C.

Porous MS prepared from PMMA, Advancel HB-2051 (Sekisui Chemical Co., Ltd., Tokyo) was used. The typical physicochemical properties of the MS are as follows: mean diameter, 20 μm; porosity, 50%; density, 0.5 g/cm^3. For the typical BM, two kinds of carboxymethylcellulose micro-pieces (Sunrose SLD-F1 and SLD-FM) were supplied from Nippon Paper Industries Co., Ltd., Tokyo. The physicochemical properties of these materials are shown in Table 1. The BM were stamped with a blender and sieved out in the size of 100 μm mesh pass.

As an organic phase in the L–L IBR and L–L IBR$_{tac}$ systems, low viscous dimethylsilicone oil (KF-96L-1CS) purchased from Shin-Etsu Chemical Co., Ltd., Tokyo, was used. The typical physicochemical properties of the solvent are as follows: viscosity, 1 cP; boiling point, 153 °C; specific gravity, 0.818 (25 °C). The solvent has superior oxygen solubility and biocompatibility. Indeed, all fungal strains tested vigorously grew on potato-dextrose agar plates overlaid by KF-96L-1CS. All other chemicals were also commercially available.

2.2. Stability test of an MS–BM layer formed on a water-surface

Eleven ml of reverse osmosis water colored with rose vengal, 300 mg of the MS (Advancel HB-2051), 0, 5,10 or 15 mg of the BM (SLD-F1 or SLD-FM), and a stirring bar (4 mmφ × 10 mm) were added into a 50 ml-glass vial (volume, 50 ml; diameter, 30 mm) and the mixture was vigorously mixed with a magnetic stirrer. After floating of the MS and BM during 24 h, 2 ml of *n*-decane calmly added onto the MS–BM layer. The vial was laid with rotation (60 rpm) for 3 days. The collapse or not of the floating layer was observed by viewing.

Water-solubility of the BM was estimated under the following conditions. Three hundred mg of the BM (SLD-F1 or SLD-FM) and a stirring bar (4 mmφ x 10 mm) were added into 15 ml of reverse osmosis water in a 50 ml-glass vial. After vigorous agitation with the magnetic stirrer at 500 rpm for 10 min, the mixture was set at room temperature for 4 days. The water-solubility of the BM was estimated by observation of an aliquot mixture with an optical microscopy.

2.3. Floating test of MS and collection test of BM in an MS layer

So as to measure the floating rate of the MS and BM, 920 mg of the MS (HB-2051) and 100 mg of the BM (SLD-F1 or SLD-FM) were added into 30 ml of reverse osmosis water. After vigorous mixing with a magnetic stirrer, turbidity (610 nm) of the mixture (5 ml) in a test tube (10 mm i.d. × 105 mm) was measured by using a photometer (PD-303, APEL Co., Ltd., Saitama) at the distance of 8.5 mm from the bottom every 1 min.

In order to estimate collection rate of the BM in the MS layer, 460 mg of the MS (HB-2051) and 50 mg of the BM (SLD-F1 or SLD-FM) were added into 15 ml of reserve osmosis water. After vigorous mixing with a magnetic stirrer, the mixture was allowed to stand at room temperature for 24 h. The dry weight of the precipitate on the bottom was determined after heating at 105 °C overnight. Four duplicates were prepared in three systems, HB-2051, HB-2051 plus SLD-F1, and HB-2051 plus SLD-FM.

2.4. Oxidation of citronellol, 2-methylcyclohexanol, and 2-octanol by R. hoagii NBRC 3730

Oxidation activities to these substrates by *R. horgii* NBRC 3730 were compared among submerged cultivation (SmC), organic–aqueous two-liquid-phase (TLP), and L–L IBR$_{tac}$ systems. The strain was cultivated in 25 ml of B-medium at 30 °C with rotation (200 rpm) for 1 day as a seed broth. Concerning the SmC, the seed broth (225 μl) was inoculated into 15 ml of B-medium prepared in a 100 ml-Erlenmeyer flask. After precultivation at 30 °C with rotation (200 rpm) for 1 day, 150 μl of citronellol, 2-methylcyclohexanol or 2-octanol was added and the incubation was continued for 14 days. Two ml of a broth was sampled, added excess NaCl, and thrice extracted with ethyl acetate. The products were determined by gas chromatography (GLC). As for the TLP, each substrate was added into the 1-day broth as 3 ml of a 5% solution in *n*-decane, and incubation was continued for 14 days. The organic phase was directly analyzed by the GLC.

Concerning the L–L IBR$_{tac}$, 1.2 ml of the 1-day broth was inoculated into 80 ml of the B-medium containing 2.5 g of the MS (HB-2051) and 100 mg of the BM (SLD-FM). After vigorous agitation, the mixture was poured into 4 glass vials (50 ml; 30 mm i.d.) by 15 ml. After stationary precultivation at 30 °C for 1 day, 3 ml of a 5% solution of the substrate in *n*-decane was added onto a cells–MS–BM layer. After the continued cultivation, the products in each organic phase were directly determined by the GLC.

2.5. Oxidation of citronellal with Candida viswanathii NBRC 10321

Microbial oxidation of citronellal to citronellic acid was compared between the S–L IBR and the L–L IBR$_{tac}$. *C. viswanathii* NBRC 10321 was cultivated in 25 ml of the modified YM-medium at 30 °C with rotation (200 rpm) for 1 day as a seed broth. Concerning the S–L IBR, 50 μl of the 1-day broth was inoculated on the modified YM-agar plate prepared in a glass vial (50 ml; 30 mm i.d.) and still precultivation was done at 30 °C for 1 day. The same four reactors were constructed. Three ml of a 5% or a 10% solution of citronellal in *n*-decane was added onto a biofilm formed on the agar plate, and the incubation was performed at 30 °C for 12 days. The products accumulated in the organic phase were directly determined by the GLC.

As for the L–L IBR$_{tac}$, 1.2 ml of the 1-day broth was inoculated into 80 ml of the modified YM-medium containing 2.5 g of the MS (HB-2051) and 170 mg of the BM (SLD-FM). After vigorous agitation, the mixture was poured 4 glass vials (50 ml; 30 mm i.d.) by 15 ml. The precultivation, cultivation, and product determination were done by methods same as those of the S–L IBR.

2.6. Transacetylation of citronellol with P. kluyveri NBRC 1165

Microbial transacetylation of citronellol by acetyl-CoA produced via glucose metabolism was compared between the S–L IBR and L–L IBR$_{tac}$. *P. kluyveri* NBRC 1165 was cultivated in 25 ml of the CA-medium at 30 °C with rotation (200 rpm) for 1 day as a seed broth.

Concerning the S–L IBR, 150 μl of the 1-day seed broth was inoculated on the CA-agar plate prepared in a glass vial (50 ml; 30 mm i.d.) and stationary precultivation was done at 30 °C for 1 day. The same four reactors were constructed. Three ml of a 10 or 20% solution of citronellol in *n*-decane was added onto a biofilm formed on the agar plate, and incubation was continued for 12 days. The products accumulated in the organic phase were directly determined by the GLC.

As for the L–L IBR$_{tac}$, 800 μl of the 1-day broth was mixed into 80 ml of the CA-medium containing 2.5 g of the MS (HB-2051) and 100 mg of the BM (SLD-FM). After vigorous agitation, the mixture was poured 4 glass vials (50 ml; 30 mm i.d.) by 15 ml. The precultivation, cultivation, and determination of the products were done by the manner same as those of the S–L IBR.

2.7. Analytical procedures

All of the conversion products, citronellal, 2-methylcyclohexanone, 2-octanone, citronellic acid, and citronellyl acetate were determined by the GLC. Stereochemistry of all products was not analyzed. Citronellal and 2-octanol were determined by below conditions: the column (0.25 mm i.d. × 60 m) contained Equity-5 (Supelco Co., Ltd., Bellefonte, PA), the column and detector temperatures were 150 and 160 °C, respectively, the carrier gas was He (20 cm/sec), and the split ratio was 100:1.

2-Methylcyclohexanone was determined by below condition: the column (0.25 mm i.d. × 60 m) contained SUPELCOWAX (Supelco Co., Ltd.), the column and detector temperatures were 90 and 160 °C, respectively, the carrier gas was He (20 m/sec), and the split ratio was 100:1.

Citronellic acid was determined by below condition: the column (0.25 mm i.d. × 60 m) contained Equity-5, the column and detector temperatures were 150 and 255 °C, respectively, the carrier gas was He (20 cm/sec), and the split ratio was 100:1. Citronellyl acetate was determined by below condition: the column (0.25 mm i.d. × 60 m) contained Equity-5, the column and detector temperatures were 200 and 210 °C, respectively, the carrier gas was He (20 cm/sec), and the split ratio was 100:1.

3. Results and discussion

The L–L IBR which is an interfacial bioconversion system with a fungal cells–MS layer generally enables to produce high accumulations of hydrophobic products [16,25–29]. However, the application of the system has been limited to well-growing fungi. The applications to slow-growing fungi and actinomycetes, and unicellular bacteria and yeasts are very difficult because of the collapse of a cells–MS layer by overlaying of hydrophobic organic solvent. To overcome the disadvantage of the former L–L IBR, the BM micro-pieces was added into the cells–MS layer in order to attach of MS to each other (L–L IBR$_{tac}$). First, the MS–BM layer was characterized in terms of the stability, floating ability and collection rate. Next, the availability of the L–L IBR$_{tac}$ was estimated by applying the system to some microbial transformations.

3.1. Stability test of an MS–BM layer formed on a water-solvent interface

In the case that a fungal mat is not enough formed on the MS layer, the fungal cells–MS layer easily collapses by overlaying with hydrophobic organic solvent as shown in Fig. 1A and B. It is expected that the collapse of the cells–MS layer could be prevented by addition of tacky BM micro-pieces (Fig. 1C and D). First, the BM having sustained stickiness was screened for the protection of the fungal cells–MS layer in the L–L IBR$_{tac}$. In a preliminary experiment, it was confirmed that starch, sodium alginate, and polyvinyl alcohol could not be used as the BM because of their water-soluble properties. The BM must be water-insoluble and maintain tacky property for a long time (Fig. 1C).

As the water-insoluble and tacky micro-pieces, two kinds of carboxymethyl cellulose, Sunrose SLD-F1 and SLD-FM, were selected. The physicochemical properties of both BMs are shown in Table 1. The supplementary effects of the BM are shown in Fig. 3. Both BMs sustained an MS–BM layer under the condition with rotation (60 rpm) for 3 days by addition more than 5 mg/vial (0.7 mg/cm^2-liquid surface). The suitable effect of the BM was largely due to the water-insoluble and tacky properties (Figs. 1D and 4). Indeed, the BM micro-pieces were present after vigorous mixing and standing for 4 days. First of all, Sunrose SLD-F1 and FM were selected as the favorable insoluble binder material for sticking MS micro-particles to each other.

3.2. Floating test of MS and collection test of BM in an MS layer

It was assumed that the collection efficiency of the BM micro-pieces in the MS layer depends on the floating rate of the MS because the specific gravity of the BM was larger than 1.0. The floating rate of the MS and BM to a liquid surface was estimated by measuring the turbidity at OD$_{610}$. As shown in Fig. 5, OD$_{610}$ values of HB-2051, HB-2051 plus SLD-F1, and HB-2051 plus SLD-FM were 0.813, 1.435, and 1.614 at 60 min, respectively. The floating rates (OD$_{610}$ decrease for first 15 min) of HB-2051, HB-2051 plus SLD-F1, and HB-2051 plus SLD-FM were 0.895, 0.191, and 0.127, respectively (Table 2). Thus, it was worried that the collection rate of the BM in the MS layer was not so high.

The collection rate of the BM micro-pieces in the MS layer was estimated by measuring sediment dry weight on a bottom of the vial. As shown in Table 2, while 22.5 ± 7.9% of SLD-F1 settled on the bottom of the vial, the sedimentation rate of SLD-FM reached 29.9 ± 2.5%. Thus, the collection rates of SLD-F1 and SLD-FM were 77.5 ± 7.9 and 70.1 ± 2.5%, respectively. In conclusion, SLD-F1 whose collection rate was higher than that of SLD-FM and it was selected as the best BM. Although the collection rate of SLD-F1 in an MS layer was limited to 77.5 ± 7.9%, the MS–BM layer was stably held for 3 days with revolution (60 rpm). Thus, SLD-F1 is efficiently effective for maintenance of the MS–BM layer.

3.3. Application of L–L IBR$_{tac}$ to bioconversion with an actinomycete

As mentioned above, Sunrose SLD-F1 can sustain its tackiness and effectively stuck the MS and microbial cells (Figs. 1,3 and 4). The MS–BM layer formed on a liquid-surface did not collapse by overlaying a hydrophobic organic solvent such as dimethylsilicone oil. Next, the novel interface cultivation system with the MS and BM, L–L IBR$_{tac}$, was applied to some microbial transformations in order to verify its wide availability.

Rhodococcus hoagii NBRC 3730 catalyzes many kinds of oxidation of alcohols [33] and sulfides [34]. The authors have also reported that this actinomycete catalyzes the oxidation of citronellol [18,32], 2-methylcyclohexanol [22], and 2-octanol [22]. First, the L–L IBR$_{tac}$ was applied to these microbial oxidations by the actinomycete, and the efficacy of the L–L IBR$_{tac}$ was compared with that of the SmC and the TLP.

As shown in Fig. 6, citronellol, 2-methylcyclohexanol, and 2-octanol were not oxidized in SmC because these substrates exhibited strong biotoxicity at the level of only 1% in the SmC. The coenzyme-regenerating cycle might be shut by killing the actinomycete cells by the toxic substrates. In the TLP system, the biotoxicity of substrates were partially alleviated except 1% citronellol because *n*-decane played as a reservoir of toxic substrate [35,36]. Five % of 2-methylcyclohexanol and 2-octanone could be efficiently oxidized in the TLP, whereas, 2-methylcyclohexanone and 2-octanone were decreased after 7th day. It was assumed that the both products might be decomposed via Bayer-Villiger oxidation [37,38]. Moreover, an organic phase in the TLP playing as an extractant of product [38] and an oxygen vector [36,39] reduced by vigorous agitation. Thus, TLP system may be also unfavorable to practical bioconversions.

On the other hand, the L–L IBR$_{tac}$ effectively catalyzed all the oxidations. The accumulation of citronellal, 2-methylcyclohexanone, and 2-octanone reached 3.1 (16 days), 2.3 (12 days), and 32.9 g/l (12 days) in spite of strong biotoxicity of the substrates and/or products without collapse of the actinomycete cells–MS–BM layer. The Bayer-Villiger oxidation of the ketones produced was partially repressed compared with the TLP system (Fig. 6). The repression of Bayer-Villiger oxidation in the L–L IBR was also observed in the oxidation of *n*-decane to 4-

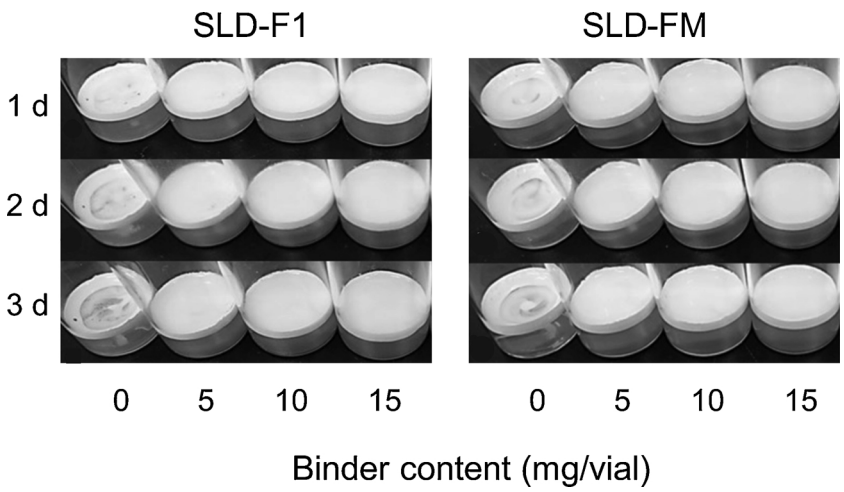

Fig. 3. Stabilization of an MS layer by adding BM. Eleven ml of reverse osmosis water, 300 mg of MS (HB-2051) 0–15 mg of BM (SLD-F1 or FM) were vigorously mixed in a 50 ml-glass vial (50 ml; diameter, 30 mm). After floating of the MS and the BM during 24 h, 2 ml of *n*-decane calmly added onto the MS–BM layer. The vial was laid with rotation (60 rpm) for 3 days. The collapse or not of the floating layer was observed.

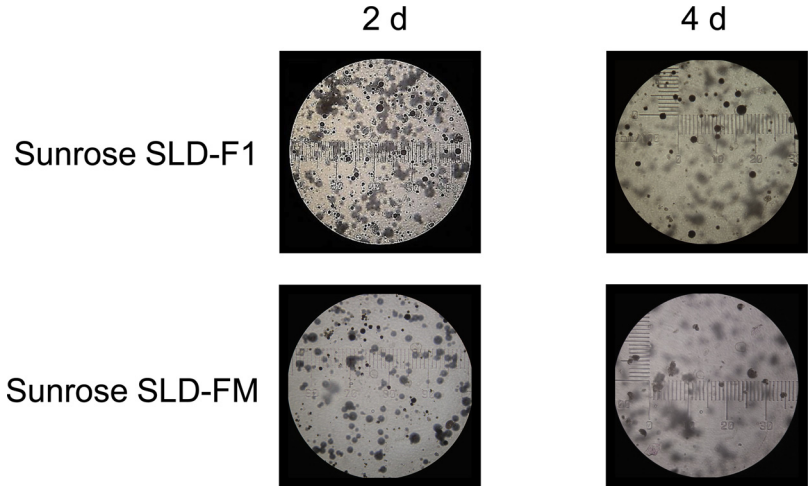

Fig. 4. Microscopic photos of BM suspended in water. Water-solubility of the BM was estimated under the following conditions. Three hundred mg of the BM (SLD-F1 or SLD-FM) and a stirring bar (4 mmφ × 10 mm) were added into 15 ml of reverse osmosis water in a 50 ml-glass vial. After vigorous agitation with the magnetic stirrer at 500 rpm for 10 min, the mixture was set at room temperature for 4 days. The water-solubility of the BM was estimated by observation of an aliquot mixture with an optical microscopy.

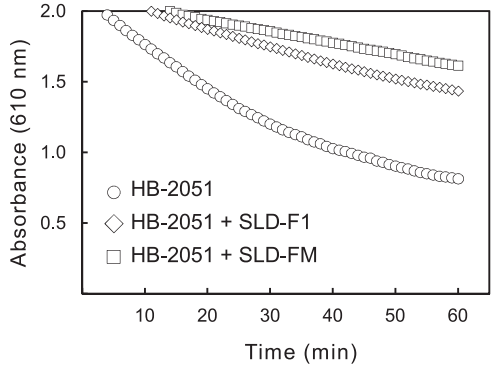

Fig. 5. Floating curves of MS and BM. The MS (HB-2051; 920 mg) and 100 mg of the BM (SLD-F1 or FM) were added into 30 ml of reverse osmosis water. After vigorous mixing with a magnetic stirrer, turbidity (610 nm) of the mixture (5 ml) in a test tube (10 mm i.d. × 105 mm) was measured by using a photometer (PD-303, APEL Co., Ltd., Saitama) at the distance of 8.5 mm from the bottom every 1 min.

decanol and 4-decanone with an isolated fungus, *Monilliera* sp. NAP 00702 [28]. It is assumed that excess oxygen supply is repressed in the L–L IBR and the L–L IBR_{tac} compared with the TLP because of their static cultivation conditions.

3.4. Application of L–L IBR_{tac} to bioconversion with yeasts

The L–L IBR_{tac} was applied to the oxidation of citronellal to citronellic acid with *C. viswanathii* NBRC 10321 and its efficacy was compared with that of the S–L IBR system. As shown in Fig. 7, although the efficacy of the L–L IBR_{tac} was slightly inferior compared with that of the S–L IBR, the oxidation of citronellal smoothly proceeded to give 6.1 g/l of citronellic acid for 12 days. The yeast cells–MS–BM layer of the L–L IBR_{tac} did not collapse by addition of Sunrose SLD-F1.

Next, the L–L IBR_{tac} was applied to the transacetyltion of citronellol by acetyl-CoA produced via glucose metabolism with *P. kluyveri* NBRC 1165. Concerning the reaction, the efficacy of the L–L IBR_{tac} was compared with that of the S–L IBR. As shown in Fig. 8, although the production of citronellyl acetate in the L–L IBR_{tac} was inferior to that in the S–L IBR, 2.8 g/l of citronellyl acetate was produced from 10% citronellol for 12 days in spite of the strong biotoxicity of citronellol. In this case, the yeast cell–MS–BM layer of the L–L IBR_{tac} did not collapse.

As mentioned above, efficiencies of the oxidation and transacetylation of citronellol in the L–L IBR_{tac} were inferior to those in the S–L IBR. Concerning the causes of the results, firstly, it is assumed that interfacial hydrophobicity given by MS (PMMA) affects on the bioconversion activities. Morisaki reported that *Escherichia coli* cells adhered onto some hydrophobic polymer surface, such as polytetrafluoroethylene (PTFE) and pyrophylite, exhibited significant increase of respiration activity and decrease of glucose uptake [40].

Furthermore, it was also reported that production of hydrophobins in *Lecanicillium lecanii* [41], pycnidiospores formation in *Phyllosticta ampelicida* [42], and corticosterone uptake in *Candida albicans* were

Table 2
Floating and collection rates of MS and BM.

	Floating rate[a]	Sediment dry wt (mg)[b]	Sedimentation rate (%)[c]	Collection rate (%)
HB-2051	0.895	0.6 ± 0.3	> 0.0	< 100.0
HB-2051 plus SLD-F1	0.191	11.2 ± 3.9	22.5 ± 7.9	77.5 ± 7.9
HB-2051 plus SLD-FM	0.127	15.0 ± 1.3	29.9 ± 2.5	70.1 ± 2.5

Measurements of sediment dry weight were performed in four replicates, and the reported results represented as the mean of quadruplicates ± standard derivation.
[a] OD_{610} decrease for first 15 min.
[b] Dry weight in a vessel.
[c] Initial dry weight of BM was 50.0 mg.

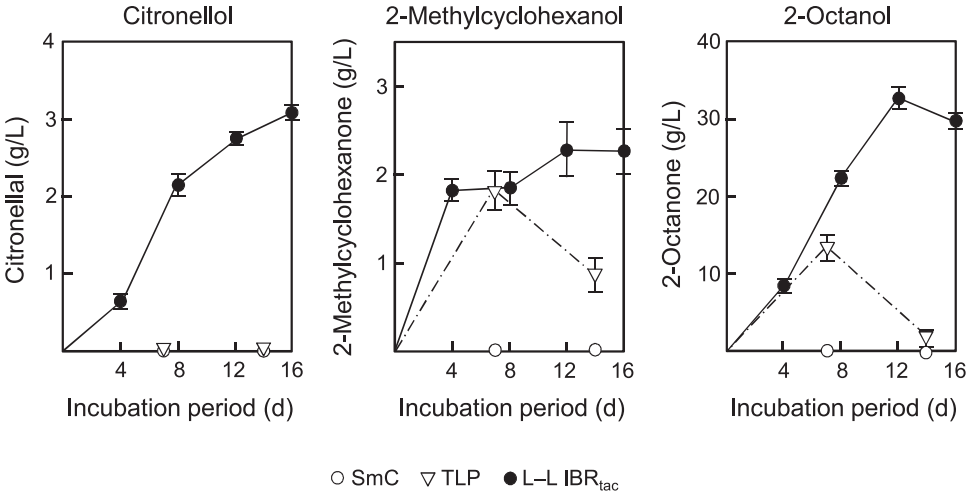

Fig. 6. Oxidation of 3 kinds of alcohol by *R. hoagii* NBRC 3730 in submerged (SmC), organic–aqueous two–liquid–phase (TLP), and L–L IBR_{tac} systems. The substrate concentrations in all systems were 1% in each reaction mixture. In the TLP and the L–L IBR_{tac}, each substrate was added in vessels as a 5% solution in *n*-decane. While the SmC and the TLP systems were incubated at 30 °C with rotation (200 rpm), the L–L IBR_{tac} was set at 30 °C without rotation.

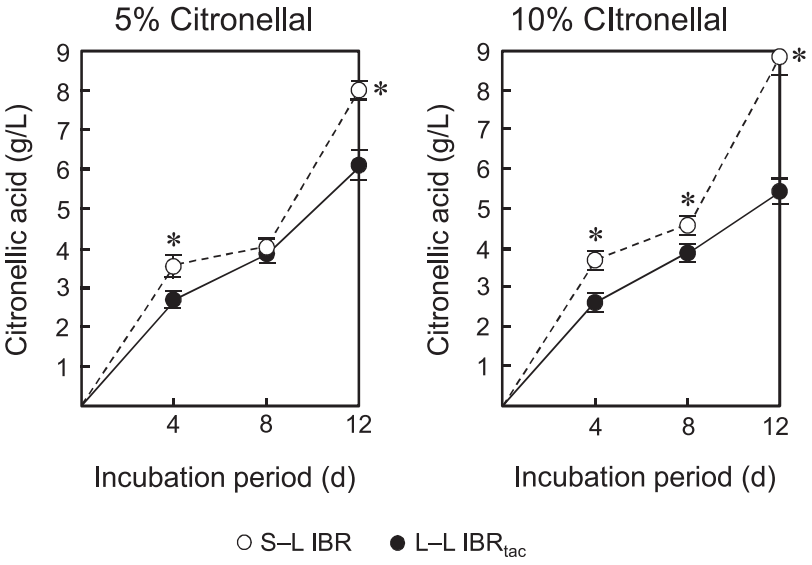

Fig. 7. Oxidation of citronellal by *C. viswanathii* NBRC 20321 in S–L IBR and L–L IBR_{tac}. Three ml of a 5% or 10% solution of citronellal in *n*-decane was added onto a biofilm formed on an agar plate (S–L IBR) or a cells–MS–BM layer (surface area, 7.1 cm^2; L–L IBR_{tac}) and incubation was performed at 30 °C without rotation. Error bars indicate standard deviation (n = 4). *Significant difference from L–L IBR_{tac} at $p < 0.05$ in *t*-test ($n = 4$).

accelerated by contact with some hydrophobic solid surface [43]. The authors have also discovered that *n*-decane subterminal hydroxylation activity of *Monilliera* sp. NAP 00702 is significantly enhanced by addition of PTFE into an MS layer of the L–L IBR [44]. Thus, the contact of microbial cells on a hydrophobic surface changes some physiological and biochemical properties of the microbial cells. It was assumed that the oxidation and transacetylation activities of yeast cells were repressed by contact on the hydrophobic surface of PMMA resin in the L–L IBR_{tac}.

Secondly, as another possible factor causing the decrease of oxidation and transacetylation activities in the L–L IBR_{tac}, it was supposed that much moisture in a biofilm formed on the surface of the MS–BM layer inhibited permeation and diffusion of hydrophobic substrates into the biofilm. Release of conversion products from cells into an organic phase might be also repressed by the moist biofilm. The authors think the presence of much water in the biofilm is an obstacle to the bioconversions in the L–L IBR_{tac} in part.

4. Conclusion

A modified liquid–liquid interface bioreactor (l–L IBR_{tac}), which comprised a hydrophobic organic solvent (upper phase), a microbial cells–floating microspheres (MS) layer reinforced by binder micro-pieces (BM) (middle phase), and a liquid medium (lower phase), was developed. The water-insoluble BM, Sunrose SLD-F1, was water-insoluble but tacky by swelling via hydration. Thus, the BM micro-pieces

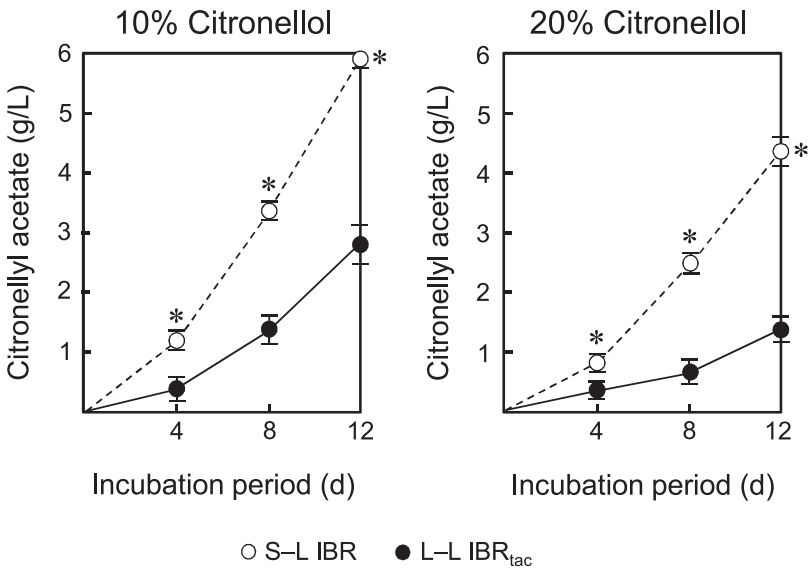

Fig. 8. Transacetyltion of citronellol by acetyl-CoA produced via glucose metabolism with *P. kluyveri* NBRC 1165 in S–L IBR and L–L IBR$_{tac}$. Three ml of a 10% or 20% solution of citronellol in *n*-decane was added onto a biofilm formed on an agar plate or a cells–MS–BM layer (surface area, 7.1 cm^2) and incubation was performed at 30 °C without rotation. Error bars indicate standard deviation (n = 4). *Significant difference from L–L IBR$_{tac}$ at $p < 0.05$ in *t*-test ($n = 4$).

stuck the MS to offer a scaffold for growth of microbial cells.

The efficacy of the L–L IBR$_{tac}$ was estimated through 5 kinds of microbial transformation. The oxidation of citronellol to citronellal, 2-methylcyclohexanol to 2-methylcyclohexanone, and 2-octanol to 2-octanone by *R. hoagii* NBRC 3730, the oxidation of citronellal to citronellic acid and the transacetylation of citronellol by acetyl-CoA produced via glucose metabolism by yeasts, *C. viswanathii* NBRC 10321 and *P. kluyveri* NBRC 1165, respectively. In all cases, the microbial cells–MS–BM layer did not collapse by supplementation of the BM micro-pieces and catalyzed all reactions effectively.

Thus, it is concluded that the L–L IBR$_{tac}$ is effective for the microbial transformation with low-growing and/or unicellular microorganisms. Furthermore, it is strongly suggested that a modified extractive liquid-surface immobilization (Ext-LSI$_{tac}$) system, an extraction fermentation system with the MS and BM, may be constructed with low-growing and/or unicellular microorganisms, such as actinomycetes.

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