Syntheses of Optically Active Citronellol, Citronellal, and Citronellic Acid by Microbial Oxidation and Double Coupling System in an Interface Bioreactor

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We have already reported that (*RS*)-citronellol [(*RS*)-**1**] can be optically resolved via a transacetylation with acetyl coenzyme A [acetyl-CoA] by the aid of alcohol acetyltransferase [AATFase] in *Pichia kluyveri* IFO 1165, which we have referred to as a double coupling system (*E* value, 30 to 40). In this system, although (*R*)-**1** is obtained at over 98% ee, (*S*)-citronellyl acetate [(*S*)-**2**] is prepared at 70 to 80% ee. In this article, we report on the conversion of (*R*)-**1** to (*R*)-citronellal [(*R*)-**3**] and (*R*)-citronellic acid [(*R*)-**4**] in high yield without racemization by the aid of *Rhodococcus equi* JCM 6817 and *Geotrichum candidum* JCM 01747, respectively. On the other hand, the low ee of (*S*)-**1** (78% ee) prepared via the alkaline hydrolysis of (*S*)-**2** is converted to optically active (*S*)-**1** and (*S*)-**4** in high yield with a repeated double coupling system with *P. kluyveri* IFO 1165 and via enantioselective oxidation with *Candida viswanathii* IFO 10321, respectively. Thus, five optically active terpenoids related to citronellol, except for (*S*)-**3**, were efficiently synthesized via microbial transformations in an interface bioreactor.

An interface bioreactor, which is a non-aqueous bioreactor using a microorganism growing on an interface between a hydrophilic carrier and a hydrophobic organic solvent, is a superior device for the production of various lipophilic products.[1—4] In this bioreactor, some useful chiral synthons, such as (*R*)-**1** and (*S*)-**4**,[5,6] (*R*)-2-benzylcyclohexanone,[7] (*R*)-sulcatol,[8] and ethyl (*R*)-2-hydroxy-4-phenylbutanoate,[9] were efficiently synthesized at high accumulation according to toxicity alleviation on the solid-liquid interface.[10] The interface bioreactor has also been applied to a new acetylation system without any acetyl donors, tentatively referred to as a double coupling system, in which various primary alcohols are quantitatively acetylated with acetyl-CoA formed via the metabolism of glucose.[11,12] Moreover, (*RS*)-**1** could be optically resolved in the system with *P. kluyveri* IFO 1165 according to (*S*)-preferential transacetylation at 30 to 40 of the *E* value.[13] In this case, although (*R*)-**1** could be obtained at high ee (over 98% ee), the ee of (*S*)-**2** was insufficient (70 to 80% ee). In this article, we describe the conversion of (*R*)-**1** to (*R*)-**3** and (*R*)-**4** via microbial oxidation without racemization, the elevation of ee of (*S*)-**1** with a repeated double coupling system, and the formation of (*S*)-**4** via enantioselective oxidation (Fig. 1).

Experimental

Microorganisms, Media, and Chemicals. *R. equi* JCM 6817 and *G. candidum* JCM 01747 were purchased from the Institute of Physical and Chemical Research (Saitama), and *P. kluyveri* IFO 1165 and *C. viswanathii* IFO 10321 were purchased from the Institute of Fermentation (Osaka). The basal medium I (pH 7.0) for *R. equi* JCM 6817 containing 10.0 g of polypeptone, 2.0 g of yeast extract, 1.0 g of $MgSO_4 \cdot 7H_2O$, and 1.0 liter of distilled water was used. The basal medium II (pH 6.0) for *G. candidum* JCM 01747 and *C. viswanathii* IFO 10321 containing 5.0 g of peptone, 3.0 g of yeast extract, 3.0 g of malt extract, 10.0 g of glucose, 1.0 g of $MgSO_4 \cdot 7H_2O$, and 1.0 liter of distilled water was used. For *P. kluyveri* IFO 1165, the basal medium II contained 200.0 g of glucose. Agar powder (15.0 g) was added to the basal media (1.0 liter) for preparing an agar plate interface bioreactor. (*R*)- and (*S*)-**1**, **3**, and **4** were the products of Aldrich Co. Ltd. Ester (*RS*)-**2** was purchased from Wako Pure Chemicals Co. Ltd., Tokyo. All other chemicals were also commercially available.

Determination of the Absolute Configuration and the Enantiomeric Excess of Citronellol, Citronellal, and Citronellic Acid. The absolute configuration and the ee of **1** were directly determined by gas chromatography: the column was β-DEX™ 225 (diameter, 0.25 mm; length, 30 m; Supelco Co. Ltd.); the column temperature was 85 °C (isothermal); the injector and detector temperatures were 210 and 220 °C, respectively; the split ratio was 1 : 100; and the carrier gas was He (linear velocity, 54 cm s^{-1}). The retention times of (*S*)- and (*R*)-**1** were 30.1 and 30.8 min, respectively. The ee of **1** was determined based on the peak areas. Aldehyde **3** and acid **4** were purified on a silica-gel column [Wakogel C-200; eluent, hexane–ethyl acetate (1 : 1)], and reduced to **1** with $LiAlH_4$. The absolute configuration and the ee of the resulting **1** were determined in the same manner.

Oxidation of (*R*)-Citronellol to (*R*)-Citronellal with *R. equi* JCM 6817. A nutrient agar plate consisting of the basal medium I was prepared in a glass petri dish (surface area, 38.5 cm^2; volume, 25 ml). A cell suspension (300 μl of 1 loopful/ml-medium) of *R.*

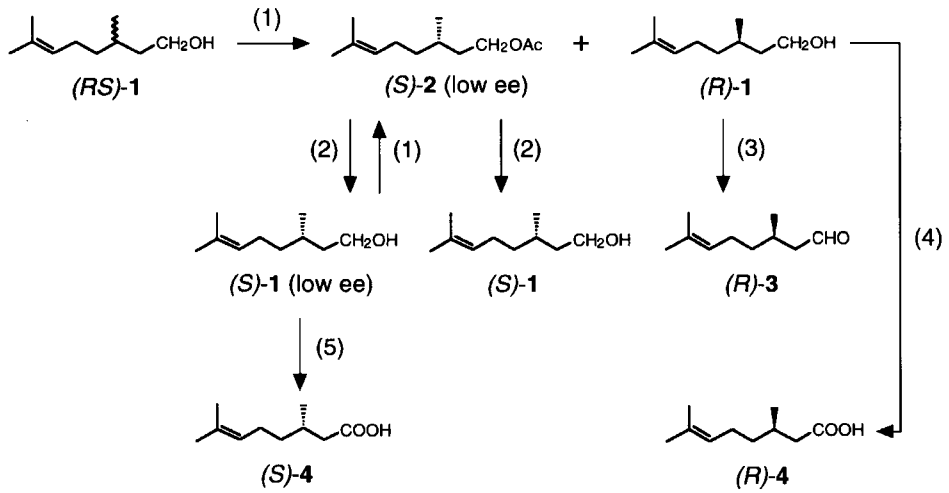


Fig. 1. Strategy for the syntheses of optically active citronellol, citronellal, and citronellic acid in an interface bioreactor. (1), *Pichia kluyveri* IFO 1165 (a double coupling system); (2), ethanolic 10 wt% NaOH solution, 90 °C, 3 h (alkaline hydrolysis); (3), *Rhodococcus equi* JCM 6817 (microbial oxidation); (4), *Geotrichum candidum* JCM 01747 (microbial oxidation); (5), *Candida viswanathii* IFO 10321 (microbial oxidation).

equi JCM 6817 was spread on the agar plate, and excess moisture was removed by allowing the plate to stand. After precultivation at 30 °C for 1 day, 8 ml of a 1 wt% solution of (*R*)-**1** (98% ee) in decane was added, and incubation was carried out at 30 °C by allowing the dish to stand for 5 days. The scheme for the conversion with the agar plate interface bioreactor is illustrated in Fig. 2.

Oxidation of (*R*)-Citronellol to (*R*)-Citronellic Acid with *G. candidum* JCM 01747. A nutrient agar plate consisting of the basal medium II was prepared in a glass petri dish (surface area, 38.5 cm^2; volume, 25 ml). A cell suspension (300 µl of 1 loopful/ml-medium) of *G. candidum* JCM 01747 was spread on the surface of the plate, and precultivation was performed at 30 °C for 1 d. After precultivation, 8 ml of a 2 wt% solution of (*R*)-**1** (98% ee) in decane was added, and incubation was carried out at 30 °C by allowing the dish to stand for 10 days.

Repeated Double Coupling of Low ee of (*S*)-Citronellol with *P. kluyveri* IFO 1165. A nutrient agar plate consisting of the basal medium II containing 20 wt% of glucose was prepared in a glass petri dish (surface area, 38.5 cm^2; volume, 25 ml). A cell suspension (300 µl of 1 loopful/ml-medium) of *P. kluyveri* IFO 1165 was spread on the surface of the plate, and precultivation was carried out at 30 °C by allowing the dish to stand for 1 day. After precultivation, 8 ml of a 1 wt% solution of low ee of (*S*)-**1** (78% ee) derived from (*S*)-**2** by alkaline hydrolysis (at 90 °C in an ethanolic 10 wt% solution of NaOH for 3 h) in decane was added, and incubation was performed at 30 °C with swaying (40 strokes min^{-1}) for 2 days.

Oxidation of Low ee of (*S*)-Citronellol to (*S*)-Citronellic acid with *C. viswanathii* IFO 10321. A nutrient agar plate consisting of the basal medium II was prepared in a glass petri dish (surface area, 38.5 cm^2; volume, 25 ml). A cell suspension (300 µl of 1 loopful/ml-medium) of *C. viswanathii* IFO 10321 was spread on the plate, and precultivation was performed at 30 °C for 1 day. Then, 8 ml of a 2 wt% solution of low ee of (*S*)-**1** (78% ee) in decane was added, and incubation was performed at 30 °C by allowing the dish to stand for 10 days.

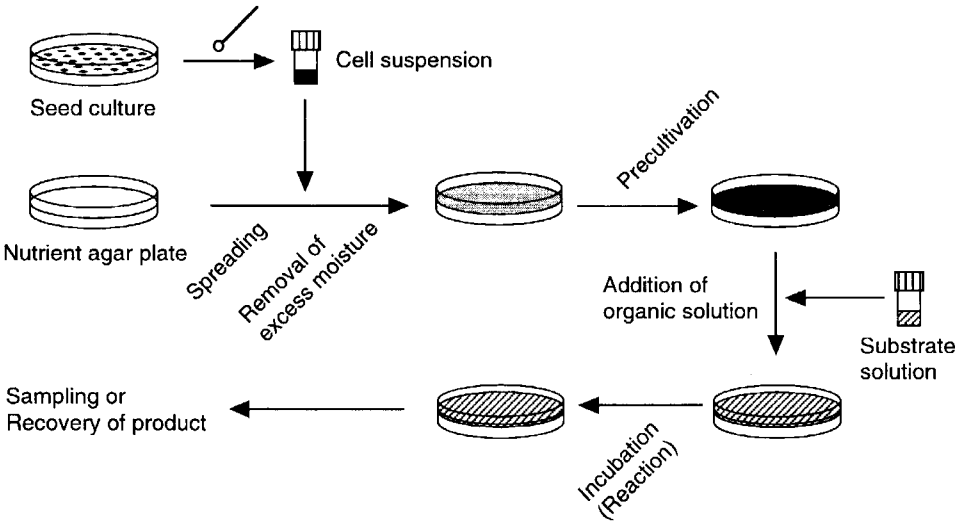


Fig. 2. Scheme for the microbial transformation with the agar plate interface bioreactor.

Results and Discussion

Optically active **1**, **3**, and **4** were used for the syntheses of various optically active natural products, such as insect pheromones[14—18] and alkaloids.[19,20] Terpene alcohols (*R*)- and (*S*)-**1** could be synthesized from geraniol and nerol with BINAP–Ru(II), respectively.[21] The alcohol (*S*)-**1** was also synthesized via enantioselective reduction of racemic **3**[22] and geraniol[23] by the aid of *Saccharomyces cerevisiae*. Aldehyde (*R*)-**3** was prepared from geranylamine via asymmetric isomerization catalyzed by BINAP–Rh(I) followed by hydrolysis of the resulting enamines.[24] Acid (*R*)-**4** was synthesized from (−)-isopulegol or (*R*)-pulegone via chemical procedures.[25—27] On the other hand, the optical resolution of (*RS*)-**1** has generally been unsuccessful by either esterification using lipases[28,29] or microbial hydrolysis.[30] Although Cambou and Klibanov reported that the optical resolution of (*RS*)-**1** via transesterification with hog liver carboxyl esterase was successful, the instability and the high cost of the enzyme were shortcomings.[31]

We have succeeded in the optical resolution of (*RS*)-**1** in a double coupling system with *P. kluyveri* IFO 1165.[13] In this system, (*S*)-**1** in an organic phase is preferentially acetylated with acetyl-CoA formed via glucose metabolism by the aid of AATFase to afford (*R*)-**1** and (*S*)-**2** at *E* value of 30—40. Although (*R*)-**1** is obtained in high ee (over 98%), the ee of (*S*)-**2** is insufficient (70—80% ee). In this study, we tried to prepare (*R*)-**3** and (*R*)-**4** from optically active (*R*)-**1**, and (*S*)-**1** and (*S*)-**4** from low ee of (*S*)-**1** induced via alkaline hydrolysis of (*S*)-**2**.

Syntheses of (*R*)-Citronellal and (*R*)-Citronellic Acid. The interface bioreactor is efficiently applicable to microbial oxidation because the organic phase highly solubilizes oxygen as compared to the aqueous phase. In many cases, the microbial oxidation smoothly proceeds, even in still cultivation.

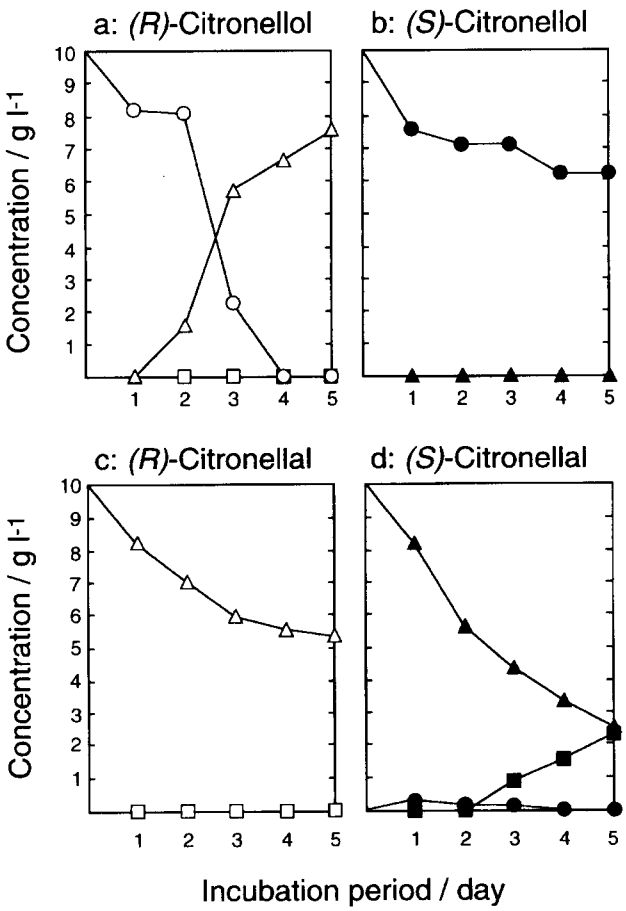


Fig. 3. Conversion of optically active citronellol and citronellal with *R. equi* JCM 6817 in the agar plate interface bioreactor. Symbols: ○, (*R*)-citronellol; ●, (*S*)-citronellol; △, (*R*)-citronellal; ▲, (*S*)-citronellal; □, (*R*)-citronellic acid; ■, (*S*)-citronellic acid. *R. equi* was inoculated on a nutrient agar plate of which surface area and volume were 38.5 cm^2 and 25 ml, respectively. After precultivation at 30 °C for 1 day, 8 ml of a 1 wt% solution of citronellol or citronellal enantiomer in decane was added, and incubation was carried out at 30 °C by allowing the device to stand.

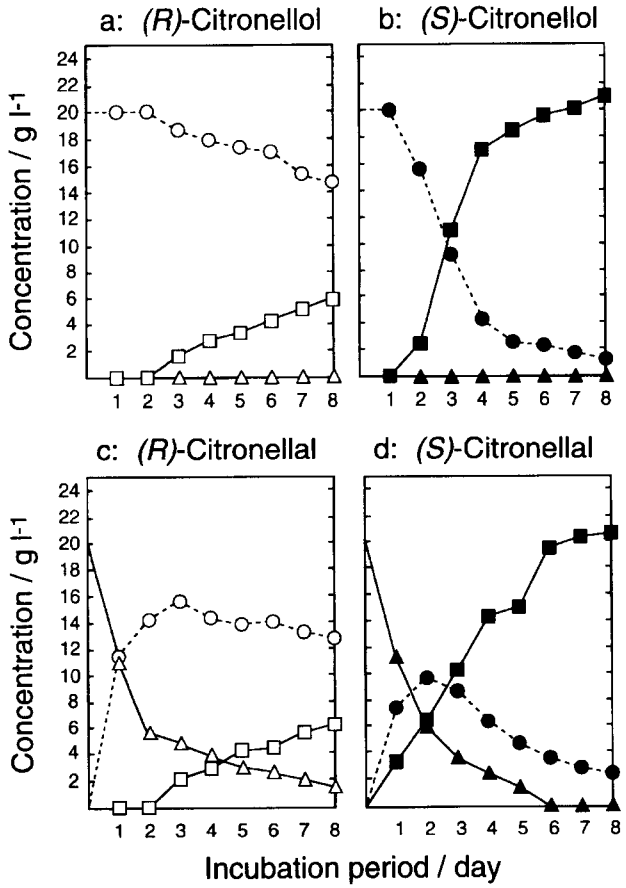


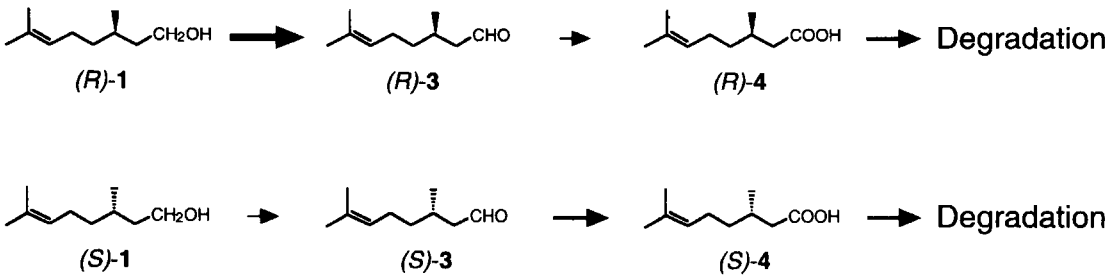
Fig. 4. Conversion of optically active citronellol and citronellal with *C. viswanathii* IFO 10321 in the agar plate interface bioreactor. Symbols: ○, (*R*)-citronellol; ●, (*S*)-citronellol; △, (*R*)-citronellal; ▲, (*S*)-citronellal; □, (*R*)-citronellic acid; ■, (*S*)-citronellic acid. *C. viswanathii* was inoculated on a nutrient agar plate of which surface area and volume were 38.5 cm^2 and 25 ml, respectively. After precultivation at 30 °C for 1 day, 8 ml of a 1 wt% solution of citronellol or citronellal enantiomer in decane was added, and incubation was carried out at 30 °C by allowing the device to stand.

R. equi JCM 6817 preferentially oxidizes (*R*)-**1** to afford (*R*)-**3** as an oxidation product.[6] Although (*R*)-**3** was formed by oxidation of (*R*)-**1** in 76% yield (Fig. 3a), the consumption of (*R*)-**1** and (*R*)-**3** without accumulation of acid **4** was observed (Figs. 3a and 3c). These results suggest that slowly produced (*R*)-**4** is smoothly degraded. On the other hand, while (*S*)-**1** was consumed at a relatively lower rate (Fig. 3b), (*S*)-**3** was smoothly converted to acid **4** (Fig. 3d). Thus, the enantioselectivities and reactivities of **1**- and **3**-oxidizing enzymes can be summarized as shown in Scheme 1. Indeed, 1 wt% of (*R*)-**1** (98% ee) was smoothly converted to (*R*)-**3** with *R. equi* JCM 6817 in 81% yield without racemization and accumulation of acid **4** in the agar plate interface bioreactor.

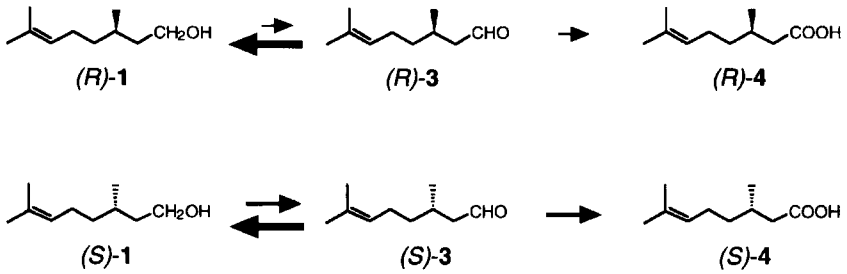
Next, 2 wt% of (*R*)-**1** (98% ee) was oxidized to (*R*)-**4** with *G. candidum* JCM 01747, which exhibits strong **1**- and **3**-oxidizing enzyme activities.[6] The oxidation smoothly proceeded without racemization and formation of by-products to afford optically active (*R*)-**4** in 89% yield. Thus, (*R*)-**1** could be efficiently converted to (*R*)-**3** and (*R*)-**4** by using two different microorganisms.

Syntheses of (*S*)-Citronellol and (*S*)-Citronellic Acid. The ee of (*S*)-**1** derived by alkaline hydrolysis of low ee of (*S*)-**2** could be elevated with the repeated double coupling system by the aid of *P. kluyveri* IFO 1165. Thus, 1 wt% of (*S*)-**1** (78% ee) was acetylated to give (*S*)-**2** (96% ee) in 87% yield. The resulting (*S*)-**2** could be hydrolyzed to (*S*)-**1** with an ethanolic 10 wt% NaOH solution without racemization.

Next, we tried to convert the low ee of (*S*)-**1** to acid **4** via microbial oxidation. As shown in Figs. 4a, 4b, 4c, and 4d, while *C. viswanathii* IFO 10321 preferentially oxidized (*S*)-



Scheme 1. Enantioselectivities and reactivities of citronellol- and citronellal-oxidizing enzymers in *Rhodococcus equi* JCM 6817.



Scheme 2. Enantioselectivities and reactivities of citronellol- and citronellal-oxidizing and citronellal-reducing enzymes in *C. viswanathii* IFO 10321.

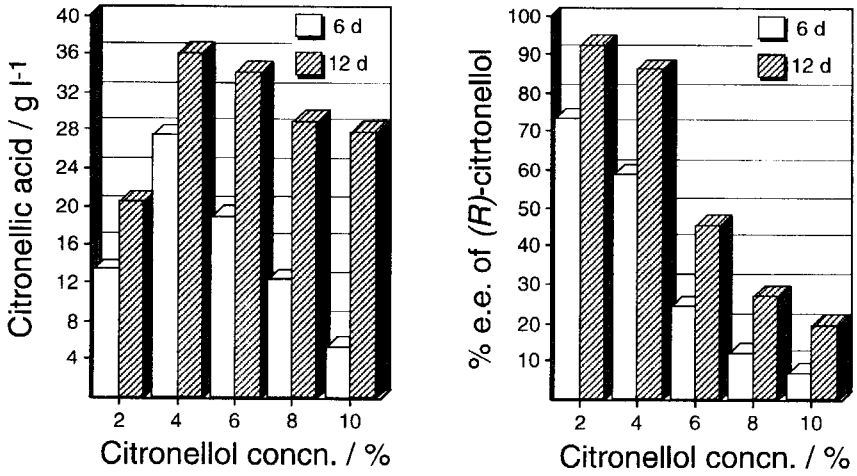


Fig. 5. Conversion of high concentrations of (*RS*)-citronellol with *C. viswanathii* IFO 10321 in the agar plate interface bioreactor. *C. viswanathii* was inoculated on a nutrient agar plate of which surface area and volume were 38.5 cm^2 and 25 ml, respectively. After precultivation at 30 °C for 1 day, 8 ml of a 2, 4, 6, 8, or 10 wt% solution of (*RS*)-citronellol in decane was added, and incubation was carried out at 30 °C by allowing the device to stand.

1 and (*S*)-**3**, as compared with (*R*)-**1** and (*R*)-**3**, the strain also reduced both (*S*)- and (*R*)-**3** to the corresponding **1** at a high reaction rate (Figs. 4c and 4d). The combination of oxidation and reduction was also observed in many yeasts, such as *Hansenula anomala* IFO 0147[6] and *H. saturnus* IFO 0809.[5] The enantioselectivities and reactivities of **1**- and **3**-oxidizing, and **3**-reducing enzymes in *C. viswanathii* IFO 10321 are summarized in Scheme 2. The strain also enantioselectively oxidized (*RS*)-**1** of as high as 10 wt% concentration in the interface bioreactor in spite of the strong toxicity of the substrate (Fig. 5).[32–34] Indeed, the low ee of (*S*)-**1** (78% ee) was oxidized to 98% ee (*S*)-**4** in 78% yield for 10 days.

Although (*S*)-**3** could not be synthesized, the excellent ee of (*R*)-**1**, **3**, **4**, (*S*)-**1** and **4** was achieved by microbial oxidation and the double coupling system in high yield using the interface bioreactor. We also tried to oxidize alcohol **1** to acid **4** by Jones' oxidation (data not shown). However, a low yield and the formation of by-products due to over oxidation of resulting **4** could not be avoided, because **4** had a reactive double bond. Thus, it is concluded that microbial transformation is favorable to oxidize optically active **1** to **3** and **4** in high yield as compared to chemical procedures, such as Jones' oxidation.

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