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Screening and characterization of microorganisms catalyzing (*S*)-selective oxidation of α,α-disubstituted propanediols

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ABSTRACT

In this study, we attempted to achieve efficient synthesis of (*S*)-form 2-(hydroxymethyl)-2-methylbutanoic acid (HMMBA) from 2-ethyl-2-methyl-1,3-propanediol (EMPD) using an enzymatic approach. *Agromyces* sp. E2 was found to produce HMMBA from soil samples through enrichment culture using EMPD as a carbon source. The microbial asymmetric oxidation was characterized, and the highest activity was observed following addition of 0.2% (w/v) 2,2-diethyl-1,3-propanediol in the culture as an inducer. After 72 h of whole-cell incubation with 40 mM EMPD under optimized conditions, strain E2 completely converted EMPD to HMMBA with 39% enantiomeric excess (*S*). *Agromyces* sp. E2 exhibited high oxidative activity and moderate enantioselectivity for α,α-disubstituted 1,3-propanediols.

1. Introduction

Optically active hydroxyalkanoic acids are beneficial as pharmaceutical intermediates, chiral synthons, or functional polymers. Efficient syntheses of valuable compounds from hydroxyalkanoic acids have been previously reported (Ren et al., 2005; De Vitis et al., 2017; Singh and Mallick, 2017). Recently, 2-hydroxybutanoic acids are expected to be a novel biodegradable polymer or molecular glue. It was reported that blends of both (*S*)- and (*R*)- 2-hydroxybutanoic acid-based co-polymer compose of monomers (Tsuji and Sobue, 2015a,b; Tsuji et al., 2017; Tsuji et al., 2018). Among 2-hydroxybutanoic acids, we focused on 2-(hydroxymethyl)-2-methylbutanoic acid (HMMBA), which potentially serves as molecular glue. The efficient production of HMMBA should increase the likelihood of the development of novel polymers. To synthesize optically active HMMBA, we selected microbial asymmetric oxidation of prochiral 2-ethyl-2-methyl-1,3-propanediol (EMPD).

Although enzymatic synthesis of α-substituted hydroxyalkanoic acid has been studied through asymmetric oxidation (Ohta et al., 1982; Leon et al., 2001; Molinari et al., 2003), reports on the synthesis of optically active α,α-disubstituted hydroxyalkanoic acid are scarce. In our previous study, we reported that *Rhodococcus* sp. 2N oxidizes EMPD to (*R*)-HMMBA with 65% enantiomeric excess (ee) (*R*) through enrichment culture using 2,2-diethyl-1,3-propanediol (DEPD) as a carbon source (Kikukawa et al., 2018). Unfortunately, in the screening, no (*S*)-HMMBA-forming strain was obtained.

In this study, we isolated a large number of propanediol-oxidizing microorganisms through enrichment culture using EMPD, which is less sterically hindered than DEPD. We selected the best strain in terms of (*S*)-chemoselectivity for a hydroxymethyl group with a high conversion rate and characterized the oxidation activity using a whole-cell reaction.

2. Materials and methods

2.1. Materials

The diol compounds EMPD and DEPD were purchased from Tokyo Chemical Industry Co., Ltd. (Japan). The following products were used: polypeptone (Nippon Pharmaceutical Co., Ltd., Japan), meat extract (Kyokuto Pharmaceutical Industrial Co., Ltd., Japan), and yeast extract (Oriental Yeast Co., Ltd., Japan). All other chemicals were certified to be of reagent grade.

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2.2. Screening of α,α-disubstituted propanediol-oxidizing microorganisms

Various soil samples collected in Gifu, Japan were placed in 5 mL of enrichment culture medium comprising 0.2% (w/v) EMPD, 0.3% (w/v) NH_4Cl, 0.2% (w/v) K_2HPO_4, 0.1% (w/v) NaCl, 0.001% (w/v) $FeSO_4{\cdot}7H_2O$, 0.01% (w/v) $MgSO_4{\cdot}7H_2O$, and 1% (v/v) metal solution. The metal solution constituted 0.04% (w/v) $CaCl_2{\cdot}2H_2O$, 0.03% (w/v) H_3BO_3, 0.004% (w/v) $CuSO_4{\cdot}5H_2O$, 0.01% (w/v) KI, 0.02% (w/v) $FeSO_4{\cdot}7H_2O$, 0.04% (w/v) $MnSO_4{\cdot}7H_2O$, and 0.02% (w/v) $NaMoO_4{\cdot}2H_2O$ in 1% (v/v) conc. HCl. The samples were incubated at 28 °C for 7 days. Next, 0.10 mL of culture broth was added to fresh medium. This step was performed eight times until the microorganisms could be isolated on an agar plate with enrichment culture medium.

The DNA of the soil isolate strain E2 was extracted and purified, and the 16S rRNA gene was amplified and cloned into the pT7Blue vector. The primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1525r (5′-AAAGGAGGTGATCCAGCC-3′) were used for 16S rRNA gene sequencing analysis. The amplified gene fragment was analyzed using GenomeLab DTCS Quick Start Kit (Sciex, USA) and sequenced using a Beckman CEQ8000 DNA sequencer (Beckman Coulter, UK).

2.3. Culture conditions of agromyces sp. E2

Agromyces sp. E2 was precultured at 28 °C with reciprocal shaking at 120 strokes/min for 24 h in 4 mL of nutrient medium containing 0.5% (w/v) polypeptone, 0.5% (w/v) meat extract, 0.2% (w/v) NaCl, 0.05% (w/v) yeast extract, and 0.3% (w/v) EMPD in a test tube. Cultivation was performed in 40 mL of culture medium containing 1% (w/v) D-glucose, 1% (w/v) bacto peptone, 0.2% (w/v) casein peptone, 0.6% (w/v) NaCl, 0.2% (w/v) yeast extract, and 0.2% (w/v) EMPD in a 500 mL shake flask at 28 °C with reciprocal shaking at 120 strokes/min for 60 h. To induce oxidative activity following addition of diol in the culture medium, the following compounds were tested: 1,3-propanediol, 2-methyl-1,3-propanediol, 2-butyl-1,3-propanediol, EMPD, 2,2-dimethyl-1,3-propanediol, DEPD, glycerol, 2-amino-1,3-propanediol, and 2-amino-2-methyl-1,3-propanediol. The optimal induction of the oxidative activity using DEPD was examined in the range of 0%–0.4% (w/v).

2.4. Propanediol oxidation and analyses of reaction products

The standard reaction mixture comprised 100 mM potassium phosphate (KPi) buffer (pH 7.0), 10 mM EMPD, and whole cells of strain E2 (10.5 mg as dry cell weight) in a total volume of 4 mL in a 50 mL sample tube. The reaction was performed at 30 °C with reciprocal shaking at 120 strokes/min. The reaction products with carboxyl group (s) were detected using 0.05% (w/v) 2,6-dichlorophenolindophenol in ethanol using thin-layer chromatography (TLC). The HMMBA formation was analyzed using high-performance liquid chromatography (HPLC) with a YMC-Triart C18 column (4.6 × 150 mm) coupled to an SPD-10A$_{VP}$ UV–Vis detector (Shimadzu Corporation, Kyoto, Japan). The eluent, 50 mM NaH_2PO_4/H_3PO_4 (pH 2.8)/acetonitrile (4:1 v/v), was applied at a flow rate of 1 mL/min, and the eluate was monitored at 210 nm.

For optimization of the oxidative reaction, EMPD oxidation using strain E2 cells was performed under the following conditions: temperature range 25–40 °C and pH range pH 5.0–11.0, with various buffers at 100 mM for 24 h.

2.5. Identification of HMMBA and measurement of optical purity

After the whole-cell reaction, the reaction mixture was adjusted to pH 3.0 with 6 M HCl. HMMBA was extracted from the reaction mixture using diethyl ether, purified using silica gel column chromatography (30 × 110 mm; Wakogel C-300) from crude products, and the eluents were run in steps: *n*-hexane/ethyl acetate = 2:1 (200 mL) and 1:1 (200 mL). HMMBA was identified using ^1H NMR and ^{13}C NMR spectroscopy.

Table 1
HMMBA formation from EMPD using whole cells.

Strain	HMMBA (mM)	Optical purity	Dicarboxylic acid formation
A4-D	3.2	34% ee (*R*)	+
A9-D	3.4	15% ee (*R*)	+
A13-F	2.9	77% ee (*R*)	+
A13-J	3.2	49% ee (*R*)	+
B6-B	3.6	52% ee (*R*)	+
B16-C	3.6	36% ee (*R*)	+
C2-D	4.3	72% ee (*R*)	+
C5-D	7.2	14% ee (*R*)	+
E2	4.6	27% ee (*S*)	−
J-E	1.8	58% ee (*S*)	+

The reaction was carried out at 30 °C for 24 h in a reaction mixture of 4 mL containing 100 mM KPi buffer (pH 7.0), 10 mM EMPD, and whole cells harvested from 5 mL of culture broth.

Table 2
Effects of various diols on the induction of EMPD-oxidizing activity in *Agromyces* sp. E2 cells[a].

Compound	Growth (OD$_{610}$)	Relative activity (%)
None	3.2	0
1,3-propanediol	4.0	0
2-methyl-1,3-propanediol	3.9	18
2-butyl-1,3-propanediol	3.8	43
2,2-dimethyl-1,3-propanediol	4.0	39
2-ethyl-2-methyl-1,3-propanediol	4.0	100[b]
2,2-diethyl-1,3-propanediol	4.3	151
2-amino-1,3-propanediol	4.2	0
2-amino-2-methyl-1,3-propanediol	4.0	0
Glycerol	3.5	15

[a] *Agromyces* sp. E2 was cultivated in a nutrient medium containing the indicated propanediols at 0.2% (w/v). The oxidation reaction was carried out by the harvested whole cells for 24 h.

[b] 2.8 mM HMMBA was formed.

The optical purity of HMMBA was measured using chiral HPLC with a ChiralPak AD-H column (4.6 × 150 mm; Daicel Corp.). The eluent, *n*-hexane/ethanol (7:3 v/v), was applied at a flow rate of 1 mL/min, and the eluate was monitored at 254 nm. The retention times of (*S*)- and (*R*)-HMMBAs were 11.2 and 13.6 min, respectively.

3. Results

3.1. Screening of EMPD-oxidizing microorganisms

A total of 288 microorganisms with potential (*S*)-hydroxyalkanoic acid-synthesizing activity were isolated from enrichment culture. Their EMPD-oxidizing activities were investigated by performing TLC analysis of the culture broth. Seventy strains exhibited EMPD-oxidizing activity, and most of them produced both monocarboxylic and dicarboxylic acids. Among these 70 strains, 10 exhibiting high HMMBA production by TLC analysis were screened using whole-cell reaction (Table 1). Two strains, E2 and J-E, demonstrated (*S*)-selective oxidation, whereas the other strains exhibited (*R*)-selectivity. Because only strain E2 synthesized monocarboxylic acid without forming dicarboxylic acid, it was selected as an (*S*)-HMMBA-producing strain. The 16S rRNA gene sequence of strain E2 showed > 99.9% identity to that of *Agromyces* sp., and strain E2 was therefore identified as *Agromyces* sp. E2.

The effects of various diols on the induction of EMPD-oxidizing activities in *Agromyces* sp. E2 were investigated (Table 2). The oxidation activity for EMPD was induced by adding different propanediol derivatives into the nutrient medium. The addition of 0.2% (w/v) DEPD into the nutrient medium induced the highest oxidation activity in

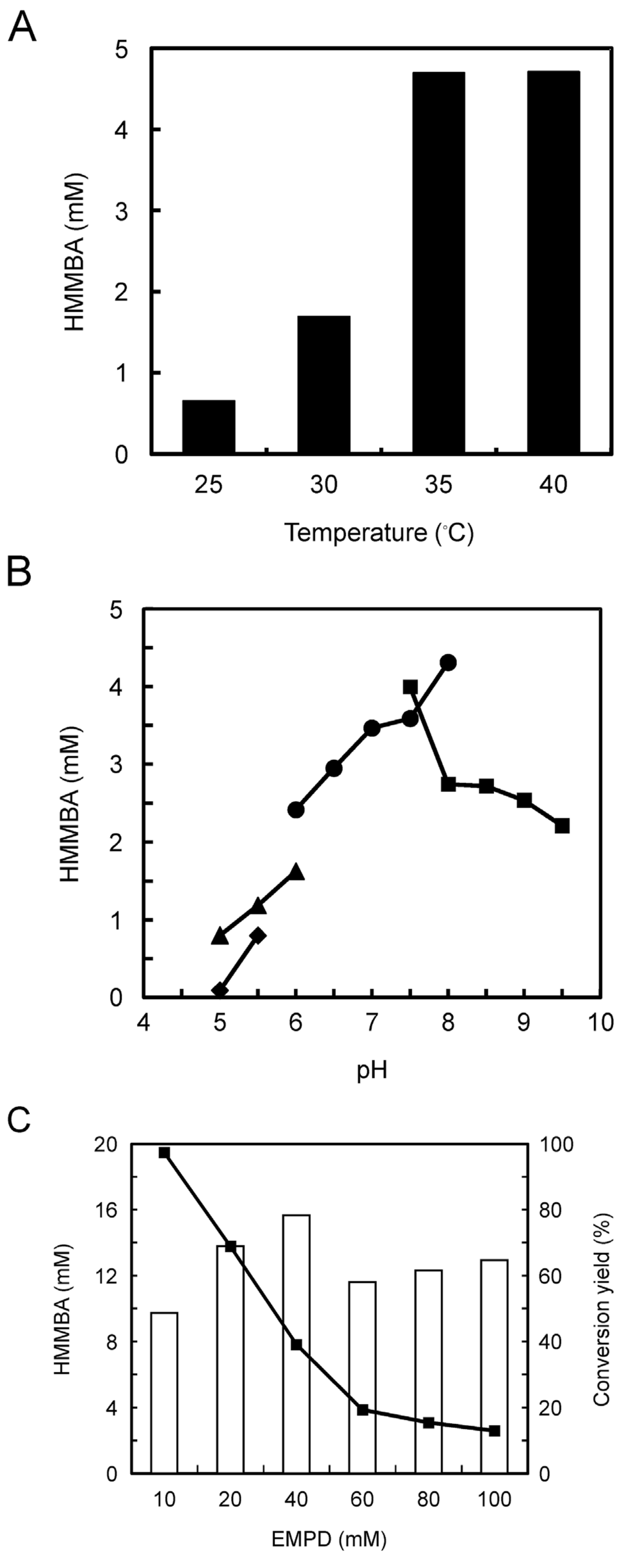

Fig. 1. Effects of temperature (A), pH (B), and EMPD concentration (C) on HMMBA production. A, HMMBA production (*solid bar*); B, acetate buffer (*diamond*), citrate buffer (*triangle*), KPi buffer (*circle*), and Tris-HCl buffer (*square*); C, HMMBA production (*open bar*) and conversion yield (*square*).

Table 3
Substrate specificity of the oxidizing enzyme(s) in *Agromyces* sp. E2.

Substrate	Relative activity (%)
2,2-dimethyl-1,3-propanediol	84
2-ethyl-2-methyl-1,3-propanediol	100[a]
2,2-diethyl-1,3-propanediol	80
2-methyl-2-propyl-1,3-propanedial	93

[a] 5.8 mM HMMBA was formed after 72 h of incubation.

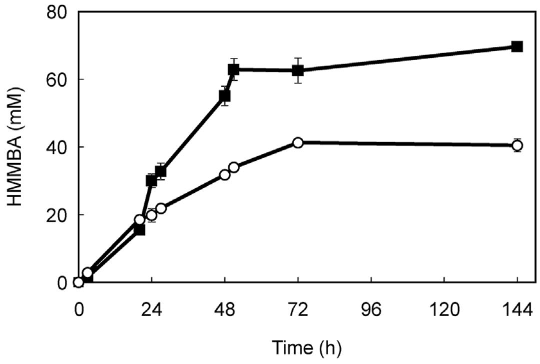

Fig. 2. Production of HMMBA from 40 mM (*open circle*) or 100 mM (*solid square*) EMPD by whole cells of *Agromyces* sp. E2. The reaction was performed in a 4 mL reaction mixture containing EMPD, *Agromyces* sp. 2N whole cells (105.4 mg as dry cell weight), and 100 mM KPi buffer (pH 8.0) at 35 °C with reciprocal shaking at 120 strokes/min. All values are means from triplicate experiments.

Agromyces sp. E2 cells, and the activity was 1.5-fold higher than that when 0.2% (w/v) EMPD was added.

3.2. Identification of reaction product

HMMBA produced by *Agromyces* sp. E2 cells was extracted and purified using column chromatography; it was obtained as a white solid (69.7 mg) from 2 mmol EMPD (236 mg). The product was identified using NMR spectroscopy. The properties of HMMBA were as follows: r.t. = 3.70 in HPLC analysis; Rf = 0.75 in TLC using ethyl acetate/methanol = 4:1. Chemical shifts of HMMBA were as follows: HMMBA: ^{1}H NMR (CDCl$_3$, 600 MHz) δ 0.93 (3H, *t*, $J = 7.6$ Hz), 1.20 (3H, *s*), 1.62 (1H, *dq*, $J = 7.6$, 14.8 Hz), 1.70 (1H, *dq*, $J = 7.6$, 15.1 Hz), 3.54 (1H, *d*, $J = 11.7$ Hz), 3.75 (1H, *d*, $J = 11.6$ Hz): ^{13}C NMR (CDCl$_3$, 150 MHz) δ 8.53, 18.90, 28.35, 47.76, 67.65, and 181.17.

In the reaction, an aldehyde intermediate 2-(hydroxymethyl)-2-methylbutanal was detected by HPLC, as in the case of the reaction using whole cells of *Rhodococcus* sp. 2N (Kikukawa et al., 2018).

3.3. Optimization of reaction conditions

The reaction conditions for HMMBA formation were optimized using whole cells of *Agromyces* sp. E2. Fig. 1 shows the effect of temperature, pH, and EMPD concentration on oxidative activity, respectively. The highest activity was observed when the reaction was performed at 35 °C in 100 mM KPi buffer (pH 8.0) with reciprocal shaking at 120 strokes/min. The EMPD-oxidizing activity of the whole cells remained at similar levels in the EMPD concentration range of 10–100 mM. The cell-free extract exhibited EMPD-oxidizing activity only upon the addition of the coenzyme NAD$^+$ but not NADP$^+$ at 1 mM.

3.4. Substrate specificity

The substrate specificity of the oxidizing enzyme(s) in *Agromyces* sp.

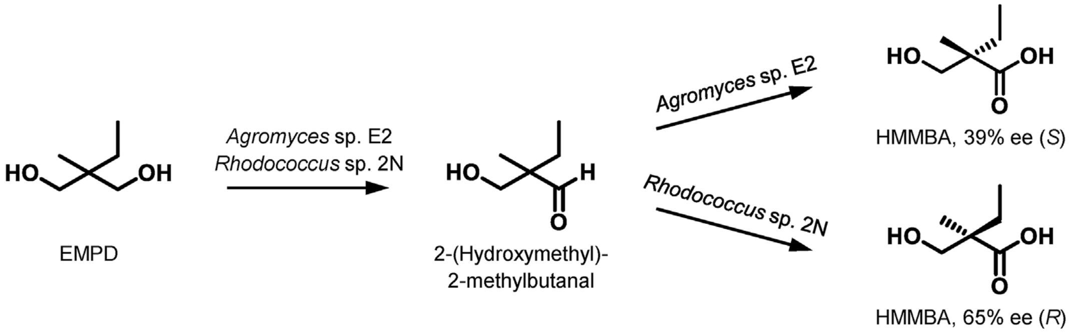

Fig. 3. Enzymatic synthesis of optically active HMMBAs by whole cells of *Rhodococcus* sp. 2N and *Agromyces* sp. E2.

Table 4
Comparison of asymmetric oxidation by strains E2 and 2N[a].

	Agromyces sp. E2	*Rhodococcus* sp. 2N[b]
Optimum inducer	0.2% (w/v) DEPD	0.3% (w/v) DEPD
Optimum pH	8.0	8.0
Optimum temperature	35 °C	30 °C
Substrate specificity[b] 	R^1 = Me, R^2 = Et [39% ee (*S*)] R^1 = Me, R^2 = Me R^1 = Et, R^2 = Et R^1 = Me, R^2 = Pr	R^1 = Me, R^2 = Et [65% ee (*R*)] R^1 = Me, R^2 = Me R^1 = Et, R^2 = Et R^1 = Me, R^2 = Pr R^1 = Me, R^2 = H

[a] Abbreviations: DEPD, 2,2-diethyl-1,3-propanediol; Me, methyl; ee, enantiomeric excess; Et, ethyl; Pr, propyl.

[b] Kikukawa et al., 2018.

E2 was investigated using whole cells and various 1,3-propanediols at a concentration of 10 mM for 72 h (Table 3). Cells of strain E2 accepted 2,2-dimethyl-1,3-propanediol, EMPD, DEPD, and 2-methyl-2-propyl-1,3-propanediol as substrates in the oxidative reaction. These substrates were converted to their corresponding hydroxycarboxylic acids. The following compounds were inert as substrates: 1,3-propanediol, 2-methyl-1,3-propanediol, 2-methylene-1,3-propanediol, 2-butyl-1,3-propanediol, 2-butyl-2-ethyl-1,3-propanediol, 2-hydroxymethyl-2-methyl-1,3-pronediol, 2-phenyl-1,3-propanediol, 2-amino-1,3-propanediol, 2-amino-2-methyl-1,3-propanediol, 2-amino-2-hydroxymethyl-1,3-propanediol, and glycerol.

3.5. HMMBA production by agromyces sp. E2 cells

The time course of HMMBA production was examined in reaction mixtures of 10 mL comprising 40 or 100 mM EMPD and whole cells of strain E2 (Fig. 2). After 72 h of incubation, 40 mM EMPD was completely converted to HMMBA with 39% ee (*S*). When 100 mM EMPD was used, the reaction achieved a conversion yield of 63% after 51 h of incubation; the optical purity of HMMBA was 38% ee (*S*).

4. Discussion

The enzymatic chemo- and stereo-selective oxidation of α,α-disubstituted 1,3-propanediols to optically active hydroxyalkanoic acids is an effective method for synthesizing pharmaceuticals, raw materials for functional polymers, or promising molecular glue of polymers for novel functional materials. However, an enzymatic approach for the asymmetric synthesis of α,α-disubstituted hydroxyalkanoic acid has barely been reported. In this study, we performed enrichment culture using EMPD to obtain α,α-disubstituted propanediol-oxidizing microorganisms and isolated *Agromyces* sp. E2, which are capable of catalyzing (*S*)-selective oxidation of EMPD. The NAD^+ dependence of the oxidizing activity in the cell-free extract of *Agromyces* sp. E2 suggests that the enzyme(s) involved in the asymmetric oxidation of EMPD to the aldehyde intermediate might be a type of dehydrogenase. Characterization of the oxidation reaction using purified enzyme(s) should be conducted.

In our previous study, we isolated *Rhodococcus* sp. 2N, which exhibited (*R*)-HMMBA-producing activity through enrichment culture using DEPD as a sole carbon source (Kikukawa et al., 2018). Thus, the enzymatic synthesis of (*S*)- and (*R*)-form HMMBAs was established (Fig. 3). Table 4 shows the characteristics of asymmetric oxidation by *Agromyces* sp. E2 cells and *Rhodococcus* sp. 2N cells. Strains E2 and 2N were isolated through enrichment culture using EMPD and DEPD as the carbon source, respectively. However, the oxidative enzyme(s) in both strains were induced most by DEPD. Furthermore, pH and temperature were not significantly different between the strains. In the present study, a large number of microorganisms exhibiting (*R*)-selective oxidative activity were obtained because of EMPD being less sterically hindered than DEPD. Only *Agromyces* sp. E2 exhibited both (*S*)-selectivity and chemoselectivity. Both strain E2 and strain 2N exhibited high substrate specificity and did not accept α,α-disubstituted 1,3-propanediols with an amino group, hydroxyl group, and aromatic group as substrates.

Under the optimized reaction conditions using whole cells, 100 mM EMPD was converted with a 63% molar conversion yield after 51 h of incubation. The oxidizing enzyme(s) were not inactivated after 51 h of incubation because the reaction proceeded in the reaction containing 40 mM EMPD after 51 h of incubation (Fig. 2). This suggests that HMMBA inhibited the enzyme activity. No substrate inhibition was observed in the presence of 100 mM EMPD as shown in Fig. 2.

For further analysis of the enzymatic asymmetric oxidation of α,α-disubstituted 1,3-propanediols and to construct systems for producing optically active hydroxyalkanoic acids in a more efficient manner, the identification of genes encoding alcohol dehydrogenases and aldehyde dehydrogenases oxidizing 1,3-propanediols is warranted.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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