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Stereoselective Hydrolysis of *dl*-Menthyl Succinate by Gel-Entrapped *Rhodotorula minuta* var. *texensis* Cells in Organic Solvent*

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Summary. *dl*-Menthyl succinate was successfully hydrolyzed stereoselectively by *Rhodotorula minuta* var. *texensis* cells entrapped within photo-crosslinked or polyurethane resin gels in water-saturated *n*-heptane. The hydrolyzed product was found to be pure *l*-menthol. The catalytic activity of the immobilized cells, especially those entrapped in urethane polymers, was far more stable than that of the free cells. The half-life of the polyurethane-entrapped cells was estimated to be 55–63 days in the organic solvent.

Introduction

l-Menthol has a peppermint flavour and is an important perfume which is obtained from peppermint or other mint oils. *dl*-Menthol is currently prepared chemically by hydrogenation of thymol, but the *dl*-menthol prepared in this way contains four isomers (*dl*-menthol, *dl*-iso-menthol, *dl*-neo-menthol and *dl*-iso-neo-menthol). Thus, separation of *l*-menthol from the isomers and racemates is industrially very important. Resolution of menthyl ester racemates by microbial carboxyesterase has been reported in aqueous systems (Oritani and Yamashita 1973; Yamaguchi et al. 1977). However, neither *dl*-menthyl ester nor *l*-menthol is soluble in water. Hence, it would be advantageous to employ organic solvents for the optically selective hydrolysis of menthyl ester racemates. This can only be done, however, if the microbial esterases are not inactivated by the organic solvents.

We have reported the successful transformations of various steroids in water-immiscible organic solvents using microbial cells entrapped in gels of appropriately hydrophobic or hydrophilic nature (Fukui et al. 1980; Omata et al. 1979a and b, 1980; Yamane et al. 1979). These steroid transformations were characterized by homogeneous reaction systems and position-specific conversion of water-insoluble substrates even in organic solvents. The techniques employed for the steroid transformations seem to be applicable to the bioconversion of various water-insoluble or strongly hydrophobic compounds.

This paper describes optically selective hydrolysis of *dl*-menthyl ester by immobilized yeast cells in organic solvent systems. The immobilized cells were much superior to the free cells.

Materials and Methods

Urethane Prepolymers

Two kinds of water-miscible urethane prepolymers (PU-3 and PU-6) (Fukushima et al. 1978; Omata et al. 1979a) were prepared by Toyo Rubber Industry Co., Japan. PU-3 gives a hydrophobic gel and PU-6 a hydrophilic gel. The structure and property of the prepolymers have been described previously (Omata et al. 1979a; Sonomoto et al. 1980).

Photo-Crosslinkable Resin Prepolymer

ENT-4000, a derivative of poly(ethylene glycol)-4000 (Tanaka et al. 1978) was synthesized by Kansai Paint Co., Ltd., Japan. This prepolymer gives a hydrophilic gel.

Cultivation of Yeast

Rhodotorula minuta var. *texensis* IFO 1102 was cultivated with shaking (220 rpm) at 30 °C in 500 ml shaking flasks each containing 100 ml of a medium of the following composition (per liter): Treacle, 50 g; corn steep liquor, 50 g; ammonium sulfate, 5 g; mineral mixture, 10 ml. The mineral mixture was (g per liter): $MgSO_4 \cdot 7H_2O$, 20; $FeSO_4 \cdot 7H_2O$, 5; $CaCl_2$, 2; $MnCl_2 \cdot 4H_2O$,

* Dedicated to the 65th birthday od Professor Dr. G. Manecke

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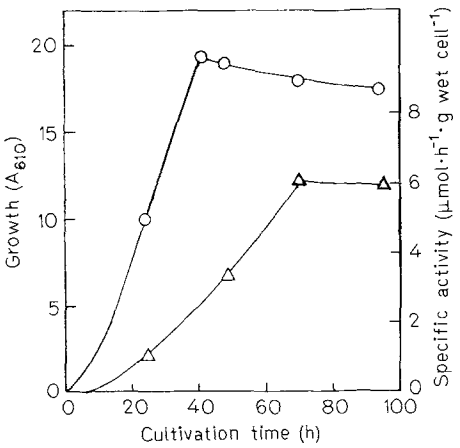

Fig. 1. Growth and specific hydrolytic activity of *R. minuta* var. *texensis*. Enzyme activity was measured with 180 m*M* *dl*-menthyl succinate ammonium salt. ○, Growth; △, specific enzyme activity

0.2; $NaMoO_4 \cdot 2H_2O$, 0.1 and NaCl, 0.1. The pH of the medium was adjusted to 7.0 with 1 N NaOH before sterilization. Cells were harvested by centrifugation after 70 h of cultivation, washed twice with 20 m*M* potassium phosphate buffer (pH 7.0), mixed thoroughly, and stored in sealed sample bottles at −20 °C. The frozen cells maintained their original hydrolytic activity for at least one month.

Immobilization of Cells

Thawed cells (1 g wet cells) were suspended in 3 ml of water and immobilized with 1 g of the hydrophilic photo-crosslinkable resin prepolymer, ENT-4000, as described previously (Omata et al. 1979a). Thawed cells (1 g wet cells) suspended in 2 ml of water were entrapped with 1 g of the urethane prepolymer, PU-3 or PU-6 or a mixture of both, as reported previously (Omata et al. 1979a).

Reaction Conditions

The reaction was carried out at 30 °C with shaking (180 strokes/min) for the indicated period. Unless stated otherwise, a two-phase system composed of 20 m*M* potassium phosphate buffer (pH 7.0) and *n*-heptane (1 : 5 by volume) was employed as a reaction medium for free cells, since the yeast cells could not be suspended well in any of the organic solvents we have tried. As mentioned in "Results", *n*-heptane saturated with water was also used as the reaction solvent in the immobilized cell system, taking into account the solubility of the substrate and the products and the minimum inactivation of the hydrolyzing enzyme (esterase) in the cells.

Analytical Methods

l-Menthol produced from *dl*-menthyl succinate was assayed by gas chromatography with a JEOL-20 KFL gas chromatograph equipped with a hydrogen flame ionization detector. The steel column (2 m x 2 mm ID) was packed with LAC-2R-446 (20% by weight) on Celite 545, 60–80 mesh (Gasukuro Kogyo, Japan). The temperatures at the injector, in the column and at the detector were 175°, 140°, and 240 °C, respectively. The flow rate of the carrier gas, helium, was 44 ml/min. γ-Valerolactone was used as the internal standard for the determination of *l*-menthol. *l*-Menthol was isolated from the reaction mixture as follows: the reaction mixture was washed with an equal volume of sodium hydroxide (0.1 N), dried on calcium chloride, and the solvent was removed in vacuo to yield white crystals which were recrystallized from a mixture of ethanol and water. The m.p. of *l*-menthol so obtained was 40–41 °C (m.p. in literature, 41–43 °C). Optical rotation of the product was measured in ethanol with a DIP-SL automatic polarimeter (Japan Spectroscopic Co.). The specific rotation $[\alpha]_D^{25}$ was estimated using the following equation.

$$[\alpha]_D^{25} = \frac{a}{l \times c},$$

where a is the observed rotation; l, light path (dm); and c, concentration of *l*-menthol (g/100 ml).

Synthesis of Menthyl Succinate

dl-Menthyl succinate was synthesized by the following method. Succinic anhydride (15.7 g) was added dropwise to *dl*-menthol (24.5 g) dissolved in *o*-xylene with stirring at 80 °C. After 3 h, the solvent was removed to obtain white crystals, which were recrystallized from benzene (yield, 86%). Identification of the product was carried out by spectrophotometric analyses (NMR, IR and Mass). The results showed that the compound was indeed *dl*-menthyl succinate. The water-soluble ammonium salt of *dl*-menthyl succinate was prepared as follows: *dl*-Menthyl succinate (8.3 g) was neutralized with 60 ml of 2.2 M ammonium hydroxide, and the pH was adjusted to 7.0 with 5.1 N HCl. The final concentration of *dl*-menthyl succinate ammonium salt was estimated to be 390 m*M*. If necessary, this solution was diluted with 20 m*M* potassium phosphate buffer (pH 7.0).

Results

Cultivation of Yeast

The relationship between the growth of *R. minuta* var. *texensis* and its ability to hydrolyze menthyl succinate ammonium salt is illustrated in Fig. 1. The yeast reached the stationary growth phase after 40 h of cultivation, while the enzyme activity continued to increase until 70 h. Exogenous compounds are not necessary to induce the hydrolyzing enzyme (esterase). In the subsequent experiments, the cells were harvested after 70 h of cultivation.

Stereoselective Hydrolysis of Menthyl Succinate by Yeast

dl-Menthyl succinate and its ammonium salt were employed as substrates in the stereoselective hydrolysis of menthyl ester by the *R. minuta* var. *texensis* cells (Fig. 2). The succinate ester was used because the succinate produced by the enzymatic reaction can be easily recovered as succinic anhydride with a high yield, and

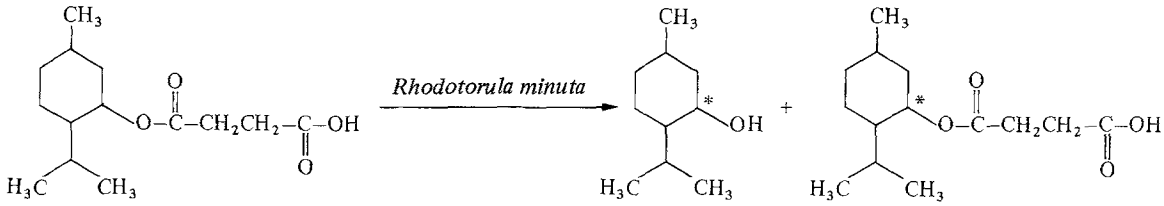

Fig. 2. Stereoselective hydrolysis of *dl*-menthyl succinate catalyzed by *R. minuta* var. *texensis*

Table 1. Effect of solvent on hydrolysis of *dl*-menthyl succinate by free cells of *R. minuta* var. *texensis*

Substrate	Solvent	*l*-Menthol formed (m*M*)	Conversion ratio (%)
dl-Menthyl succinate ammonium salt	KPB[a]	13.9	71.1
	KPB : Methanol (75 : 25)	9.3	47.9
	KPB : Dimethylformamide (50 : 50)	2.7	13.6
	KPB : CH_3CN (50 : 50)	1.0	5.2
	KPB : CH_3CN (65 : 35)	2.7	13.6
	KPB : CH_3CN (80 : 20)	5.8	29.9
dl-Menthyl succinate	Water-saturated benzene	0.1	0.6
	Water-saturated chloroform	0	0
	Two-phase system (KPB-Organic solvent, 1 : 5)		
	t-Butyl acetate	0[b]	0
	Benzene	4.4[b]	22.4
	Benzene-*n*-Heptane (50 : 50)	4.9[b]	24.9
	Benzene-*n*-Heptane (40 : 60)	6.3[b]	32.5
	Benzene-*n*-Heptane (30 : 70)	7.4[b]	38.0
	Benzene-*n*-Heptane (20 : 80)	8.7[b]	44.6
	Benzene-*n*-Heptane (10 : 90)	10.4[b]	53.1
	n-Heptane	14.2[b]	72.6

[a] 20 m*M* potassium phosphate buffer (pH 7.0)
[b] Concentration in organic solvent phase
The cells (1 g wet cell) were incubated for 48 h in 10 ml of KPB, KPB-water-miscible solvents or water-saturated organic solvents (39m*M* substrate), or in two-phase systems composed of 10 ml of water-immiscible organic solvent (39 m*M* substrate) and 2 ml of KPB

because a water-soluble derivative, menthyl succinate ammonium salt, is easily prepared.

The free yeast cells (1 g wet cells) were suspended in 10 ml of 20 m*M* potassium phosphate buffer (pH 7.0) containing 39 m*M* *dl*-menthyl succinate ammonium salt. A linear relationship was observed between the yield of *l*-menthol and the reaction time until 48 h of incubation. Most of the *l*-menthol formed did not dissolve in the aqueous buffer and accumulated on the surface of the cells, thereby decreasing their apparent activity. To prevent the accumulation of *l*-menthol on the cell surface, various kinds of water-miscible organic solvents were tested for the cosolvent of the buffer. As shown in Table 1, the hydrolytic activity of the yeast cells was reduced by the presence of organic cosolvents, probably due to denaturation of the enzyme (esterase) in the cells. Thus, the use of menthyl succinate ammonium salt as substrate in the aqueous reaction system was not necessarily convenient for the production of *l*-menthol.

Hydrolysis of menthyl succinate in organic solvents by the free cells was not successful, because the cells could not be suspended well in such solvents. Therefore, two-phase systems composed of the phosphate buffer and water-immiscible organic solvents (1 : 5 by volume) were tested for the optically selective hydrolysis of *dl*-menthyl succinate by the free cells of *R. minuta* var. *texensis*. The results are summarized in Table 1. The yield of *l*-menthol after 48 h incubation in a two-phase system composed of 20 m*M* potassium phosphate buffer (pH 7.0) and *n*-heptane (1 : 5 by volume) was almost the same as in the phosphate buffer containing *dl*-menthyl succinate ammonium salt. Hence, *n*-heptane was selected as the reaction solvent in the subsequent experiments using the phosphate buffer-*n*-heptane two-phase system for the free cells and water-saturated *n*-heptane for the entrapped cells.

In the immobilized cell-catalyzed systems, water-saturated *n*-heptane was employed as reaction solvent instead of the buffer-*n*-heptane two-phase system to construct a

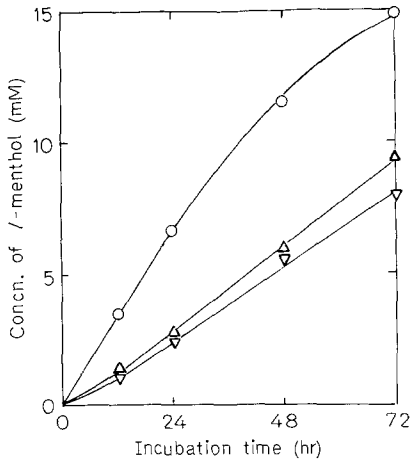

Fig. 3. Time-course of *l*-menthol production by *R. minuta* var. *texensis*. The free yeast cells (1 g wet cell) were incubated at 30 °C in a two-phase system composed of 10 ml of *n*-heptane containing 39 m*M* *dl*-menthyl succinate and 2 ml of 20 m*M* potassium phosphate buffer (pH 7.0). The concentration of *l*-menthol is given as that in the organic solvent phase. The immobilized cells (1 g wet cell) were incubated in 10 ml of water-saturated *n*-heptane containing 39 m*M* of substrate. ○, Free cells; △, PU-3-entrapped cells; ▽, PU-6-entrapped cells

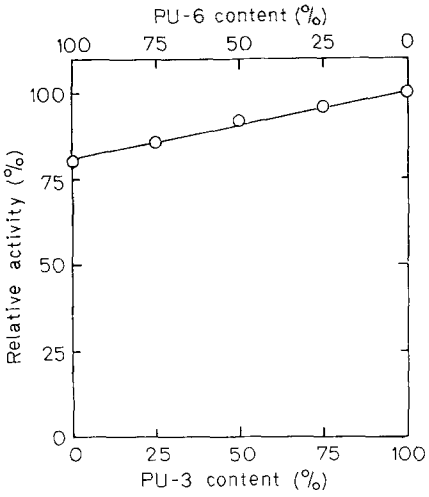

Fig. 4. Effect of gel hydrophobicity on relative hydrolytic activity. The activity of the PU-3-entrapped cells was expressed as 100%. The reaction was carried out in water-saturated *n*-heptane containing 39 m*M* *dl*-menthyl succinate

homogeneous system. Figure 3 illustrates the time-course of *l*-menthol production by the free and immobilized cells in the buffer-*n*-heptane system and water-saturated *n*-heptane, respectively. The hydrolytic activity of the PU-3-entrapped cells was slightly higher than that of the PU-6-entrapped cells and the rate of *l*-menthol formation was 2.5 and 2.0 μmol · h^{-1} · g wet $cell^{-1}$ respectively, while that of the free cells was 6.1 μmol · h^{-1} · g wet $cell^{-1}$.

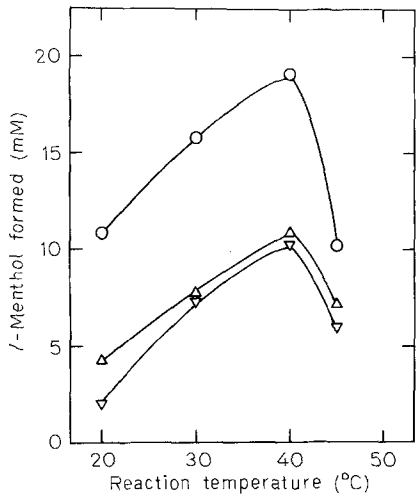

Fig. 5. Temperature-dependency of hydrolytic activity. The enzyme reaction was carried out for 24 h as in Fig. 3, except for the reaction temperature and the amount of cells used (2 g wet cell). ○, Free cells; △, PU-3-entrapped cells; ▽, PU-6-entrapped cells

Table 2. Specific rotation of *l*-menthol produced from *dl*-menthyl succinate by free and gel-entrapped cells

Cells	$[\alpha]_D^{25}$	Optical purity
Free cells	−51°	100
PU-3-entrapped cells	−51°	100
PU-6-entrapped cells	−51°	100

Figure 4 shows the relationship between the relative hydrolytic activity of the cells and the hydrophobicity of the gels prepared by mixing hydrophilic PU-6 and hydrophobic PU-3 in different ratios. The activity increased as the content of the hydrophobic PU-3 increased.

The temperature-dependency of the hydrolytic activity of the cells is illustrated in Fig. 5. The optimal temperature for the free and immobilized cells was 40 °C. However, subsequent experiments were carried out at 30 °C to minimize the inactivation of the enzyme during long periods of operation.

Optical Purity of l-Menthol Produced

The specific rotation of *l*-menthol produced from *dl*-menthyl succinate by the free and immobilized cells is summarized in Table 2. The results showed that the product was pure *l*-menthol. The initial optical yields were maintained even after 10 reaction batches (total operational period, 200 h) in every case.

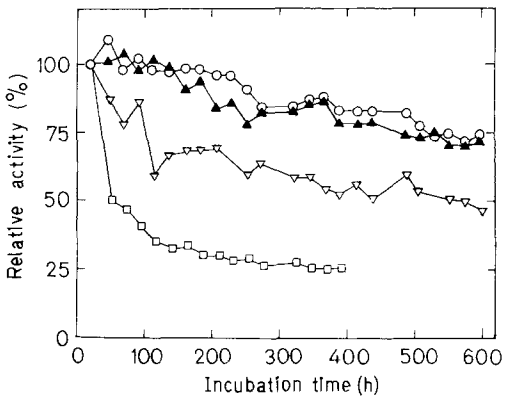

Fig. 6. Repeated use of free and gel-entrapped *R. minuta* var. *texensis* cells in hydrolysis of *dl*-menthyl succinate. Each reaction was carried out at 30 °C with shaking (180 strokes/min) for 24 h as in Fig. 3. □, Free cells; ○, PU-3-entrapped cells; ▲, PU-6-entrapped cells; ▽, ENT-4000-entrapped cells

Stability of Hydrolytic Activity of Free and Immobilized Cells

When menthyl succinate ammonium salt was hydrolyzed by the free cells, about 80% of *l*-menthol produced was accumulated on the cell surface. Extraction of *l*-menthol by organic solvent, such as benzene, significantly damaged the hydrolytic activity of the cells, thus rendering repeated use of the cells difficult. Addition of 10% methanol to the aqueous reaction mixture improved the productivity of *l*-menthol by the free cells, and their activity was stable for at least 10 reaction batches (total operational period, 240 h). Figure 6 illustrates the repeated use of the free cells in the buffer-*n*-heptane system and of PU-3-, PU-6- and ENT-4000-entrapped cells in water-saturated *n*-heptane for the hydrolysis of menthyl succinate. The free cells were recovered from reaction mixture by centrifugation, and the immobilized cells by filtration. The half-life of the activity of the free cells was 50 h, while that of the ENT-4000-, PU-6- and PU-3-entrapped cells was estimated to be 560, 1,315, and 1,520 h, respectively. Thus, immobilization greatly improved the operational stability of the hydrolytic enzyme in the yeast cells.

Discussion

Optically specific hydrolysis of *dl*-menthyl esters to yield *l*-menthol by microorganisms is industrially important. However, this microbial transformation has some problems; for example the low solubilities of both substrates (*dl*-menthyl esters) and the product (*l*-menthol) in the aqueous reaction media; the different optical purity of the hydrolysis products depending on the kinds of substrates, microorganisms and reaction conditions; and the instability of the hydrolytic enzyme (esterase) in the microorganisms.

In the study presented here, *R. minuta* var. *texensis* was selected since it showed a high stereoselectivity in the hydrolysis of *dl*-menthyl succinate. Use of an organic solvent such as *n*-heptane, either in a two-phase system or a water-saturated homogeneous system, permitted high solubilities of *dl*-menthyl succinate and *l*-menthol. Another product, succinic acid, was also soluble even in the homogeneous system up to a concentration of 39 m*M*. Furthermore, entrapment of the yeast cells within a suitable gel improved the stability of the hydrolytic enzyme in the cells.

The acids used to form the *dl*-menthyl esters have to be carefully selected because of its influence on the optical purity of the hydrolysis product (Yamaguchi et al. 1976). Recovery of the corresponding acids from the reaction mixture also should be high. We employed the succinate ester as the substrate because of the high optical purity (100%) of *l*-menthol produced by the enzyme of *R. minuta* var. *texensis*, and of the high recovery (75%) of succinic acid. The remaining *d*-menthyl succinate can be racemized by heating in the presence of certain catalysts.

The free yeast cells could not be suspended in water-immiscible organic solvents, such as benzene, carbon tetrachloride, chloroform and *n*-heptane. Therefore, a two-phase system composed of potassium phosphate buffer and organic solvent (1 : 5 by volume) was tested in the free cell system. Hydrolytic activity was significantly affected by the kind of solvents, while optical selectivity on hydrolysis of *dl*-menthyl succinate was not.

In the water-saturated *n*-heptane system, the activity of the immobilized cells increased with the hydrophobicity of the gels, although there was little difference in activity between the hydrophilic gel (PU-6)-entrapped cells and the hydrophobic gel (PU-3)-entrapped cells. This may be due to the less hydrophobic nature of *dl*-menthyl succinate than cholesterol (Omata et al. 1979a).

During long-term repeated hydrolysis of *dl*-menthyl succinate, the entrapped cells were more stable than the free cells and retained most of their initial activity even after 600 h of the reaction. The optical selectivity was completely maintained even after 200 h. These results indicate that denaturation of some parts of the enzyme by the organic solvent does not affect the optical yield of *l*-menthol but only the hydrolytic activity.

As described above, a possibility of the practical application of microbial cells immobilized with a photo-cross-linkable resin prepolymer or urethane prepolymer has been presented for the production of optically pure *l*-menthol from *dl*-menthyl succinate in a water-saturated organic solvent system. The flow diagram for the large scale production of *l*-menthol is shown in Fig. 7.

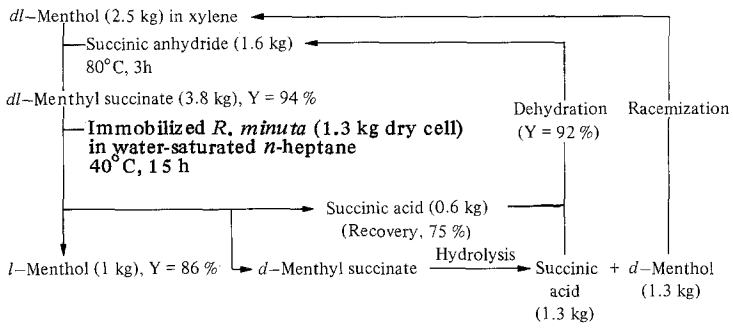

Fig. 7
Flow sheet of *l*-menthol production by gel-entrapped cells

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