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Microbial production of (*S*)-1-phenyl-1,3-propanediol by stereospecific reduction of 3-hydroxy-1-phenylpropane-1-one

Ikuo Kira*, Takayuki Suzuki, Norimasa Onishi, Kazuhiro Watanabe, Eizi Nakanishi

AminoScience Laboratories, Ajinomoto Co., Inc., 1-1 Suzuki-cho, Kawasaki-ku, Kawasaki 210-8681, Japan

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ABSTRACT

A novel microbial method of synthesizing (*S*)-1-phenyl-1,3-propanediol [(*S*)-PPD] was developed in this study. Our laboratory stock cultures were screened for microorganisms that stereospecifically produced (*S*)-PPD from 3-hydroxy-1-phenylpropane-1-one (HPPO) using an intact cell system. Of the 828 strains examined (321 bacteria, 233 yeasts and 274 molds), certain strains of *Williopsis saturnus* var. *mrakii* and *Cryptococcus albidus* were found to produce (*S*)-PPD with over 99% enantiomeric excess (e.e.). Screening identified *W. saturnus* var. *mrakii* AJ-5620 as the most productive strain, and this strain was used for further experiments. The (*S*)-PPD-producing reaction using intact *W. saturnus* var. *mrakii* AJ-5620 cells was carried out by successive feeding of HPPO. A total (*S*)-PPD yield of 9.9 g/l was produced in 20 h. The molar yield was 81% and the optical purity of the (*S*)-PPD produced was over 99% e.e.

1. Introduction

Recent investigations have clearly established that the individual enantiomers contained in racemic mixtures of pharmaceutical drugs can have different pharmacokinetic and bioavailability profiles. The manufacturing of the active form of the drug is consequently becoming the norm in the industry [1]. Chiral alcohols are very important precursors of a large number of pharmaceuticals, and (*S*)-1-phenyl-1,3-propanediol [(*S*)-PPD] is an important intermediate in the synthesis of therapeutic agents such as the serotonin-uptake inhibitor (*S*)-fluoxetine (Fig. 1).

A number of chemical and chemoenzymatic methods of synthesizing (*S*)-PPD have been reported including synthesis of (*S*)-PPD by asymmetric epoxidation of cinnamyl alcohol followed by reduction [2], or by asymmetric allylboration of benzaldehyde followed by ozonolysis and reduction [3], or by asymmetric aldol reaction of silylketene acetals with benzaldehyde followed by reduction [4]. However, these chemical methods require optically active catalysts and are too complicated to be suitable for the industrial production of (*S*)-PPD. Teshima et al. [5] and Boaz [6] reported synthesis of (*S*)-PPD by reduction of optically active 3-hydroxy-3-phenylpropionic acid prepared by enzymatic optical resolution. However, the above mentioned chemoenzymatic methods have the disadvantage that the yield of the desired enantiomer is less than 50% unless the residual substrates are racemized.

Several microbial methods of synthesizing of (*S*)-PPD have also been reported, including synthesis of (*S*)-PPD by hydrolysis of optically active 3-hydroxy-3-phenylpropionic acid ethyl ester prepared by microbial asymmetric reduction of ethyl benzoylacetate [7], or by microbial optical resolution of racemic PPD [8]. Though these microbial methods are excellent procedures, the molar yield of (*S*)-PPD produced is not high (40–60%).

Therefore, with industrial production in mind, we attempted to produce (*S*)-PPD by a microbial method involving the stereospecific reduction of 3-hydroxy-1-phenylpropane-1-one (HPPO), which is easily synthesized from 4-phenyl-1,3-dioxane (Fig. 2).

Several alcohol dehydrogenases and aldehyde reductases have been isolated and applied for the preparation of chiral aryl alcohols [9–13]. Various microorganisms such as *Saccharomyces cerevisiae* [14], *Corynebacterium* sp. [15], *Cryptococus macerans* [16], *Hansenula capsulate* [17], *Geotrichum* sp. [18] and *Leifsonia* sp. [19] have also been used for the preparation of optically pure aryl alcohols. However, there have been no reports regarding (*S*)-PPD production by bioreduction.

In this report, we described screening for microorganisms which are able to produce (*S*)-PPD from HPPO, and the optimization of culture conditions and reaction conditions to maximize (*S*)-PPD production.

* Corresponding author. Tel.: +81 952 47 2214; fax: +81 952 47 6286.
E-mail address: ikuo_kira@ajinomoto.com (I. Kira).

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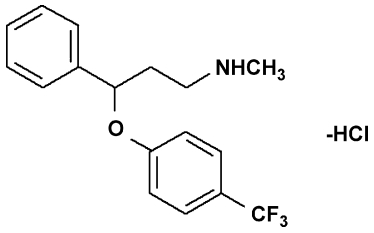

Fig. 1. Structure of (*S*)-fluoxetine.

2. Materials and methods

2.1. Materials

Both (*S*)- and (*R*)-PPD were purchased from Chisso Corporation (Japan). HPPO was prepared as follows. To a solution of 4-phenyl-1,3-dioxane (0.82 g, 4.99 mmol) in 5 ml of CH_2Cl_2 and 5 ml of water, Br_2 (0.29 ml, 5.63 mmol) was added and stirred for 2 h at 5 °C. After an addition of 10 ml of saturated Na_2SO_3 solution, the mixture was extracted with CH_2Cl_2 (2× 10 ml). The combined organic layer was washed with saturated NaCl solution, dried over anhydrous Na_2SO_4 and concentrated at reduced pressure. The residue was purified by silica-gel column chromatography to obtain HPPO (0.53 g, 3.53 mmol, 70.7%). The product was confirmed as HPPO by ^{1}H nuclear magnetic resonance spectroscopy (^{1}H NMR) with the following conditions and results: (300 MHz, $CDCl_3$) δ_{ppm} 7.9–8.0 (m, 2H), 7.5–7.6 (m, 1H), 7.4–7.5 (m, 2H), 4.04 (dt, J = 5.3 Hz, 6.6 Hz, 2H), 3.24 (t, J = 5.3 Hz, 2H), 2.66 (t, J = 6.6 Hz, 1H,-OH). All other chemicals used were obtained commercially and were of analytical grade.

2.2. Microorganisms

Microorganisms from stock cultures kept in our laboratory were examined for (*S*)-PPD-producing activity, and a representative strain, *Williopsis saturnus* var. *mrakii* AJ-5620, was subjected to further investigations.

2.3. Media

Medium I contained 20 g glucose, 5 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 3 g K_2HPO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.01 g $FeSO_4{\cdot}7H_2O$, 0.01 g $MnSO_4{\cdot}4H_2O$, 10 g yeast extract and 10 g polypeptone made up to a total volume of 1 l with distilled water, and was adjusted to pH 7.0. Medium II had the same composition as medium I except that it contained 70 g fructose and 5 g $NaNO_3$ instead of 20 g glucose and 5 g $(NH_4)_2SO_4$, and was supplemented with 20 g $CaCO_3$. The fructose and $CaCO_3$ used in the preparation of medium II were sterilized separately.

2.4. Screening for (S)-PPD-producing strains

2.4.1. First screening

Each strain was inoculated into 3 ml of medium I in a test-tube and incubated at 30 °C with reciprocal shaking for 24 h (for bacteria) or 48 h (for yeasts and molds). Subsequently, 30 μl of 10% HPPO solution (100 mg of HPPO dissolved in 900 μl of ethyl alcohol) and 300 μl of 5% glucose solution were added to the culture broth and incubation was continued for a further 24 h. After the incubation, 900 μl of ethyl alcohol was added to 100 μl of the culture fluid, and the mixture was centrifuged (8000 × *g*, 10 min) to remove any insoluble materials. The supernatant was analyzed for PPD and HPPO, and the ratio of (*S*)- to (*R*)-PPD was determined as described below.

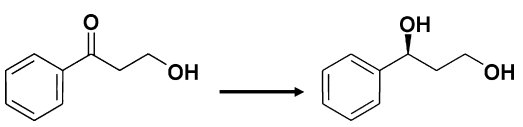

Fig. 2. Stereospecific reduction of 3-hydroxy-1-phenylpropane-1-one to (*S*)-1-phenyl-1,3-propanediol.

2.4.2. Second screening

A second screening was performed on the microorganisms selected by the first screening. Each strain was inoculated into 5 ml of medium I in a test-tube and incubated at 30 °C for 24 h with reciprocal shaking. Then, 2 ml of the culture broth was transferred to 50 ml of medium I in a 500-ml Sakaguchi flask and incubated at 30 °C for 24 h with reciprocal shaking. The cells were harvested by centrifugation (8000 × *g*, 10 min), and washed with 200 mM potassium phosphate buffer (pH 7.0). The final reaction mixture contained 5 mg of HPPO, 100 mg of glucose and the cells harvested from 5 ml of the culture broth in a total volume of 5 ml of 200 mM potassium phosphate buffer (pH 7.0). The reaction was carried out at 30 °C for 5 h with reciprocal shaking.

2.5. Preparation of intact W. saturnus var. mrakii AJ-5620 cells

One loopful of *W. saturnus* var. *mrakii* AJ-5620 cells was inoculated into 5 ml of medium I in a test-tube and incubated at 30 °C for 24 h with reciprocal shaking. Then, 2 ml of the culture broth was transferred to 50 ml of medium II in a 500-ml Sakaguchi flask and incubated at 30 °C for 18 h with reciprocal shaking. The cells were harvested by centrifugation (8000 × *g*, 10 min), washed with 200 mM potassium phosphate buffer (pH 7.0) and resuspended in 20 mM potassium phosphate buffer (pH 7.0) to produce a concentrated cell suspension, which was subsequently used for (*S*)-PPD production.

2.6. Production of (S)-PPD by intact microbial cells

The standard reaction mixture contained 25 mg HPPO, 250 mg glucose, 25 mg $(NH_4)_2SO_4$, 2.5 mg $MgSO_4{\cdot}7H_2O$ and 50 μl of vitamin mixture solution (20 mg thiamine hydrochloride, 100 mg riboflavin, 25 mg pyridoxine hydrochloride, 400 mg nicotinamide, 40 mg folic acid, 250 mg pantothenic acid and 3 mg biotin, dissolved in 1 l of distilled water), plus the washed cells harvested from 5 ml of the culture broth, in a total volume of 5 ml of 200 mM potassium phosphate buffer (pH 7.0). The reaction was carried out at 30 °C for 2 h with reciprocal shaking.

2.7. Analytical methods

The amounts of PPD produced and HPPO consumed were determined by high-pressure liquid chromatography (HPLC) under the following conditions: column, Chiralcel OB (4.6 mm × 250 mm; Daicel Chemical Industries, Japan); mobile phase, hexane–isopropanol (4/1) at a flow-rate of 0.7 ml/min; detector, Hitachi L-4000 UV detector (λ = 210 nm). Under these conditions, (*S*)-PPD, (*R*)-PPD and HPPO eluted after 8.72, 9.72 and 17.45 min, respectively.

The amount of glucose consumed was analyzed by using a glucose analyzer (model; DIAGLUCA HEK-30, TOYOBO).

Table 1
Reduction of HPPO to PPD by the microorganisms selected by initial screening

Strain	Molar yield of PPD (%)	e.e.[a] of (*S*)-PPD (%)
Cryptococcus albidus AJ-4297	37.8	>99
Cryptococcus albidus AJ-4298	27.9	>99
Cryptococcus albidus AJ-4332	17.7	>99
Cryptococcus albidus AJ-4334	17.2	>99
Cryptococcus albidus AJ-4336	20.2	>99
Williopsis saturnus var. *mrakii* AJ-5619	18.3	>99
Williopsis saturnus var. *mrakii* AJ-5620	54.3	>99

The cultivation and reaction conditions are described in Section 2 (second screening).

[a] e.e., enatiomeric excess.

2.8. Isolation and identification of the reaction product

The intact *W. saturnus* var. *mrakii* AJ-5620 cells harvested from 50 ml of culture broth were suspended in a total volume of 50 ml of the reaction mixture. The reaction mixture contained 250 mg HPPO, 2.5 g glucose, 250 mg $(NH_4)_2SO_4$, 25 mg $MgSO_4{\bullet}7H_2O$, and 500 μl of vitamin mixture solution described above in 200 mM potassium phosphate buffer (pH 7.0).

The reaction was carried out at 30 °C for 24 h with reciprocal shaking, and the reaction mixture was centrifuged (8000 × *g*, 10 min) to remove the cells. The resulting supernatant (30 ml) was extracted with ethyl acetate (2× 30 ml). The combined organic layer was concentrated at reduced pressure. The residue was purified by thin layer chromatography using a hexane–ethyl acetate (1/1) solvent system. PPD was detected using phosphomolybdic acid solution (7 g phosphomolybdic acid dissolved in 100 ml ethyl alcohol) [20].

3. Results and discussion

3.1. Screening for (*S*)-PPD-producing strains

A total of 828 strains, selected from stock cultures kept in our laboratory and comprising 321 strains of bacteria (42 genera), 233 strains of yeasts (37 genera) and 274 strains of molds (67 genera), were tested for their ability to produce (*S*)-PPD from HPPO. In the first screening, 2 strains of *W. saturnus* var. *mrakii* and 5 strains of *Cryptococcus albidus* were found to produce (*S*)-PPD with both a high molar yield (90%) and high enantioselectivity (enantiomeric excess [e.e.] > 99%). *Trichosporon fermentans*, *Aspergillus oryzae* (7 strains), *Aspergillus usami*, *Fusarium oxysporum* and *Neocosmospora vasinfecta* produced (*R*)-PPD with an e.e. of over 80%; however, the molar yield was low (11–24%).

The seven strains selected by the first screening were then tested for their (*S*)-PPD-producing activity. As shown in Table 1, *W. saturnus* var. *mrakii* AJ-5620 exhibited the highest activity in the second screening. On the basis of these results, *W. saturnus* var. *mrakii* AJ-5620 was selected as the best (*S*)-PPD-producing strain and used in further experiments.

W. saturnus var. *mrakii* AJ-5620 showed higher conversion rate and stereoselectivity in converting HPPO to (*S*)-PPD.

3.2. Isolation and identification of the reaction product

The reaction product produced by *W. saturnus* var. *mrakii* AJ-5620 was isolated by the procedures described in Materials and Methods and was found to have the same retention time as authentic (*S*)-PPD in HPLC analysis. It was identified by its FAB-MS profile: *m*/*z* 152 (M+H) and its ^{1}H NMR spectrum: (300 MHz, $CDCl_3$) δ_{ppm} 7.26–7.40 (5H, m), 4.95–4.99 (1H, m), 3.84–3.91 (2H, m), 2.74 (1H, d, *J* = 3.3 Hz), 2.27 (1H, t, *J* = 5.2 Hz), 1.89–2.10 (2H, m).

Table 2
Effects of various carbon sources on the (*S*)-PPD-producing activity of *W. saturnus* var. *mrakii* AJ-5620

Carbon source (g/l)	Cultivation		Reaction
	Final pH	Growth[a]	(*S*)-PPD produced (g/l 2 h)
Citrate (20)	8.0	2.08	0.11
Fumarate (20)	7.9	2.08	0.11
Succinate (20)	8.2	3.90	0.05
Lactose (20)	7.5	2.34	0.04
Maltose (20)	7.5	2.34	0.09
Sucrose (20)	7.6	3.38	0.09
Galactose (20)	7.4	1.82	0.09
Xylose (20)	6.8	4.16	0.13
Glycerol (20)	6.2	14.3	0.34
Glucose (20)	6.3	14.6	0.31
Fructose (20)	6.5	14.6	0.51
Fructose (30)	6.2	14.6	0.68
Fructose (50)	5.9	14.6	1.00
Fructose (70)	5.4	14.6	1.22
Fructose (100)	5.2	14.0	1.20

W. saturnus var. *mrakii* AJ-5620 was cultivated with the carbon sources indicated at 30 °C for 18 h, using medium I (see Section 2) without glucose as the basal medium. When fructose concentrations greater than 30 g/l were used, the medium was supplemented with 20 g/l $CaCO_3$. The reaction was carried out as described in Section 2.

[a] Growth is expressed as the optical density at 562 nm.

3.3. Establishment of the optimal cultural conditions for (*S*)-PPD production by *W. saturnus var. mrakii AJ-5620*

Since intact cells were used as a direct enzyme source for (*S*)-PPD production, we considered it necessary to establish optimal culture conditions for preparing cells with high (*S*)-PPD-producing activity.

To establish the best cultural conditions for maximizing the (*S*)-PPD- producing activity of *W. saturnus* var. *mrakii* AJ-5620, the effects of varying the components of the medium and the culture period were examined.

The effects of various carbon sources on (*S*)-PPD-producing activity were examined by using medium I without glucose as the basal medium. As shown in Table 2, organic acids such as citrate, fumarate and succinate, and disaccharides such as lactose, maltose and sucrose, were unfavorable for cell growth. In contrast, glucose, glycerol and fructose were suitable carbon sources. Of the carbon sources tested, fructose was the most effective for increasing (*S*)-PPD-producing activity. Although cell growth did not increase according to the fructose concentration of the culture medium, the (*S*)-PPD- producing activity increased according to the concentration of this sugar and the optimal concentration was 70 g/l.

The effects of various inorganic nitrogen sources such as $(NH_4)Cl$, CH_3COONH_4, $(NH_4)_2SO_4$ and $NaNO_3$ on (*S*)-PPD-producing activity were examined by using medium II without $NaNO_3$ as the basal medium. As shown in Table 3, all inorganic nitrogen sources tested were suitable for cell growth and the (*S*)-PPD-producing activity was highest when $NaNO_3$ was used as the inorganic nitrogen source. Thus, medium II (containing fructose and $NaNO_3$ as the carbon and inorganic nitrogen sources, respectively) was used to prepare *W. saturnus* var. *mrakii* AJ-5620 cells for further experiments.

The effects of various culture periods on (*S*)-PPD- producing activity were investigated using medium II. As shown in Fig. 3, (*S*)-PPD-producing activity changed markedly depending on the growth phase. It reached its maximum at the late exponential phase

Table 3
Effects of various inorganic nitrogen sources on the (*S*)-PPD-producing activity of *W. saturnus* var. *mrakii* AJ-5620

Inorganic nitrogen source	Cultivation		Reaction
	Final pH	Growth[a]	(*S*)-PPD produced (g/l 2 h)
NH_4Cl	5.1	14.6	0.85
CH_3COONH_4	5.8	15.6	1.22
$(NH_4)_2SO_4$	5.2	14.6	1.22
$NaNO_3$	5.3	14.6	1.38

W. saturnus var. *mrakii* AJ-5620 was cultivated with the inorganic nitrogen sources indicated at 30 °C for 18 h, using medium II (see Section 2) without $NaNO_3$ as the basal medium. Fructose was used as carbon source. The reaction was carried out as described in Section 2.

[a] Growth is expressed as the optical density at 562 nm.

(after 16–20 h of growth), after which it decreased in a similar manner as the keto-ester reductases from *Candida parapsilosis* [21] and *Microbacterium* sp. [22] and ketone reductase from *Rhodotorula* sp. [23]. The reasons for this correspondence are not clear yet, but probably result from the cessation of reductase synthesis in the aged cells and the degradation of the reductase by proteases in vivo during the later period of growth. The optical purity of the (*S*)-PPD produced was not altered by these changes in the cultivation conditions. This is in contrast to findings with other reductases from *Trichosporon captitatum* [24] and *Candida magnoliae* [25]. From an industrial point of view, this provides an advantage in that the preparation of (*S*)-PPD-producing cells with high enantioselectivity is easy.

3.4. Establishment of the optimal reaction conditions

The optimal reaction conditions were investigated using intact *W. saturnus* var. *mrakii* AJ-5620 cells grown under optimal cultural conditions.

The effect of pH on the production of (*S*)-PPD from HPPO was examined at various pH values from 5.0 to 8.0 in several buffers. Sodium acetate buffer was used for pH 5.0 and pH 5.5; potassium phosphate buffer for pH 5.5–7.5; and Tris–HCl buffer for pH 7.5 and pH 8.0. As shown in Fig. 4, pH 5.5–7.5 in potassium phosphate buffer seemed to be preferable and the optimal pH was 7.0 at 30 °C for a 2-h incubation period.

As regards the effect of temperature on the production of (*S*)-PPD from HPPO, 28 to 32 °C seemed to be preferable and the optimal temperature was 30 °C at pH 7.0 for a 2-h incubation period (Fig. 5).

The effect of the HPPO concentration on (*S*)-PPD production was examined at pH 7.0 and 30 °C. As shown in Table 4, when the concentration of HPPO in the reaction mixture was 2.5 or 5 g/l, 1.33 g/l of (*S*)-PPD was produced. When the concentration of HPPO was higher than 7.5 g/l, the amount of (*S*)-PPD production decreased according to the increase of the concentration of HPPO, and 20 g/l of HPPO caused a dramatic decrease of the amount of (*S*)-PPD production.

As shown in Table 4, the amount of glucose consumption decreased according to the increase of the concentration of HPPO. This suggests the possibility that HPPO inactivates an enzyme-recycling coenzyme system such as NADPH/NADH in a similar manner as the keto-ester reductases from *Candida parapsilosis* [21] and *Microbacterium* sp. [22].

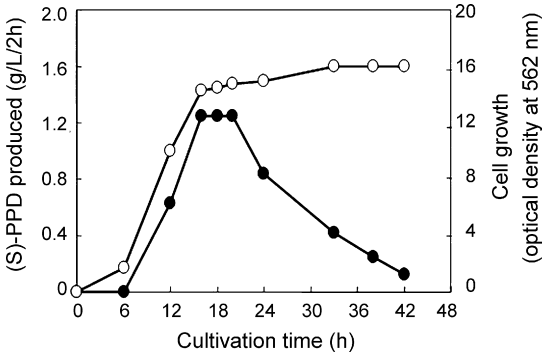

Fig. 3. Effect of culture period on (*S*)-PPD-producing activity. *W. saturnus* var. *mrakii* AJ-5620 was cultivated at 30 °C in medium II (as described in Section 2) for the culture periods indicated. The reaction was carried out as described in Section 2: (○) growth; (●) (*S*)-PPD produced.

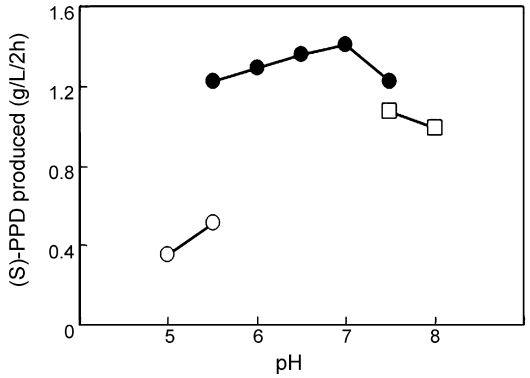

Fig. 4. Effect of pH on the production of (*S*)-PPD. The intact cells used as the enzyme source were obtained as described in Section 2. The reaction was carried out at 30 °C for 2 h as described in Section 2, except that the pH was varied as indicated: (○) 0.2 M sodium acetate buffer; (●) 0.2 M potassium phosphate buffer; (□) 0.2 M Tris–HCl buffer.

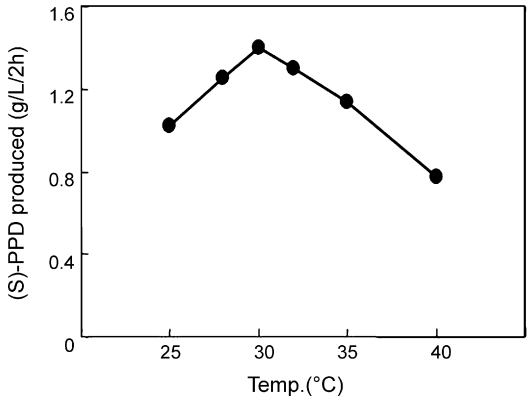

Fig. 5. Effect of temperature on the production of (*S*)-PPD. The intact cells used as the enzyme source were obtained as described in Section 2. The reaction was carried out at pH 7.0 for 2 h as described in Section 2, except that temperature was varied.

Table 4
Effect of HPPO concentration on the production of (*S*)-PPD

HPPO concentration (g/l)	(*S*)-PPD produced (g/l 2 h)	Glucose consumed (g/l 2 h)
2.5	1.33	2.45
5.0	1.33	1.55
7.5	1.24	1.15
10.0	1.22	0.75
20.0	0.15	0.10

The procedure for obtaining the intact cells used as the enzyme source was carried out as described in Section 2. The reaction was carried out at 30 °C and pH 7.0 for 2 h as described in Section 2, except that the HPPO concentration was varied.

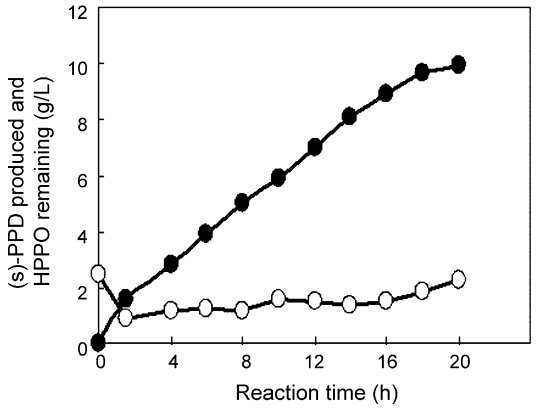

Fig. 6. Time course of (*S*)-PPD production. The intact cells used as the enzyme source were obtained as described in Section 2. The reaction mixture contained 2.5 g of glucose and the washed cells harvested from 50 ml of culture broth, in a total volume of 50 ml of 200 mM potassium phosphate buffer (pH 7.0). The reaction was carried out at 30 °C as described in Section 2, except that the HPPO concentration was kept below 0.25% by successive feeding of HPPO: (●) (*S*)-PPD; (○) HPPO.

3.5. Time course of the production of (S)-PPD

The production of (*S*)-PPD from HPPO was examined by using intact *W. saturnus* var. *mrakii* AJ-5620 cells. As shown in Fig. 4, since a high concentration of HPPO inhibited (*S*)-PPD production, the reaction was carried out under at pH 7.0 and 30 °C for 20 h, keeping the HPPO concentration below 0.25% by successive feeding of HPPO. The time course of (*S*)-PPD production is shown in Fig. 6. A total of 9.9 g/l of (*S*)-PPD was produced after a reaction time of 20 h. The molar yield was 81% and the optical purity of the (*S*)-PPD produced was over 99% e.e.

Thus we established a method of synthesizing for (*S*)-PPD based on the stereospecific reduction of HPPO by *W. saturnus* var. *mrakii*. Our method requires neither optically active catalysts nor complicated reactions. In addition to the above advantages, the (*S*)-PPD produced by our method had high optical purity, and HPPO, which is the substrate of the bioreduction, can be cheaply synthesized. It is not as yet clear which oxidoreductase group (*S*)-PPD-producing enzyme(s) from *W. saturnus* var. *mrakii* belong to. Our interest in the properties of the (*S*)-PPD-producing enzyme of this strain continues, and we are now proceeding with the purification and characterization of this enzyme.

4. Conclusion

Williopsis saturnus var. *mrakii* AJ-5620 was selected as the best strain for (*S*)-PPD production through the screening of a large number of candidates strains. *W. saturnus* var. *mrakii* AJ-5620 produced (*S*)-PPD from HPPO with high optical yield and chemical yield. We succeeded in establishing a method of synthesizing for (*S*)-PPD based on the bioreduction of HPPO.

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