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A new enzymatic method of stereoselective oxidation of racemic 1,2-indandiols

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Abstract

Microorganisms degrading indan derivatives were screened from soil for stereoselective oxidation of racemic *cis*- or *trans*-1,2-indandiol into optically active 2-hydroxy-1-indanone. Three strains, which were identified taxonomically as *Arthrobacter* sp. strain 1HB and 1HE and *Pseudomonas aeruginosa* strain IN, showed high activity and stereoselectivity on the reaction. All the strains produced inducible enzymes responsible for the oxidation reaction, recognizing the stereochemistry of 1- or 2-positions of the diol regardless of their *cis* and *trans* geometry. By using the resting cells of the strains, which had been grown under the optimized conditions, both enantiomers of 2-hydroxy-1-indanone were synthesized in enantiomerically pure form only by selecting *cis*- or *trans*-1,2-indandiol as the substrate.

Keywords: Screening; Stereoselective oxidation; Microorganism; Optically active; Indandiol; 2-Hydroxy-1-indanone; *Arthrobacter*; *Pseudomonas*

1. Introduction

Optically active *cis*-1-amino-2-indanol **1** is a key precursor of the chiral ligand **2**, and the chiral auxiliary **3** for asymmetric synthesis [1] and an important intermediate to a leading HIV protease inhibitor, Crixivan® **4** [2] (Fig. 1). The aminoalcohol **1** was synthesized by a Ritter reaction of racemic 1,2-epoxyindene followed by a crystallization/resolution [2] or by a direct Ritter reaction of optically active 1,2-indandiol [3,4]. Boyd et al. reported the one-step synthesis of **1** by toluene dioxygenase-catalyzed stereoselective benzylic hydroxylation of 2-substituted indane derivatives [5]. However, these methods have drawback in which the aminoalcohol is unavailable in large quantities. Recently, it is reported that **1** was readily synthesized from optically active 2-hydroxy-1-indanone **5** through oxime formation and diastereoselective hydrogenation [6]. Although the method is much easy to scale-up, the preparation of the hydroxyketone **5** is rather laborious and the reaction is carried out in harmful organic solvents: **5** is prepared in five steps via an intramolecular Friedel–Crafts reaction of 2-acetoxy-3-phenylpropanoyl chloride, which had been derived from optically active phenylalanine. Construction of a simple preparation of optically active **5** under mild conditions would attract attentions in the field of applied microbiology and organic chemistry.

In recent years, microbial/enzymatic transformation has been extensively evaluated from the viewpoint of synthetic organic chemistry since these methods are usually highly regio- and stereoselective and carried out under mild conditions [7–9]: some of the reactions proved to be both scientifically and industrially

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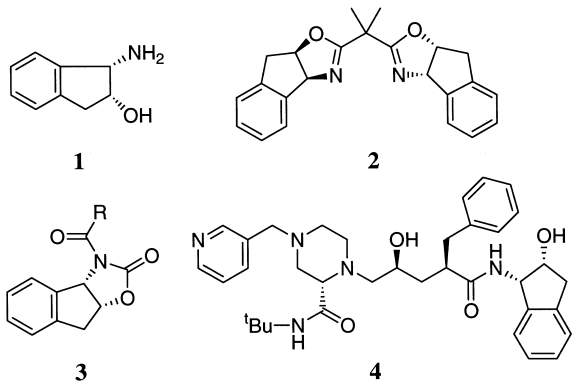

Fig. 1. Optically active *cis*-1-amino-2-indanol as a chiral template in organic synthesis.

important [10,11]. However, a surprisingly limited amount of information exists on microbial and/or enzymatic synthesis of optically active α-hydroxyketone by stereoselective oxidation of racemic 1,2-diol which can be easily prepared from olefins [12–15]. It is of our interest to accumulate biochemical informations on the enzymatic stereoselective oxidation reaction and to apply the reaction to organic syntheses.

We therefore started to discover a novel method to synthesize optically active **5** by a microbial stereoselective oxidation of racemic *cis*-1,2-indandiol **6** or *trans*-one **7**, which were easily prepared from indene [16] (Fig. 2). We screened for microorganisms having an ability to stereoselectively oxidize the racemic diol **6** or **7** into optically active **5** among various indan derivatives-degrading microorganisms, identified the active strains, and optimized the conditions for the enzyme formation during microbial cultivation and the microbial oxidation of the diols. This is the first report on stereoselective microbial synthesis of both for enantiomers of the hydroxyketone **5** from the diols.

2. Materials and methods

2.1. Analytical methods

^{1}H and ^{13}C-NMR spectra were recorded by JEOL LA-400 spectrometer (Tokyo, Japan) with tetramethylsilane as an internal standard. Melting point was determined on a Yanaco MP-J3 (Yanagimoto, Tokyo, Japan) hot plate micro melting point apparatus. Optical rotations were measured on a Horiba SEPA-300 polarimeter (Tokyo, Japan).

2.2. Materials

HPLC columns Chiralcel OB and ODS-80Ts were purchased from Daicel Chem. Ind. (Tokyo, Japan) and Tosoh (Tokyo, Japan), respectively. Meat and malt extracts were obtained from Kyokuto (Tokyo, Japan). Polypepton and yeast extract were purchased from Nippon Seiyaku (Tokyo, Japan). Beef extract was from Difco (Detroit, USA). Corn steep liquor (CSL) was from Sanei Touka (Aichi, Japan). NZ AMINE® and NZ CASE® were obtained from Wako Pure Chem. (Osaka, Japan). 1-Indanol, 2-indanol, 1-indanone, 2-indanone, indan, indene, and *trans*-2-bromo-1-indanol were from Tokyo Kasei Kogyo (Tokyo, Japan). Racemic *cis*-1,2-indandiol-2-formate was from Kanto Chem. (Tokyo, Japan). 1,2-Epoxyindene was kindly donated from Nippon Steel Corp. (Kawasaki, Japan). All other chemicals were from commercial sources and used without further purification.

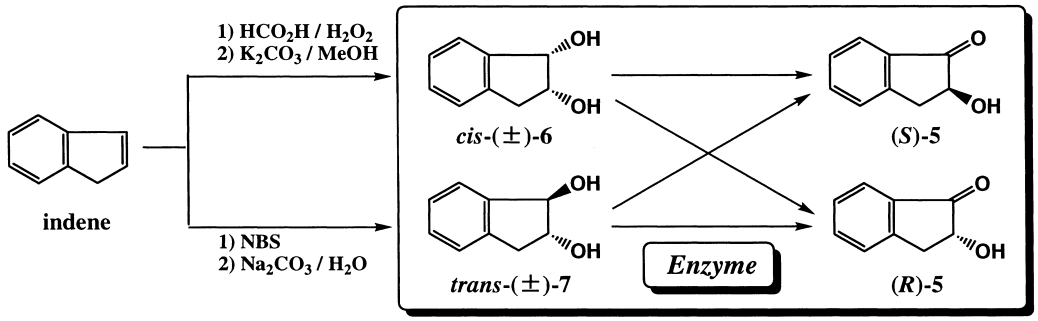

Fig. 2. Microbial stereoselective oxidation of racemic *cis*- and *trans*-1,2-indandiol into optically active 2-hydroxy-1-indanone.

2.3. Chemical synthesis of indan-derivatives

2.3.1. Racemic cis-1,2-indandiol **6**

To a stirred suspension of K_2CO_3 (90 mmol) in MeOH (300 ml) was added racemic *cis*-1,2-indandiol-2-formate (165 mmol) and the mixture was stirred at room temperature for 2.5 h. After insoluble materials were filtered-off, the filtrate was evaporated to dryness and was partitioned between water and ethyl acetate. The reaction product was extracted with ethyl acetate for three times and the combined organic layer was dried over anhydrous Na_2SO_4. After evaporation of the solvent, the crude product was recrystallized from ethyl acetate/petroleum ether to give racemic **6** as colorless needles (24.5 g, 99.2%). ^{1}H-NMR (DMSO-d_6) δ_{ppm} 7.15–7.32 (m, 4H), 4.98 (d, 1H, $J = 6.9$ Hz), 4.78 (d, 1H, $J = 5.4$ Hz), 4.55 (t, 1H, $J = 4.9$ Hz), 2.92 (dd, 1H, $J = 5.4$, 15.6 Hz), 2.76 (dd, 1H, $J = 3.4$, 15.6 Hz); ^{13}C-NMR (DMSO-d_6) δ_{ppm} 143.8, 140.4, 127.5, 126.1, 124.7, 124.6, 74.9, 72.7, 38.2; mp 98–99°C.

2.3.2. Racemic trans-1,2-indandiol **7**

Racemic *trans*-2-bromo-1-indanol (117 mmol) was added to a 440 ml of 0.62 M aqueous solution of Na_2CO_3, and the mixture was refluxed for 7 h. After cooling to room temperature, the mixture was filtered and evaporated to dryness. The residue was suspended in 2-propanol and filtered to remove insoluble materials. The filtrate was concentrated and was recrystallized from ethyl acetate afforded racemic **7** as colorless needles (5.89 g, 33.6%). ^{1}H-NMR (DMSO-d_6) δ_{ppm} 7.21–7.24 (m, 1H), 7.10–7.16 (m, 3H), 5.34 (d, 1H, $J = 6.3$ Hz), 5.13 (d, 1H, $J = 4.9$Hz), 4.66 (dd, 1H, $J = 5.4$, 6.3 Hz), 4.05 (m, 1H), 3.03 (dd, 1H, $J = 6.8$, 15.6 Hz), 2.56 (dd, 1H, $J = 7.0$, 15.6 Hz); ^{13}C-NMR (DMSO-d_6) δ_{ppm} 144.29, 139.61, 127.70, 126.62, 124.72, 124.39, 80.79, 80.17, 38.22; mp 155–160°C.

2.3.3. (R)- and (S)-2-Hydroxy-1-indanone ((R)- and (S)-**5**)

(*R*)-hydroxyketone **5** was prepared from (*R*)-phenylalanine by the methods as described [5]: (1) conversion of (*R*)-phenylalanine to (*R*)-phenyllactic acid by a Van-slyke reaction; (2) *O*-acetylation of the hydroxy acid with acetic anhydride; (3) conversion of the acetoxy acid to acid chloride with $SOCl_2$; (4) intramolecular Friedel–Crafts cyclization of the acid chloride with $AlCl_3$ to obtain (*R*)-2-acetoxy-1-indanone and (5) hydrolysis of an acetyl group of the acetoxyketone by $Sc(OTf)_3$ in an aqueous MeOH. (*R*)-**5** was obtained as pale brown needles (34.9% yield, five steps) by recrystallization from CH_2Cl_2/hexane. ^{1}H-NMR ($CDCl_3$) δ_{ppm} 7.77 (d, 1H, $J = 7.0$ Hz), 7.64 (t, 1H, $J = 7.0$ Hz), 7.46 (d, 1H, $J = 7.1$ Hz), 7.40 (t, 1H, $J = 7.3$ Hz), 4.58 (dd, 1H, $J = 4.9$, 8.0 Hz), 3.59 (dd, 1H, $J = 8.0$, 16.1 Hz), 3.47 (br, 1H), 3.03 (dd, 1H, $J = 4.9$, 16.6 Hz); ^{13}C-NMR ($CDCl_3$) δ_{ppm} 206.6, 150.9, 135.8, 134.0, 128.0, 126.7, 124.4, 74.2, 35.1; mp 82°C; $[\alpha]_D^{20} = -57$ ($c = 1.0$, MeOH). (*S*)-**5** was also prepared from (*S*)-phenylalanine by the same methods; mp 84°C; $[\alpha]_D^{20} = +56$ ($c = 1.0$, MeOH).

2.3.4. cis-(1S, 2R)-1,2-Indanediol ((1S, 2R)-**6**)

To a MeOH (200 ml) solution of (*R*)-2-acetoxy-1-indanone (26.3 mmol), prepared by the methods as described in Section 2.3.3, was added 1.25 g of wet-type 10% Pd/C (NEM-Cat., Tokyo, Japan) and hydrogenated at room temperature for 1 h. After filtration, the reaction mixture was concentrated and recrystallized from CH_2Cl_2/hexane. *cis*-(1*S*, 2*R*)-2-Acetoxy-1-indanol was obtained as colorless needles in a yield of 2.53 g (50.2%). ^{1}H-NMR ($CDCl_3$) δ_{ppm} 7.46 (m, 1H), 7.28 (m, 3H), 5.46 (m, 1H), 5.21 (m, 1H), 3.20 (dd, 1H, $J = 5.6$, 16.6 Hz), 3.06 (dd, 1H, $J = 3.4$, 16.6 Hz), 2.33 (d, 1H, $J = 7.6$ Hz), 2.05 (s, 3H); ^{13}C-NMR ($CDCl_3$) δ_{ppm} 170.9, 141.8, 139.1, 128.8, 127.3, 125.0, 124.6, 76.0, 75.2, 36.1, 21.0. The acetyl group of the acetoxyalcohol was removed under the transesterification conditions as described in Section 2.3.1. After recrystallization with ethyl acetate/diisopropylether/hexane, *cis*-(1S, 2R)-**6** was obtained as colorless needles (1.60 g, 80.8%). ^{1}H-NMR (DMSO-d_6) δ_{ppm} 7.30 (m, 1H), 7.18 (m, 3H), 4.98 (d, 1H, $J = 6.8$ Hz), 4.77 (t, 1H, $J = 5.6$ Hz), 4.56 (d, 1H, $J = 4.6$ Hz), 4.26 (m, 1H), 2.92 (dd, 1H, $J = 5.4$, 15.6 Hz), 2.76 (dd, 1H, $J = 3.6$, 15.6 Hz); ^{13}C-NMR (DMSO-d_6) δ_{ppm} 143.9, 140.5, 127.6, 126.2, 124.8, 124.7, 75.0, 72.9, 38.2; $[\alpha]_D^{20} = -50.5$ ($c = 0.95$, $CHCl_3$); mp 98°C.

2.3.5. trans-(1R, 2R)-1,2-Indanediol ((1R, 2R)-**7**)

(*R*)-2-Acetoxy-1-indanone (26.3 mmol) was hydrogenated in 200 ml of concentrated HCl/MeOH = 1/5 for 2 h in the presence of 2 g of 10% Pd/C. After recrystallization from diisopropylether/hexane, *trans*-(1*R*, 2*R*)-2-acetoxy-1-indanol (2.89 g, 57.5%) was

obtained as colorless crystals. ^{1}H-NMR ($CDCl_3$) δ_{ppm} 7.40 (m, 1H), 7.24 (m, 3H), 4.69 (m, 1H), 4.56 (m, 1H), 3.31 (dd, 1H, J = 6.6, 17.1 Hz), 2.89 (dd, 1H, J = 3.7, 17.1 Hz), 2.93 (br, 1H), 2.05 (s, 3H); ^{13}C-NMR ($CDCl_3$) δ_{ppm} 172.4, 141.1, 138.4, 128.8, 127.5, 125.0, 124.5, 84.0, 80.2, 35.7, 21.0. Transesterification of the acetoxyalcohol afforded *trans*-(1*R*, 2*R*)-**7** as colorless plates in a yield of 2.19 g (96.7%) ^{1}H-NMR (DMSO-d_6) δ_{ppm} 7.26 (m, 1H), 7.17 (m, 3H), 5.35 (d, 1H, J = 6.4 Hz), 5.14 (d, 1H, J = 4.9 Hz), 4.68 (t, 1H, J = 5.6 Hz), 4.07 (m, 1H), 3.06 (dd, 1H, J = 7.1, 15.4 Hz), 2.58 (dd, 1H, J = 6.8, 15.4 Hz); ^{13}C-NMR (DMSO-d_6) δ_{ppm} 144.1, 139.4, 127.5, 126.4, 124.5, 124.2, 80.6, 79.9, 38.0; $[\alpha]_D^{20} = -28.8$ (c = 1.08, E*t*OH); mp 157°C.

*2.3.6. cis-(1R, 2S)-1,2-Indanediol ((1R, 2S)-**6**)*

It was prepared from (*S*)-2-acetoxy-1-indanone as described in Section 2.3.4. $[\alpha]_D^{20} = -50.4$ (c = 1.03, $CHCl_3$); mp 88°C.

*2.3.7. trans-(1S, 2S)-1,2-Indanediol ((1S, 2S)-**7**)*

It was prepared from (*S*)-2-acetoxy-1-indanone as described in Section 2.3.5. $[\alpha]_D^{20} = -28.7$ (c = 1.00, E*t*OH); mp 159°C.

2.4. Screening for microorganisms which stereoselectively oxidize cis- and/or trans-1,2-indandiol

2.4.1. Media

A basal medium (pH 7.0) used for cultivation was composed of 2 g of K_2HPO_4, 1 g of NaCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of yeast extract, 10 ml of vitamin mixture, and 10 ml of trace element solution in 1 l of tap water. The vitamin mixture consisted of 4 mg thiamine·HCl, 2 mg riboflavin, 4 mg calcium pantothenate, 4 mg pyridoxine·HCl, 20 μg biotin, 2 mg *p*-aminobenzoic acid, 4 mg nicotinic acid, 0.1 mg folic acid, and 20 mg inositol in 1 l of water. The trace element solution was composed of 500 mg of Titriplex IV (CyDTA; DOTITE, Japan), 200 mg $FeSO_4 \cdot 7H_2O$, 10 mg $ZnSO_4 \cdot 7H_2O$, 3 mg $MnCl_2 \cdot 4H_2O$, 30 mg H_3BO_4, 20 mg $CoCl_2 \cdot 6H_2O$, 1 mg $CuCl_2 \cdot 2H_2O$, 2 mg $NiCl_2 \cdot 6H_2O$, and 3 mg Na_2MoO_4 in 1 l of water.

2.4.2. Isolation of indan derivatives-degrading microorganisms

Soil samples were taken from different places in Toyama Prefecture, Japan. The acclimation or enrichment culture technique [8] was used to screen for various indan derivatives-degrading microorganisms. Soil (1 g) sample was added to 5 ml of the basal medium containing 0.2% $(NH_4)_2SO_4$, 0.05–2.0% of yeast extract, and 0.2% of indan derivatives, and was incubated with shaking at 30°C. After various transfers, a loopful was streaked onto agar plates containing the same medium, and the single colonies that formed were transferred to agar slants.

2.4.3. Enzyme assays

Cells from 5 ml of culture collected by centrifugation (3800 × *g*, 25 min) were suspended in 500 μl of 0.1 M potassium phosphate buffer (pH 7.0) and to this was added a 5 mM of the diol **6** or **7** as a substrate. After incubation at 30°C with shaking, the reaction mixture was sampled at appropriate intervals and centrifuged (18 000 × *g*, 3 min) at 4°C. The supernatant obtained was analyzed with HPLC (Shimadzu) equipped with an ODS-80Ts column at a flow rate of 0.5 ml/min with 30% CH_3CN as a solvent by monitoring at 254 nm. *cis*-Diol **6**, *trans*-diol **7**, and the hydroxyketone **5** were detected at retention times of 4.5, 5.3, and 6.5 min, respectively. The optical purity and an absolute configuration of the hydroxyketone **5** was analyzed by HPLC using Chiralcel OB column at a flow rate of 0.5 ml/min with hexane/*i*PrOH = 9/1 as a solvent. Under these conditions, (*R*)- and (*S*)-5 were eluted at 18.7 and 27.7 min, respectively.

*2.4.4. Preparative scale-synthesis of (R)-**5***

Arthrobacter sp. strain 1HE was subcultured in a medium consisting 0.5% polypepton, 0.5% yeast extract, 0.1% glucose, and 0.1% K_2HPO_4, pH 7.0, for 24 h at 30°C. The subculture (500 ml) was inoculated into a 90 l jar-fermentor (OBS-90, Chiyoda Seisakusho, Nagano, Japan) containing 50 l of the optimized medium. Incubation was carried out at 30°C with aeration (50 l/min) and agitation (250 rpm) until its growth reached maximum levels (24 h). The cells from 25 l of culture harvested by continuous centrifugation (18 000 × *g*) were suspended in 1.5 l of 0.1 M potassium phosphate buffer (pH 7.0) and to this was added 75 mmol (11.3 g) of *cis*-diol **6** in 150 ml of DMSO, and the mixture was incubated with shaking at 30°C for 12 h. The reaction product was extracted with ethyl acetate (150 ml × 3) and the combined organic layer was dried over anhydrous Na_2SO_4.

Purification with column chromatography on silica gel (hexane/ethyl acetate = 3/2) afforded the hydroxyketone **5** as colorless plates (3.6 g, 32%). ^{1}H-NMR ($CDCl_3$) δ_{ppm} 7.76 (d, 1H, J = 7.8 Hz), 7.64 (t, 1H, J = 7.5 Hz), 7.46 (dd, 1H, J = 0.8, 7.5 Hz), 7.40 (t, 1H, J = 7.6 Hz), 4.57 (t, 1H, J = 5.8 Hz), 3.58 (dd, 1H, J = 8.0, 16.5 Hz), 3.45 (br, 1H), 3.03 (dd, 1H, J = 5.1, 16.5 Hz); ^{13}C-NMR ($CDCl_3$) δ_{ppm} 206.6, 151.0, 135.9, 134.0, 128.0, 126.8, 124.5, 74.3, 35.2; mp 84°C; $[\alpha]_D^{20} = -58$ (c = 1.0, MeOH); optical purity >99.9% e.e.

*2.4.5. Preparative scale-synthesis of (S)-***5**

Arthrobacter sp. strain 1HB was cultured in the optimized medium for 24 h at 30°C. The cells from 100 ml of culture harvested by centrifugation (7000 × g, 15 min) were suspended in 10 ml of 0.1 M potassium phosphate buffer (pH 7.0) and to this was added 0.5 mmol (75 mg) of *trans*-diol **7** in 1 ml of DMSO, and the mixture was incubated with shaking at 30°C for 12 h. The reaction product was extracted with ethyl acetate (5 ml × 3) and the combined organic layer was dried over anhydrous Na_2SO_4. Purification with column chromatography on silica gel (hexane/ethyl acetate = 3/2) afforded the hydroxyketone **5** as colorless plates (21.5 mg, 29%). ^{1}H-NMR ($CDCl_3$) δ_{ppm} 7.75 (d, 1H, J = 7.7 Hz), 7.64 (t, 1H, J = 7.6 Hz), 7.45 (dd, 1H, J = 0.8, 7.6 Hz), 7.42 (t, 1H, J = 7.7 Hz), 4.57 (t, 1H, J = 5.5 Hz), 3.58 (dd, 1H, J = 8.0, 16.9 Hz), 3.48 (br, 1H), 3.03 (dd, 1H, J = 5.3, 16.9 Hz); ^{13}C-NMR ($CDCl_3$) δ_{ppm} 206.2, 151.3, 136.2, 134.6, 127.8, 126.8, 124.6, 74.9, 34.9; mp 83°C; $(\alpha)_D^{20}$ = +57 (c = 1.0, MeOH); optical purity >99.9% e.e.

3. Results and discussion

3.1. Screening for microorganisms which stereoselectively oxidize cis- or trans-1,2-indandiol from microorganisms degrading various indan derivatives

We examined to isolate microorganisms for an ability to degrade 0.2% of various indan-derivatives, i.e. 1-indanol, 2-indanol, 1-indanone, 2-indanone, *cis*-diol **6**, *trans*-diol **7**, indan, indene, and 1,2-epoxyindene, in the medium containing $(NH_4)_2SO_4$ and yeast extract as nitrogen source and a nutrient to decrease a toxicity of indan derivatives, respectively. The soil samples collected in Toyama Prefecture were acclimated [8] with the indan-derivatives by transferring half of the medium every other day. During several months acclimation, we isolated 3, 10, 2, 5, 2, and 19 bacterial strains from the media containing 1-indanol, 2-indanol, 1-indanone, 2-indanone, indan, and indene, respectively, and screened the strains which stereoselectively oxidize the racemic diol **6** or **7** into the optically active hydroxyketone **5**. Among of them, 2-indanol-assimilating Gram-positive bacteria, strains 1HB and 1HE, isolated by 2 months acclimation, and Gram-negative bacterium, strain IN, which was isolated by 3 months acclimation in the medium containing indene but did not utilize it, were picked up and used for further studies. No strain in our stock cultures (407 strains of 45 genera of bacteria, 133 strains of 11 genera of actinomyces, 333 strains of 22 genera of yeasts, and 102 strains of 37 genera of fungi) could oxidize the diol **6** nor **7**.

3.2. Identification of the active strains

Table 1 shows the taxonomical characteristics of the Gram-positive strains 1HB and 1HE. Major fatty acids present in the strains were 13-methyltetradecanoic (3.9 and 22.8% of the total fatty acid of strains 1HB and 1HE, respectively), 12-methyltetradecanoic (50.0 and 56.9%), 14-methylpentadecanoic (32.1 and 5.1%), and 14-methylhexadecanoic (8.0 and 6.3%) acids. Both the strains contained lysine as a diamino acid in the cell wall. No acid and gas was produced from the sugars, such as glycerol, sucrose, maltose, lactose, and xylose. Taxonomic studies of the strain based on morphology, cultural appearance, and biochemical and physical characteristics identifies both strains as an *Arthrobacter* sp. according to "Bergey's Manual of Systematic Bacteriology" [17].

The taxonomical characteristics of the Gram-negative bacteria, strain IN, are as follows. The cells were rods occurring singly, non-motile, and non-sporeforming. The Hugh–Leifson reaction [18] with glucose was negative. Strictly aerobic. Fluorescent pigment was produced in King's A and B, and Pseudomonas F and P medium; catalase and oxidase: positive. Growth temperature ranged between 10 and 45°C. Acid without gas was produced only from arabinose but not from the other sugars. Nitrate and nitrite were reduced; denitrification, methylred

Table 1
Taxonomical characteristics of strains 1HB and 1HE

Characteristics	Strain 1HB	Strain 1HE
Gram stain	+	+
Shape	Coryneform rods	Coryneform rods
Acid fast	−	−
Spore formation	−	−
Motility	−	−
Aerobiosis	Aerobic	Aerobic
Growth temperature	15–45°C	10–45°C
Growth pH range	6–9	6–9
Growth in 10% NaCl	−	+
Hugh–Leifson reaction (glucose)	No reaction	No reaction
Litmus milk	No reaction	Aggregated by acid
Catalase	+	+
Oxidase	+	−
Citrate use (Christensen)	−	−
Starch hydrolysis	+	+
Gelatin liquefaction	−	+
NO_2 and NO_3 reduction	+	+
Methylred reaction	−	+
Voges–Proskauer reaction	−	−
Indole formation	−	−
H_2S formation	−	−
Urease	−	−
Lysine decarboxylase	−	−
Ornithine decarboxylase	−	−
Arginine dihydrolase	−	−
Phenylalanine deaminase	−	−
DNase	+	−
β-Glucosidase	−	+
Esculin hydrolysis	−	+
Presence of mycolic acid	−	−

and Voges–Proskauer: negative; indole and hydrogen sulfide formations: negative; ornithine dihydrolase: negative; lysine and arginine decarboxylases: positive; citrate utilization (Simmons and Christensen): positive; Urease, DNase, and acetamidase: positive; gelatin liquefaction: positive; esculin hydrolysis and β-galactosidase: negative. The strain assimilated glucose, mannitol, gluconate, caprate, adipate, and malate, but did not arabinose, mannose, maltose, and phenylacetate. Thus, strain IN is identified as *Pseudomonas aeruginosa* [19].

The strains 1HB, 1HE, and IN were deposited in the Toyama Prefectural University Culture Collection with the accession numbers TPU 5449, 5450, and 7174, respectively.

3.3. Culture conditions for the formation of 1,2-indandiol-oxidizing activity by the active strains

The effect of various substances added to a cultivation medium on the formation of the oxidizing activity in intact cells was investigated. These strains were grown at 30°C for 48 h and the activity was measured as described in Section 2.

For all the active strains, the oxidation activity was induced by various indan-derivatives, such as 1-indanol, 2-indanol, *cis*-diol **6**, and *trans*-diol **7**. No activity was seen in the cells of the strains grown without these compounds. Among of them, 1-indanol was selected as the best one for the enzyme formation. The optimum concentration of 1-indanol was estimated to be 0.05%. Higher concentrations (>0.25%) of 1-indanol inhibited their cell growth.

The strains were cultivated on the basal medium containing 0.2% $(NH_4)_2SO_4$, 0.05% of 1-indanol, and 0.5% of various sugars or sodium salt of organic acids. For *Arthrobacter* sp. stain 1HB, sugars, such as glucose, mannitol, sorbitol, raffinose, and rhamnose, and organic acids, such as gluconate, acetate, succinate, malonate, tartrate, lactate, and pyruvate, were effective in promoting cell growth and the activity, while, butyrate, and fumarate did not enhance the activity although the cells grew well in their presence. On the other hand, *Arthrobacter* sp. stain 1HE could grow on only fructose, galactose, and succinate. *P. aeruginosa* strain IN utilized the sugars, glycerol, fructose, and mannitol, and the organic acids, gluconate, malonate, lactate, and pyruvate, without decrease of the activity.

Supplementation with 0.2% of various organic and inorganic nitrogen sources to the basal medium containing 0.05% of 1-indanol was investigated. *Arthrobacter* sp. strain 1HB grew well on $(NH_4)_2SO_4$, $NaNO_3$, NH_4Cl, $(NH_4)_2HPO_4$, yeast extract, Polypepton, meat extract, malt extract, CSL, NZ CASE®, and beef extract without decrease of the activity. For *Arthrobacter* sp. stain 1HE, organic nitrogen sources, such as CSL, yeast extract, meat extract, and beef extract, were effective in promoting cell growth and the activity, but inorganic nitrogen ones, $NaNO_3$ and NH_4Cl, did not enhance the formation of the activity although the cells grew well in their presence. *P. aeruginosa* strain IN could grow on the organic nitrogen sources, yeast extract and NZ AMINE®, while inorganic nitrogen ones inhibited its cell growth.

Based on these results, we optimized the medium for the strains: (1) 1HB; (2) 1HE and (3) IN to be the basal medium (pH 7.2) containing 0.05% of 1-indanol with: (1) 0.5% of sodium gluconate and 0.2% of yeast extract; (2) 0.5% of fructose and 0.25% of CSL and (3) 0.5% of sodium pyruvate and 0.2% of yeast extract, respectively.

3.4. Reaction conditions for the hydroxyketone **5** *production*

The active strains were grown in the optimized medium and the conditions for the hydroxyketone **5** production from *cis*-diol **6** was examined. Fig. 3A shows the effect of temperature on the production of **5**. *Arthrobacter* sp. strain 1HB and 1HE showed their highest productivity at 25 and 35°C, respectively, while *P. aeruginosa* strain IN showed that at around 35–40°C. Fig. 3B shows the optimum pH on the reaction. *Arthrobacter* sp. strain 1HE and *P. aeruginosa* strain IN exhibited the highest productivities at around pH 8.5 and 9.0, respectively. While, *Arthrobacter* sp. strain 1HB had two activity maximum; at around pH 5.5 and 10.0. The result presented here do not exclude the possibility of the existence of two enzymes acting on the diol **6** in the strain 1HB: one has its pH optimum at acidic region and the other at an alkaline one. The details will be clarified by purifying and characterizing the enzymes.

The reaction product, chiral hydroxyketone **5**, was very easy to racemize even at weak alkaline conditions: the enantiomeric excess (% e.e.) of optically pure 5 was decreased to a half after 4 and 2 h incubations at pH 8.0 and 9.0, respectively. Therefore, the enzyme reaction was carried out at around neutral pH, which is little far below the optimum pHs of the enzymes.

The diol concentrations were varied (1–250 mM) and the productivity of **5** was examined. For all the strains, the oxidation activity was strongly decreased when the diol concentrations were greater than

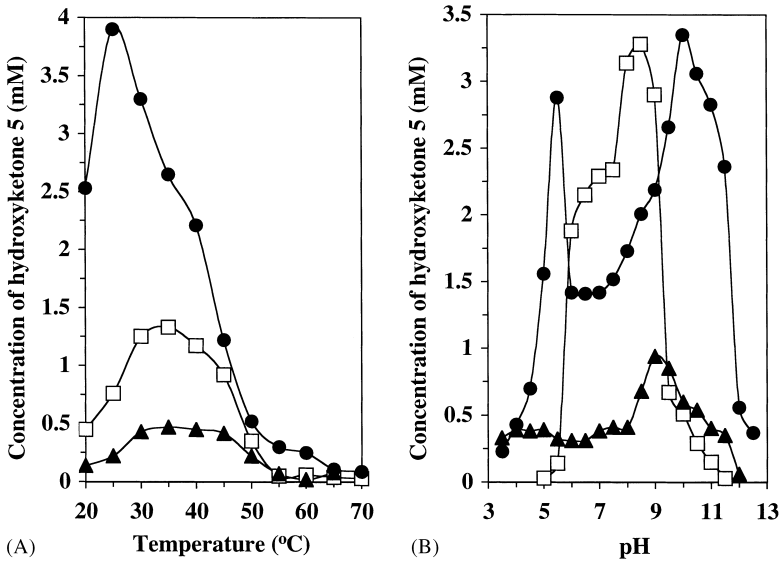

Fig. 3. Optimum temperature (A) and pH (B) on the formation of the hydroxyketone **5** from *cis*-diol **6** by *Arthrobacter* sp. strain 1HB (●), *Arthrobacter* sp. strain 1HE (□) and *P. aeruginosa* strain IN (▲). Collected cells of the strain grown with the optimized medium (5 ml) were suspended in 500 μl of (A) 0.1 M potassium phosphate buffer (pH 7.0) or (B) 0.1 M several buffers at various pHs. Ten millimolar of *cis*-diol **6** was added as a substrate and the reaction mixture was incubated for 12 h at (A) various temperatures or (B) 30°C with shaking. The concentration of the hydroxyketone **5** was analyzed with HPLC. The buffers used were: sodium acetate buffer (pH 3.5–6.0), potassium phosphate buffer (pH 6.5–8.5), MOPS–NaOH buffer (pH 7.0–7.5), HEPES–NaOH buffer (7.5–8.0), Tris–HCl buffer (pH 7.5–9.0), ethanolamine–HCl buffer (pH 8.5–11.0) and glycine–NaCl–NaOH buffer (pH 8.5–11.5).

100 mM: at 100 mM concentration of **6**, the activities of the strains 1HB, 1HE, and IN were decreased to 30, 20, and 25% to those at 50 mM concentrations, respectively. Therefore, the enzyme reaction was done at the diol concentrations of 50 mM.

Based on the observations, the optimum reaction condition for all the strains was established as follows; 50 mM of the substrate was incubated at 30°C in 100 mM potassium phosphate buffer (pH 7.0).

3.5. Stereoselectivity of the oxidation of the diols **6** *and* ***7***

To examine the stereoselectivity of the diol oxidation by the active strains, possible four stereoisomers of 1,2-indandiol were prepared from (*R*)- and (*S*)-2-acetoxy-1-indanone. We found that hydrogenation of the acetoxyketone in MeOH reduced the carbonyl group *cis* selectively and afforded *cis*-2-acetoxy-1-indanol as a sole product. On the other hand, the *trans*-isomer was obtained when the hydrogenation reaction was done in acidic MeOH (Fig. 4). Similar observations were reported that the stereoselectivity of hydrogenation of ketone was influenced by the pH of the reaction solvent [20,21]. After removal of the acetyl group of the *cis*- and *trans*-acetoxyalcohols, diastereo- and enantiomerically pure form the corresponding diols **6** and **7** were obtained. The microbial oxidation was carried out with 50 mM of the diols by using the resting cells of the strains. As shown in Fig. 5A, *Arthrobacter* sp. strain 1HB oxidized *cis*-(1*S*, 2*R*)-**6** at the highest rate but rarely oxidized its enantiomer, *cis*-(1*R*, 2*S*)-6. The strain also oxidized *trans*-(1*S*, 2*S*)-**7** at a rate of 30% to that for *cis*-(1*S*, 2*R*)-**6**; *trans*-(1*R*, 2*R*)-**7** was not transformed. *Arthrobacter* sp. strain 1HE was more stereospecific than the strain 1HB. It selectively acted on *cis*-(1*S*, 2*R*)-**6**; only *trans*-(1*S*, 2*S*)-**7** was oxidized at a rate of 10% compared with that for *cis*-(1*S*, 2*R*)-**6** (Fig. 5B). As shown in Fig. 5C, *P. aeruginosa* strain IN oxidized *trans*-(1*R*, 2*R*)-**7**, *cis*-(1*S*, 2*R*)-**6**, and *cis*-(1*R*, 2*S*)-**6** at relative rates of 100, 50, and 5%, respectively. All the strains only produced the hydroxyketone **5** as a sole product: no formation of its regio-isomer, 1-hydroxy-2-indanone, was seen at any reaction period.

Based on the results, the oxidation rate for the each stereoisomer of the diols are as follows: those of the strain 1HB are *cis*-(1*S*, 2*R*)-**6** > *trans*-(1*S*, 2*S*)-**7** ≫ *cis*-(1*R*, 2*S*)-**6**, *trans*-(1*R*, 2*R*)-**7**; those of the strain 1HE are *cis*-(1*S*, 2*R*)-**6** ≫ *trans*-(1*S*, 2*S*)-**7** ≫ *cis*-(1*R*, 2*S*)-**6**, *trans*-(1*R*, 2*R*)-**7**; and those of the strain IN are *trans*-(1*R*, 2*R*)-**7** > *cis*-(1*S*, 2*R*)-**6** > *cis*-(1*R*, 2*S*)-**6** ≫ *trans*-(1*S*, 2*S*)-**7**. It is reasonable to suggest that *Arthrobacter* sp. strain 1HE strictly recognizes (*S*)-stereochemistry of benzylic hydroxyl group and preferentially acts on *cis*-isomer of the diols. The strain 1HB also recognizes the same stereochemistry as well as the strain 1HE but it can also accept *trans*-isomer. It is interesting that the both strains selectively oxidizes benzylic hydroxyl group of the diols **6** and **7** although they had been isolated as 2-indanol-degrader. While, *P. aeruginosa* strain IN recognizes (*R*)-stereochemistry of homobenzylic

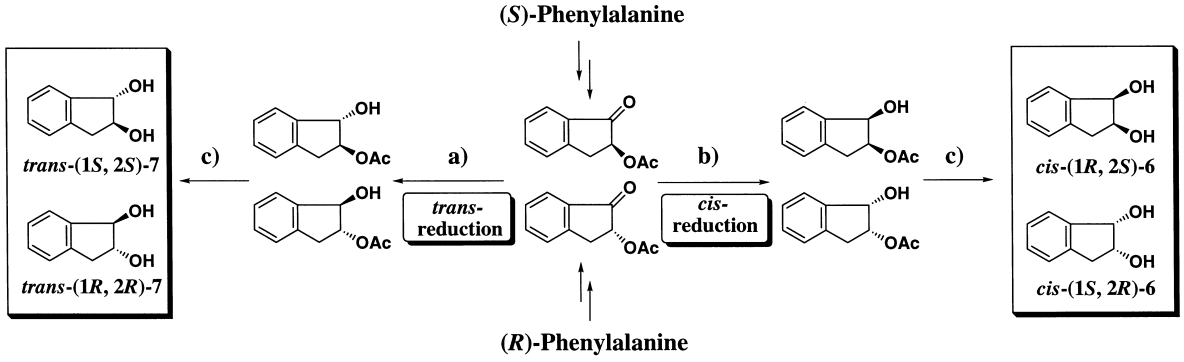

Fig. 4. Preparation of possible four stereoisomers of 1,2-indandiol: (a) H_2/Pd–C in MeOH; (b) H_2/Pd–C in HCl/MeOH; (c) K_2CO_3/MeOH.

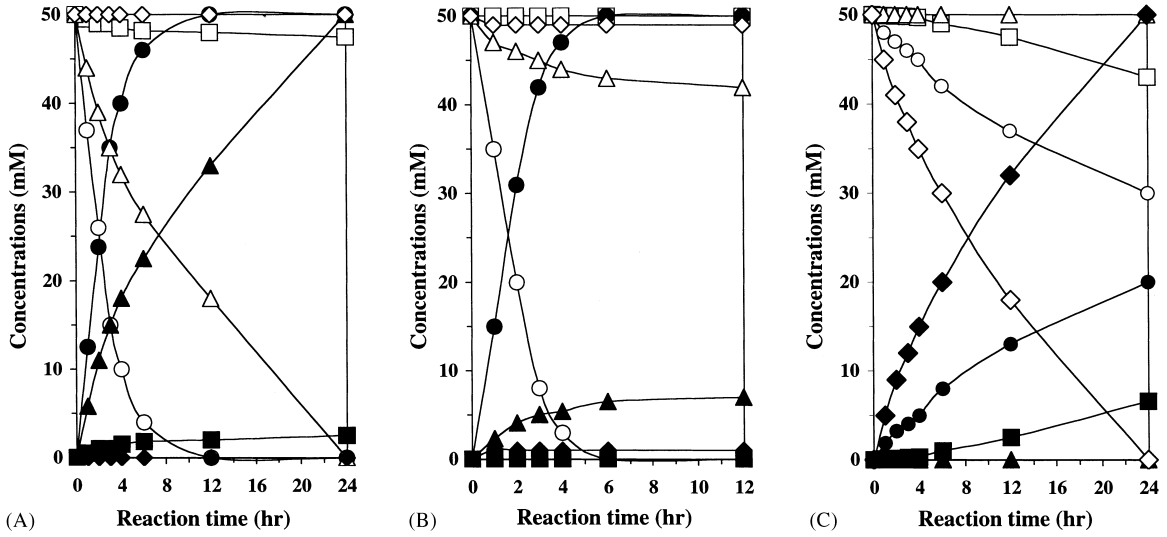

Fig. 5. Stereoselective oxidation of the possible four stereoisomers of 1,2-indandiol by the resting cells of: (A) *Arthrobacter* sp. strain 1HB; (B) *Arthrobacter* sp. strain 1HE and (C) *P. aeruginosa* strain IN. The reaction mixture (2 ml) contained 200 μmol of potassium phosphate buffer (pH 7.0), 0.1 g of the cells of the strain grown under the optimized condition, and 100 μmol of *cis*-(1*S*, 2*R*)-**6** (○, ●), *cis*-(1*R*, 2*S*)-6 (□, ■), *trans*-(1*R*, 2*R*)-**7** (△, ▲) and *trans*-(1*S*, 2*S*)-**7** (◇, ◆). Filled symbols, hydroxyketone concentrations; open symbols, diol concentrations.

hydroxyl group regardless to the stereochemistry of benzylic ones although it also selectively oxidized benzylic hydroxyl group.

3.6. Stereoselective synthesis of both enantiomers of the hydroxyketone **5**

Since the enzymes which responsible for the oxidation of the diols **6** and **7** were highly regio- and stereoselective, we examined to synthesize both enantiomers of the hydroxyketone **5** by changing the *cis* and *trans* forms of the substrate (Table 2). By using *Arthrobacter* sp. strains 1HB and 1HE, optically pure (*R*)- and (*S*)-**5** were synthesized from *cis*-diol **6** and *trans*-diol **7**, respectively. (*R*)-**5** was also prepared in an enantiomerically pure form from the *trans*-diol **7** by the cells of *P. aeruginosa* sp. strain IN. Elongation of the reaction time more than 24 h caused a decrease of the yield of the hydroxyketone **5** due to its further metabolism by the strains. This problem could be solved by using the partially purified enzymes.

Table 2
Stereoselective oxidation of racemic *cis*-diol **6** or *trans*-diol **7** into optically active 2-hydroxy-1-indanone **5**[a]

Strain	Diol	Reaction time (h)	Yield (%)	Enantiomeric excess (% e.e.)	Absolute configuration of **5**
Arthrobacter sp. 1HB	**6**	4	46	>99.9	*R*
	7	12	35	>99.9	*S*
Arthrobacter sp. 1HE	**6**	4	47	>99.9	*R*
	7	24	8	>99.9	*S*
P. aeruginosa IN	**6**	5	7	82.5	*R*
	7	24	40	>99.9	*R*

[a] The reaction mixture (2 ml) contained 200 μmol of potassium phosphate buffer (pH 7.0), 0.1 g of the cells of the strain grown under the optimized condition and 100 μmol of the diol. The reaction was carried out with shaking at 30°C.

3.7. Preparative scale-synthesis of both enantiomers of **5**

Preparative scale-synthesis of (*R*)- and (*S*)-**5** were examined by using the resting cells of *Arthrobacter* sp. strain 1HE and 1HB, respectively, grown under the optimized conditions. Both enantiomers of the hydroxyketone **5** were obtained as colorless plates in their optically pure form and their spectral characteristics were good agreement with those of authentic samples.

Of particular interest and novelty in this study is the demonstration of the one step synthesis of the both enantiomers of the optically pure hydroxyketone **5** by selecting *cis*- or *trans*-diol as the substrate for the microbial oxidation reaction. This process will make a new route to the chemo-enzymatic production of the aminoalcohol **1**.

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