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Biosynthesis of 3-hydroxypropionic acid from glycerol in recombinant *Escherichia coli* expressing *Lactobacillus brevis dhaB* and *dhaR* gene clusters and *E. coli* K-12 *aldH*

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HIGHLIGHTS

- Two *Lactobacillus brevis dhaB* and *dhaR* gene clusters of were cloned.
- *In vitro*/*in vivo* assays confirmed that DhaR is a DhaB reactivation factor.
- Recombinant *E. coli* expressing *dhaB*, *dhaR* and *aldH* produced 14.3 g/L 3-HP from glycerol in the two-step fed-batch fermentation.

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ABSTRACT

3-Hydroxypropionic acid (3-HP) is a value-added chemical for polymer synthesis. For biosynthesis of 3-HP from glycerol, two *dhaB* and *dhaR* clusters encoding glycerol dehydratase and its reactivating factor, respectively, were cloned from *Lactobacillus brevis* KCTC33069 and expressed in *Escherichia coli*. Coexpression of *dhaB* and *dhaR* allowed the recombinant *E. coli* to convert glycerol to 3-hydroxypropionaldehyde, an intermediate of 3-HP biosynthesis. To produce 3-HP from glycerol, fed-batch fermentation with a two-step feeding strategy was designed to separate the cell growth from the 3-HP production stages. Finally, *E. coli* JHS00947 expressing *L .brevis dhaB* and *dhaR*, and *E. coli aldH* produced 14.3 g/L 3-HP with 0.26 g/L-h productivity, which were 14.6 and 8.53 times higher than those of the batch culture. In conclusion, overexpression of *L. brevis dhaB* and *dhaR* clusters and *E. coli aldH*, and implementation of the two-step feeding strategy enabled recombinant *E. coli* to convert glycerol to 3-HP efficiently.

1. Introduction

3-Hydroxypropionic acid (3-HP, CAS No. 503-66-2) is a non-chiral three-carbon carboxylic acid containing a β-hydroxyl group. 3-HP can be converted to several valuable chemicals such as acrylic acid, acrylonitrile, malonic acid, and 1,3-propanediol (1,3-PDO) (Raj et al., 2008). 3-HP can also serve as a starting compound for the ring opening polymerization of a degradable polyester, poly(3-hydroxypropionic acid). Poly(3-HP) with a high molecular weight has promising mechanical and thermal properties such as attractive rigidity, ductility and exceptional tensile strength in drawn films (Zhang et al., 2004), and high glass transition temperature (Jiang et al., 2009). 3-HP was selected as one of the top value-added chemicals from biomass by the US Department of Energy (Bozell and Petersen, 2010), and its global market size was estimated at 2420 million kg per year (Paster et al., 2004).

For biological production of 3-HP, glucose and glycerol have been used as feedstocks and various microorganisms were metabolically engineered. Yeast strains were genetically engineered to possess several 3-HP production pathways via β-alanine, glycerate, lactate, malonyl-CoA or glycerol from glucose (Barnhart et al., 2012). It was also reported that recombinant *E. coli* expressing acetyl-CoA carboxylase, biotinidase and malonyl-CoA reductase could produce 1.6 mM 3-HP from glucose (Rathnasingh et al., 2012). Glycerol is a by-product of biodiesel production using plant oils and animal fats and is an attractive carbon feedstock for biochemical production (Yazdani and Gonzalez, 2007).

Glycerol dehydratase and aldehyde dehydrogenase are involved in the metabolic pathway for 3-HP production from glycerol. Glycerol dehydratase is a coenzyme B_{12}-dependent diol dehydratase (EC 4.2.1.30) catalyzing dehydration of glycerol to 3-hydroxypropionaldehyde (3-HPA) and is present as a dimer of heterotrimer ($\alpha_2\beta_2\gamma_2$) with adenosylcobalamin which is bound between α and β subunits

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(Yamanishi et al., 2002). Glycerol dehydratase undergoes irreversible inactivation when its substrate is glycerol (Toraya et al., 1976). The inactivation process is induced by the conversion of enzyme-bound adenosylcobalamin to an alkyl or thiol cobalamin (Bachovchin et al., 1977); however, the inactivated glycerol dehydratase can be reactivated by expression of reactivating proteins (Honda et al., 1980). Genes encoding reactivating enzymes have been identified in *Klebsiella pneumoniae* (Mori et al., 1997), *Klebsiella oxytoca* (Tobimatsu et al., 1999), *Citrobacter freundii* (Seifert et al., 2001), and *Lactobacillus reuteri* (Talarico and Dobrogosz, 1990). Aldehyde dehydrogenase converts 3-HPA to 3-HP and is specific for NAD^+ cofactor. To date, aldehyde dehydrogenases with 3-HPA oxidation activity have been identified in *Saccharomyces cerevisiae* (ADH4) (Suthers and Cameron, 2005), *E. coli* K-12 (AldH) (Jo et al., 2008), *L. reuteri* (PduP) (Luo et al., 2011), *K. pneumoniae* (PuuC) and *Azospirillum brasilense* (KGSADH) (Ko et al., 2012).

Recombinant *E. coli* expressing *dhaB* of *K. pneumoniae* and *aldH* of *E. coli* K-12 was constructed to produce 31 g/L 3-HP with 0.35 mol yield (Mohan Raj et al., 2009). Expression of glycerol dehydratase reactivase (GDR) and α-ketoglutaric semialdehyde dehydrogenase (KGSADH), and an aerobic fed-batch fermentation resulted in a 3-HP concentration of 38.7 g/L with a yield of 35% (Rathnasingh et al., 2009). *K. pneumoniae* has endogenous metabolic pathways from glycerol to 1,3-propanediol (1,3-PDO). By expression of an aldehyde dehydrogenase gene, recombinant *K. pneumoniae* produced 3-HP only or 3-HP and 1,3-PDO simultaneously (Ko et al., 2012).

Glycerol dehydratase and its reactivating factor from Gram negative bacteria have been well characterized. Among the corresponding enzymes from Gram positive bacteria, only those from *L. reuteri* have been studied (Mohan Raj et al., 2009; Mori et al., 1997; Talarico and Dobrogosz, 1990). In the present study, *dhaB* and *dhaR* gene clusters coding for glycerol dehydratase (DhaB) and its reactivating factor (DhaR), respectively, were cloned from a Gram positive bacterium of *Lactobacillus brevis* KCTC 33069, characterized and expressed in *E. coli* BL21 star (DE3). Batch and fed-batch fermentations of a recombinant *E. coli* strain expressing *L. brevis dhaB* and *dhaR*, and *E. coli* K-12 *aldH* were carried out to produce 3-HP using glycerol and glucose as co-substrate.

2. Methods

2.1. Strains and plasmids

E. coli TOP10 and BL21 star (DE3) (Invitrogen, Carlsbad, CA, USA) were used as host strains for DNA manipulation and 3-HP production, respectively. The pET-29b(+) vector (Novagen, Germany) was used for the expression of the glycerol dehydratase gene (*dhaB*) and reactivating factor gene (*dhaR*) cluster from *L. brevis* KCTC 33069. Plasmid pCDF/*aldH* containing the aldehyde dehydrogenase gene (*aldH*) from *E. coli* K-12 was provided by Professor Sunghoon Park at Pusan National University (Raj et al., 2008). The *T7* promoter was used for expression of the *dhaB* and *dhaR* gene cluster, and the *aldH* gene. Strains and plasmids used in this study are listed in Table 1.

2.2. Genetic manipulation

Genomic DNA of *L. brevis* KCTC 33069 was used as a template for polymerase chain reaction (PCR) to amplify two *dhaB* and *dhaR* gene clusters. Two DNA fragments containing *dhaB1*, *dhaB2*, and *dhaB3* were PCR-amplified using PCR primer sets, F_Lb_dhaB1 and R_Lb_dhaB1 for *dhaB1*, and F_Lb_dhaB23 and R_Lb_dhaB23 for *dhaB2* and *dhaB3*. A full-length *dhaB* cluster was constructed by an overlap-extension PCR using the two PCR products with *dhaB1*, *dhaB2* and *dhaB3*, and primers F_Lb_dhaB1 and R_Lb_dhaB23. The full length of the *dhaR* cluster was amplified by PCR using the F_Lb_dhaR12 and R_Lb_dhaR12 primers. All PCRs were performed with the nPfu-forte DNA Polymerase kit (Enzynomics, Korea) and GeneAmp PCR System 2400 (Applied Biosystems, CA, USA). The PCR primer sequences designed in this study are listed in Table 1.

The amplified DNA fragments of *dhaB* (2961 bp) and plasmid pET-29b(+) were digested with *Xba*I and *Bam*HI, and combined, yielding plasmid pET29b/*dhaB123* (called pJHS00638). The *dhaR* gene (2205 bp) and plasmid pJHS00638 were treated with *Bam*HI and *Not*I, and ligated. Finally, an expression vector harboring the *dhaB* and *dhaR* gene clusters, pET29b/*dhaB123-dhaR12* (pJHS00640) was constructed. The expression of these genes was controlled by the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *T7* promoter. The maps of plasmid pJHS00638 and pJHS00640 were illustrated in Supplemental Fig. 1. Plasmids pJHS00638 and pJHS00640 were transformed into *E. coli* BL21 star (DE3), resulting in the *E. coli* JHS00943 and JHS00945 strains respectively. The *E. coli* JHS00947 strain was constructed by transforming pCDF/*aldH* into the JHS00945 strain. The $CaCl_2$-based bacterial transformation method was used as previously described (Kim et al., 2011). An appropriate amount of kanamycin or streptomycin was added to select transformants.

2.3. Culture conditions

To confirm the production of 3-HPA *in vivo*, a batch fermentation was performed in a 500-mL baffled flask (Nalgene, Rochester, NY, USA) with 100 mL of LB medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl, and suitable antibiotics) at 37 °C and 200 rpm. At the logarithmic growth phase (~0.6 OD), IPTG, glycerol and coenzyme B_{12} were added to final concentrations of 0.5 mM, 4.5 g/L and 20 μM, respectively. A batch culture for 3-HP production was carried out in a baffled flask containing 100 mL of defined R medium (Kim et al., 2011) and 50 μg/mL kanamycin at 25 °C and 200 rpm. At the logarithmic growth phase, IPTG, coenzyme B_{12} and glycerol were added to final concentrations of 0.2 mM, 20 μM and 11 g/L, respectively.

A fed-batch culture was performed in a 2.5-L jar fermentor (Kobiotech, Korea) with a 1-L working volume of R medium at 25 °C and pH 6.8. To maintain a dissolved oxygen level above 10%, agitation speed and aeration rate were set at 1200 rpm and 1 vvm of air supply, respectively. After the depletion of 20 g/L glucose, the feeding solution A (800 g/L glucose) was fed by the pH-STAT mode of operation (Kim et al., 2011). When the cell mass reached an OD of 100, IPTG and coenzyme B_{12} were added to final concentrations of 0.2 mM and 20 μM, respectively. The jar was covered with aluminum foil and solution A was replaced with solution B consisting of 400 g/L glycerol and 400 g/L glucose.

2.4. Glycerol dehydratase assay

E. coli strains JHS00943 and JHS00945 were cultured in 100 mL LB medium at 37 °C and 200 rpm. At the logarithmic growth phase, IPTG was added and the culture temperature was changed to 20 or 30 °C. After 12 h, the cells were harvested at 8000*g* and 10 min, washed twice with 100 mM potassium phosphate buffer (pH 7.0) and suspended in the same phosphate buffer. The dry cell concentration in the suspension was adjusted to be 7.3 g/L for SDS–PAGE and 11 g/L for the glycerol dehydratase activity assay. After cell disruption by an ultrasonic processor (Cole-Parmer, IL, USA), the samples were denoted as total protein fraction and subjected to centrifugation for 15 min at 8000*g* and 4 °C to collect soluble (supernatant) and insoluble (pellet) fractions. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with 12%

Table 1
Strains, plasmids, and primers used in this study.

Name	Description	Sources
Strain		
L. brevis KCTC 33069	Source for *dhaB* and *dhaR* gene cluster	This study
E. coli TOP10	*F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(araleu)7697 galU galK rpsL (StrR) endA1 nupG*	Invitrogen
E. coli BL21 star (DE3)	*F- ompT hsdSB (rB-mB-) gal dcm rne131 (DE3)*	Invitrogen
Plasmid		
pET-29b(+)	pBR322 origin, Kan^r, *lacI*, *T7* promoter	Novagen
pJHS00638	pET-29b(+)-based plasmid containing *L. brevis dhaB* cluster under the T_7 promoter	This study
pJHS00640	pET-29b(+)-based plasmid containing *L. brevis dhaB*, *dhaR* cluster under the T_7 promoter	This study
pCDF/*aldH*	CDF origin, Sm^r, *lacI*, *E. coli* K-12 *aldH* under the *T7* promoter	Raj et al. (2008)
Primer		
F_Lb_dhaB1	5′-GCTCTAGATAAAGGGGGATTTTTAA**a**TG	This study
R_Lb_dhaB1	5′-TTCTTGAGCCA**t**AGTGTTAAC	This study
F_Lb_dhaB23	5′-GTTAACACT**a**TGGCTCAAGAA	This study
R_Lb_dhaB23	5′-CGGGATCCCTAGTTATCACCCTTCAG	This study
F_Lb_dhaR12	5′-CGGGATCCTTAGGAGTCTTCGTATGCAA	This study
R_Lb_dhaR12	5′-ATAAGAATGCGGCCGCCCACCTAATCTAATGTCTTAA	This study

*The underlined sequences indicate the recognition sites of the corresponding restriction enzymes. The lower case letters in the nucleotide sequences correspond to the mutagenesis sites.

polyacrylamide and Coomassie brilliant blue staining were used to analyze the total, soluble, and insoluble fractions.

For the glycerol dehydratase activity assay, the soluble fraction was used as crude enzyme extract. The reaction solution was composed of 10 mM glycerol and various concentrations of coenzyme B_{12} in 100 mM potassium phosphate buffer (pH 6.4–7.6). After addition of the extract, the reaction solution was incubated at 37 °C, and the enzyme reaction was terminated by addition of the same volume of 100 mM citrate solution. The concentration of 3-HPA in the reaction solution was determined indirectly by the tryptophan–HCl method (Raj et al., 2008), in which 3-HPA is dehydrated into acrolein by heating under acidic condition and acrolein is analyzed colorimetically. Briefly, 150 μL of properly diluted reaction sample or acrolein standard was mixed with 750 μL of 37% HCl and 150 μL of tryptophan reagent (2.05 g/L DL-tryptophan in 0.417% (v/v) HCl, and 0.25% (v/v) toluene). After the solution was incubated at 40 °C for 20 min, absorbance was measured at 560 nm. Protein concentration was analyzed using a protein assay kit (Bio-Rad, Hercules, CA, USA). One unit (U) of glycerol dehydratase activity is defined as the amount of an enzyme able to produce 1 μmol 3-HPA per min under the reaction condition. The enzyme activity assay was performed in triplicate.

2.5. Determination of cell and metabolite concentrations

Optical density (OD) of the cells was measured at 600 nm using a spectrophotometer (UV-1601, Shimadzu, Japan). Dry cell mass was obtained by multiplication of OD with a pre-determined conversion factor, 0.365 g/L/OD.

The 3-HPA concentration in the culture broth was measured by the modified tryptophan–HCl method (Raj et al., 2008). The amounts of glucose, glycerol, 3-HP, 1,3-PDO and acetate were determined by a high performance liquid chromatography (1200 series, Agilent, Santa Clara, CA, USA) equipped with an Aminex HPX-87H ion exchange column (Bio-Rad, CA, USA) heated at 60 °C. Detection was made with a reflective index detector (RID) and an UV detector (UVD) at 210 nm. A mobile phase of 5 mM H_2SO_4 was used at a flow rate of 0.5 mL/min. For the 3-HPA assay,

Table 2
Amino acid sequence identities and similarities between *L. brevis* KCTC 33069 glycerol dehydratase and its reactivation factor and corresponding protein sequences from other bacteria.

Strain			Glycerol dehydratase			Reactivation factor	
			Large	Medium	Small	Large	Small
L. brevis ATCC 367	Protein ID		ABJ64692.1	ABJ64691.1	ABJ64690.1	ABJ64689.1	ABJ64688.1
	Identity		100	100	100	99.5	96.6
	Similarity	%	100	100	100	99.7	97.4
L. reuteri	Protein ID		BAG26151.1	BAG26150.1	BAG26149.1	BAG26148.1	BAG26147.1
	Identity		81.7	65.3	57.1	77.7	46.9
	Similarity	%	90.5	78.7	77.1	88.0	60.9
L. collinoides	Protein ID		CAC82541.1	CAC82542.1	CAD01091.1	CAD01092.2	CAD01093.2
	Identity		75.8	71.0	57.3	80.7	53.4
	Similarity	%	87.3	81.3	76.4	90.0	67.8
K. pneumonia	Protein ID		ABR78884.1	ABR78883.1	ABR78882.1	ABO37963.1	ABO37964.1
	Identity		63.1	39.8	38.6	56.0	28.9
	Similarity	%	78.5	50.0	55.7	72.3	38.0
S. typhimurium	Protein ID		CBW18112.1	CBW18113.1	CBW18114.1	CBW18115.1	CBW18116.1
	Identity		69.5	55.6	48.0	66.5	43.3
	Similarity	%	81.7	71.1	69.5	81.1	54.2

*This analysis was conducted using EMBOSS Needle tool of European Bioinformatics Institute (http://www.ebi.ac.uk). Protein ID represents the identification number of each protein in the GenBank database.

a gas chromatography–mass spectrometer (GC–MS) composed of a GC 6890N and an MSD 5975 instruments (Agilent, CA, USA) and equipped with a HP-VOC column (Agilent, CA, USA) was employed. Helium was used as a carrier gas. The 3-HPA standard solution was synthesized by Professor Sunghoon Park at Pusan National University, Korea.

3. Results and discussion

3.1. Genetic analysis of L. brevis dhaB and dhaR gene clusters

The *dhaB1*, *dhaB2* and *dhaB3* genes in the *dhaB* cluster from *L. brevis* KCTC 33069 consist of 1677, 720 and 528 bp and their deduced peptides of 558, 239 and 175 amino acids, respectively. When comparing the nucleotide sequences with the corresponding sequences from *L. brevis* ATCC 367 (Genbank No. CP000416), the only difference was that two cytosines were replaced with thiamine at 537 and 559 in *dhaB1*. The amino acid sequences of *L. brevis* KCTC 33069 DhaB1 (large subunit) showed high identities (>75%) and similarities (>87%) to the glycerol dehydratases of *L. reuteri* and *Lactobacillus collinoides* (Table 2) and 63% identity and 78% similarity with those of *K. pneumoniae* and *S. typhimurium*. Even though the large subunit of DhaB protein had high identity and similarity among all microorganisms, their medium and small subunits exhibited moderate or low values of identify and similarity. It is estimated that the large subunit of DhaB plays a crucial role in the action of glycerol dehydration; glycerol can be converted to 3-HPA with the essential cofactor K^+ in the active site of the glycerol dehydratase large subunit (Yamanishi et al., 2002).

The *dhaR* genes coding for the glycerol dehydratase reactivating factor of *L. brevis* KCTC 33069 were found by sequence alignment with the amino acid sequences of the putative reactivating factor

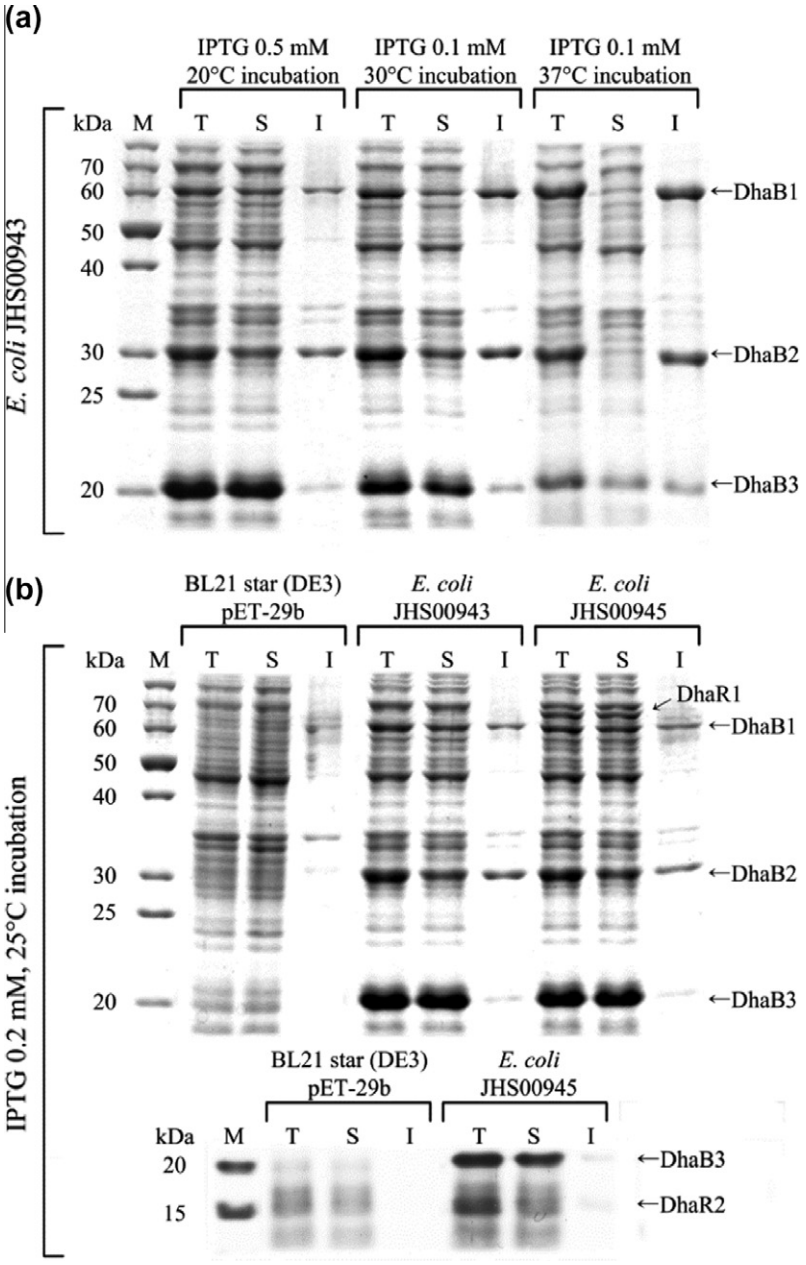

Fig. 1. Effects of IPTG concentration and culture temperature on the expression of *L. brevis* DhaB and DhaR proteins in recombinant *E. coli* JHS00943 (a and b) and JHS00945 (b). The samples were collected after 14 h of IPTG induction. The arrows indicate the bands of the corresponding proteins. SDS–PAGE with 12% polyacrylamide was used for protein analysis. Lane names are denoted as follows: M, protein marker; T, total protein; S, soluble protein; I, insoluble protein.

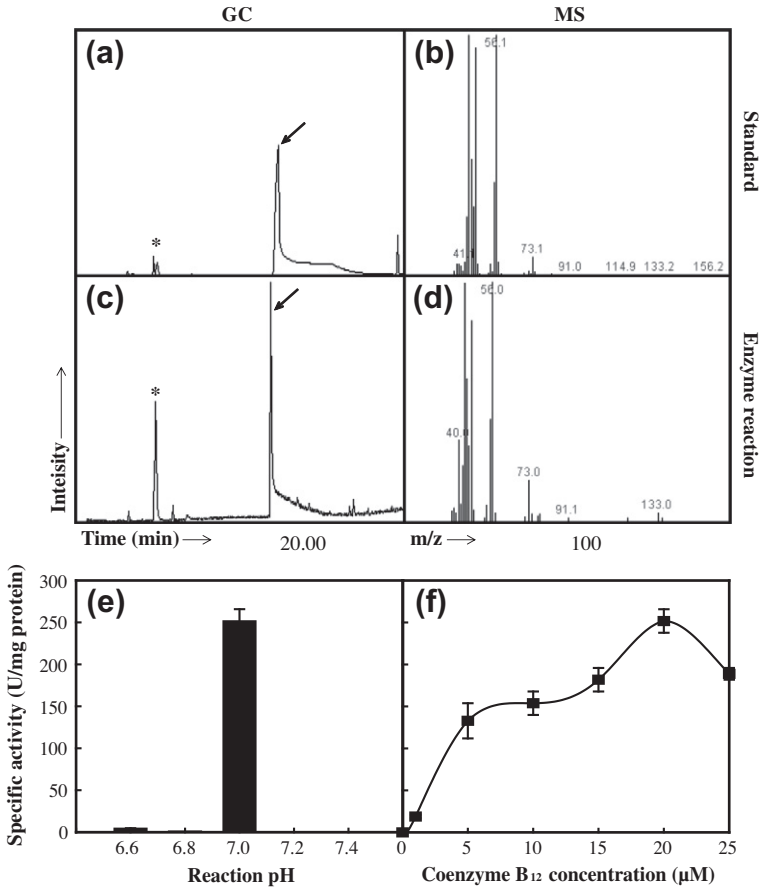

Fig. 2. Gas chromatograms (a and c) and mass spectra (b and d) of 3-HPA standard (a and b) and the DhaB reaction solution (c and d), and effects of pH (e) and coenzyme B_{12} concentration (f) on *in vitro* DhaB enzyme activity. The crude enzyme sample was prepared from the batch cultivation of *E. coli* JHS00943 at 37 °C and 200 rpm. The arrow indicates the peak of 3-HPA, and the peak for acrolein is asterisked.

encoded by two coding sequences (Genbank locus tag: LVIS_1612 and LVIS_1611) in *L. brevis* ATCC 367 and the glycerol dehydratase reactivase in *K. pneumoniae* (GdrA-GdrB). The *dhaR* cluster was composed of two open reading frames (*dhaR1* and *dhaR2*), of which 20 nucleotides overlapped each other. Two putative ribosomal binding sites were located in front of each coding region. The sequences from *L. brevis* KCTC 33069 and *L. brevis* ATCC 367 differed by 35 nucleotides and six amino acids. The large subunits of reactivase or reactivating factor showed higher similarity and identity than the small subunits. The *dhaR* cluster was located at 15 bp downstream of the *dhaB* cluster, different from the location of the reactivase genes of *K. pneumoniae*, which are positioned bidirectionally upstream (*gdrB*) and downstream (*gdrA*) of the glycerol dehydratase genes (Tobimatsu et al., 1999).

The nucleotide sequences of *dhaB* and *dhaR* gene clusters are available under Accession Number JX227982 in Genbank.

3.2. Production of DhaB and DhaR in recombinant E. coli strains

Lactobacillus sp. uses three translational initiation codons of ATG, GTG and TTG, whereas *E. coli* uses ATG only. Since *dhaB1* and *dhaB2* have TTG and GTG of the initiation codons, PCR primers of F_Lb_dhaB1, R_Lb_dhaB1 and F_Lb_dhaB23 were designed to change them to ATG codon in order to express the *dhaB* gene cluster in recombinant *E. coli*. As shown by SDS-PAGE for *E. coli* JHS00943 (Fig. 1(a)), the protein bands of DhaB1, DhaB2 and DhaB3 were identified at the theoretical molecular weights of 61, 26 and 19 kDa, respectively. A decrease in culture temperature enhanced soluble expression of the DhaB proteins, but reduced the cell growth rate considerably (data not shown). Most DhaB proteins formed insoluble inclusion bodies at 37 °C. When the culture of *E. coli* JHS00945 was grown at 25 °C and induced at an IPTG concentration of 0.2 mM, all proteins of interest were expressed in soluble form, and DhaR1 and DhaR2 were detected at estimated molecular weights of 66 and 13 kDa, respectively (Fig. 1(b)).

3.3. Activity of L. brevis glycerol dehydratase

To confirm the glycerol dehydration activity of the recombinant DhaB protein from *L. brevis* KCTC 33069, the enzyme reaction solution of the DhaB protein extract was subjected to GC–MS analysis. As shown in Fig. 2 (a and c), GC peaks of the 3-HPA standard and the reaction solution were detected at the same retention time. The two GC peaks at 17 min retention time showed the same mass spectra (Fig. 2 (b and d)), indicating that DhaB encoded by the *dhaB* gene cluster of *L. brevis* KCTC 33069 possesses glycerol dehydratase activity.

As shown in the *in vitro* activity assay of DhaB expressed in JHS00943 (Fig. 2 (e and f)), the optimum pH and coenzyme B_{12} concentration were 7.0 and 20 μM, respectively. DhaB showed no glycerol dehydratase activity except at pH 7.0. The optimum pH conditions for other glycerol dehydratases are 7.2 (*L. reuteri*) (Talarico and Dobrogosz, 1990), and 8.5 (*K. pneumoniae*) (Wang et al., 2007). The optimum coenzyme B_{12} concentration for *K. pneumoniae* glycerol dehydratase was determined at 15 μM (Honda et al., 1980).

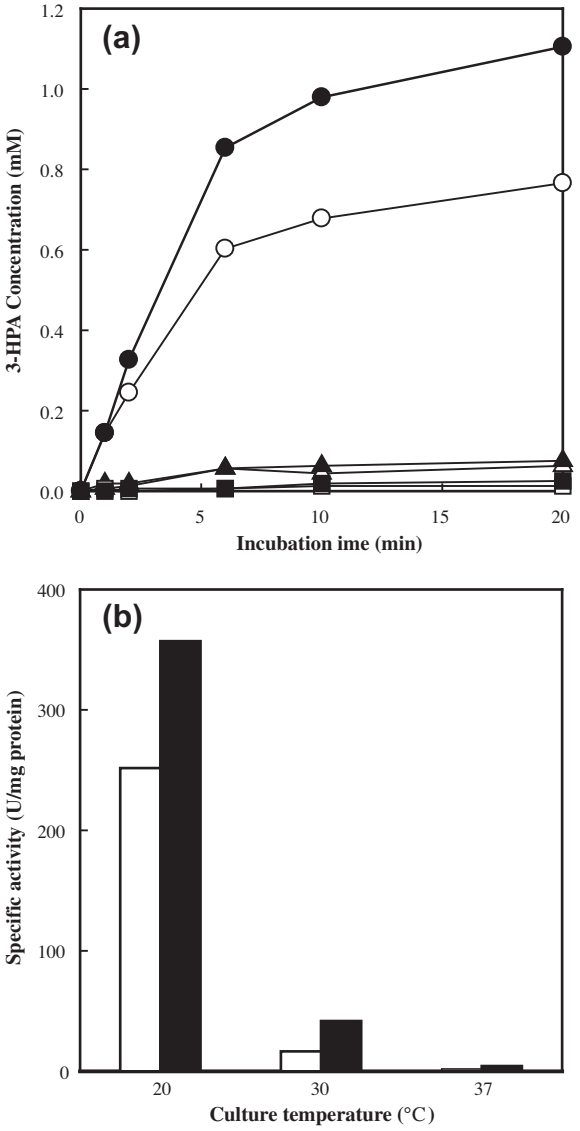

Fig. 3. Effects of culture temperature on *in vitro* 3-HPA production (a) and DhaB specific activity (b) of *E. coli* JHS00943 (white symbol and bar) and JHS00945 (black symbol and bar). The cells were cultivated at 20 °C (circle), 30 °C (triangle) and 37 °C (square) after 0.2 mM IPTG induction.

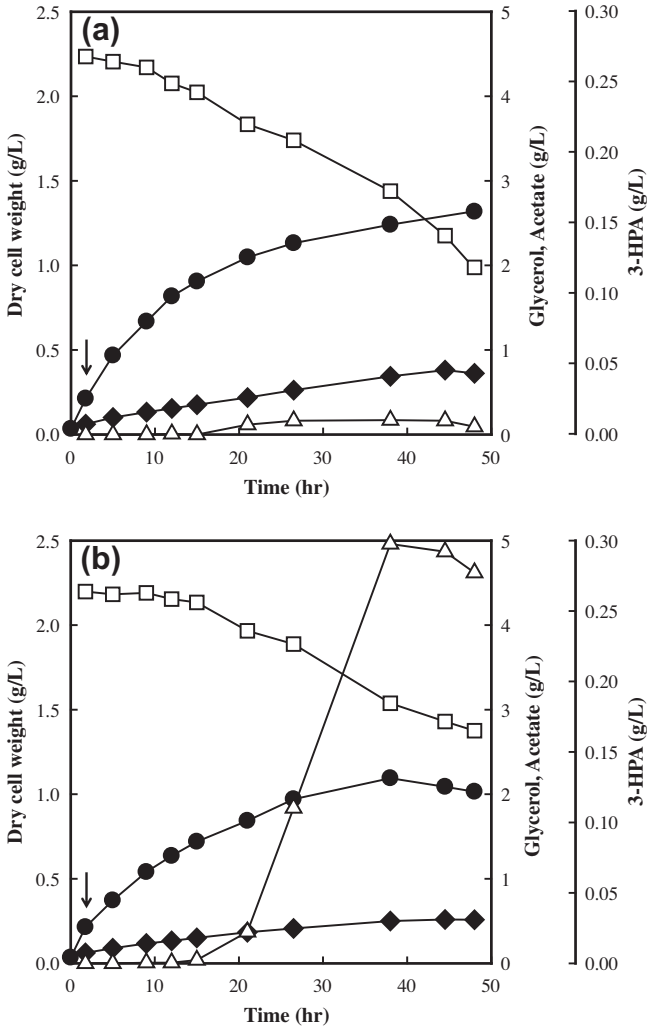

Fig. 4. Batch fermentations of *E. coli* JHS00943 (a) and JHS00945 (b) in LB medium with 4.5 g/L glycerol at 25 °C and 200 rpm. Symbols are denoted as follows: dry cell weight, ●; glycerol, □; 3-HPA, △; acetic acid, ♦; arrow, 0.2 mM IPTG induction.

3.4. Effects of culture temperature and dhaR expression on glycerol dehydratase activity

As shown in Fig. 1, the production of the DhaB and DhaR proteins was significantly influenced by culture temperatures. As shown in Fig. 3, the protein samples from cells grown at 30 and 37 °C showed a basal activity of glycerol dehydratase irrespective of the expression of DhaR. At 20 °C, the specific activity for JHS00943 expressing DhaB only was 252 U/mg protein, as soluble expression of DhaB protein could be achieved at 25 °C as shown in Fig. 1. The *E. coli* JHS00945 strain expressing both DhaB and DhaR protein showed a specific glycerol dehydratase activity of 357 U/mg protein, which was 1.4 times higher than that of *E. coli* JHS00943 expressing DhaB. The activity increment indicated that DhaR encoded by *dhaR1* and *dhaR2* might be a reactivating factor. The specific glycerol dehydratase activity for *E. coli* JHS00945 was 2.3 times higher than that for *E. coli* SH-BG2 harboring genes coding for *K. pneumoniae* glycerol dehydratase and its reactivase (Rathnasingh et al., 2009). It was reported that several 3-HPA producing microorganisms harbored the genes encoding diol or glycerol dehydratase reactivating proteins such as *gdrA-gdrB* in *K. pneumoniae* (Tobimatsu et al., 1996), *ddrA-ddrB* in *K. oxytoca* (Mori et al., 1997) and *pduG-pduH* in *L. reuteri* (Morita et al., 2008). Expression of the *K. pneumoniae* GdrA-GdrB complex in *E. coli* increased glycerol dehydratase activity by more than 30 times compared to the corresponding activity without expression of the complex (Tobimatsu et al., 1999). Overexpression of the DdrA-DdrB complex of *K. oxytoca* and GDR complex of *K. pneumoniae* (Mori et al., 1997; Rathnasingh et al., 2009), expression of *L. brevis* KCTC 33069 *dhaR* prolonged 3-HPA production after a sharp increase in 3-HPA concentration. Without the reactivating factor such as *E. coli* JHS00943, the large amount of active glycerol dehydratase was inactivated by tight binding of an inactivation form of cobalamin (Bachovchin et al., 1977), and only a small amount of 3-HPA was produced. Considering the sequence similarity and DhaB activity enhancement, it is certain that DhaR protein encoded by the *dhaR* gene cluster is a glycerol dehydratase reactivating factor of *L. brevis* KCTC 33069.

3.5. Production of 3-HPA in batch fermentation

To investigate the effect of DhaR expression on *in vivo* production of 3-HPA, batch fermentations of *E. coli* JHS00943 and

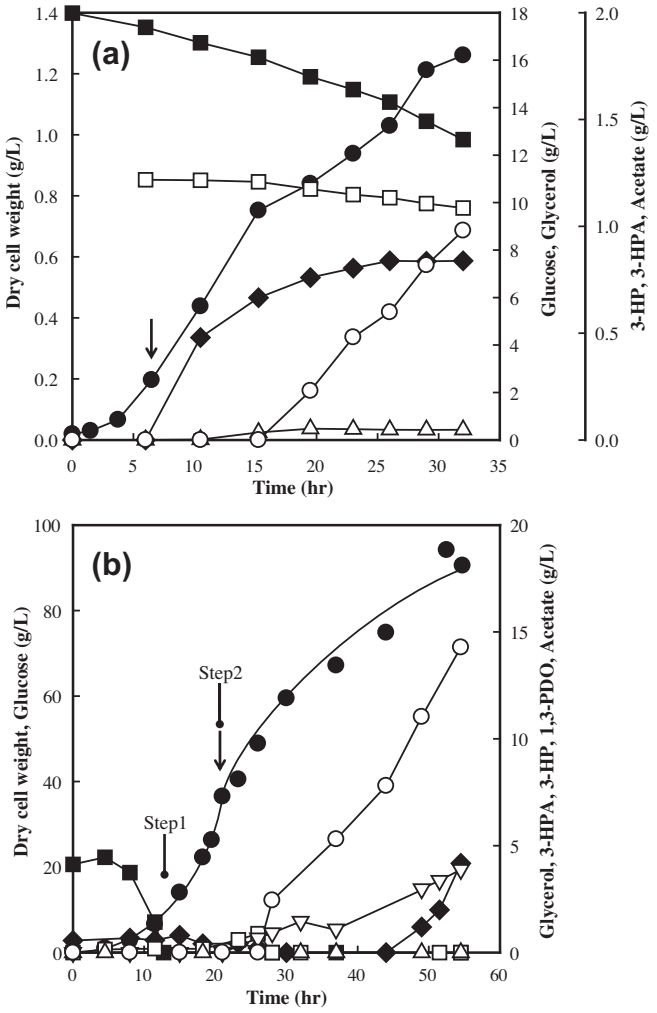

Fig. 5. Batch (a) and two-step fed-batch (b) fermentations of *E. coli* JHS00947 producing *L. brevis* DhaB and DhaR, and *E. coli* K-12 AldH in a defined medium with glycerol and glucose as co-substrate. For batch fermentation at 25 °C and 200 rpm, 11 g/L glycerol and 18 g/L glucose co-substrate were initially added. For fed-batch culture, step 1 indicates the starting point of feeding of 800 g/L glucose (feeding solution A). Step 2 presents the change of the feed solution A to a mixture of 400 g/L glycerol and 400 g/L glucose (feeding solution B), and 0.2 mM IPTG induction. The two feeding solution were fed by the pH-STAT mode of operation. Symbols are denoted as follows: dry cell weight, ●; glycerol, □; 3-HPA, △; 3-HP, ○; 1,3-PDO, ▽; acetic acid, ♦; arrow, 0.2 mM IPTG induction.

JHS00945 were carried out in LB medium with 4.5 g/L glycerol at 25 °C and 200 rpm (Fig. 4). During 38-h cultivation of JHS00943, 1.59 g/L glycerol was consumed to produce 1.21 g/L dry cell mass and 0.57 g/L acetate as by-product. JHS00943 produced less than 0.01 g/L 3-HPA only. Under the same conditions, JHS00945 produced 1.06 g/L dry cell mass and 0.30 g/L 3-HPA from 1.32 g/L consumed glycerol. Compared to the results for JHS00943, the cell mass decreased by 12%, but the 3-HPA concentration increased by 29-fold.

3.6. Batch and fed-batch production of 3-HP

In a preliminary cultivation under aerobic conditions using glycerol only, JHS00947 did not produce 3-HP and 3-HPA accumulation in the culture broth likely caused the cessation of cell growth (data not shown) (Barbirato et al., 1996). Since glycerol is metabolized for both cell growth and 3-HP production, a batch fermentation was designed to supply a co-substrate able to be used for cell growth and NAD^+ regeneration. A batch fermentation of *E. coli* JHS00947 with a mixture of 11 g/L glycerol and 18 g/L glucose resulted in 1.26 g/L dry cell mass and 0.98 g/L 3-HP concentration (Fig. 5 (a)). 3-HPA was detected at a basal level throughout the cultivation. *E. coli* K-12 aldehyde dehydrogenase encoded by *aldH* requires NAD^+ (Jo et al., 2008). High initial concentrations of glycerol inhibited cell growth at the early exponential phase (Zhu et al., 2002), and hence reduced glycerol consumption rate and 3-HP productivity. To minimize the inhibitory effect of glycerol on cell growth, fed-batch fermentation with a two-step feeding strategy was carried out by separating the cell growth phase from the 3-HP production stage. As shown in Fig. 5 (b), the two-step fed-batch fermentation of *E. coli* JHS00947 resulted in 94.2 g/L dry cell weight, 14.3 g/L 3-HP concentration and 4.18 g/L acetate by-product concentration. An overall productivity of 0.26 g/L-hr and a yield of 0.14 g 3-HP/g glycerol (equivalent to 0.15 mol 3-HP/mol glycerol) were obtained. Interestingly, 3.88 g/L 1,3-PDO was also produced in the fed-batch cultivation, possibly due to the presence of the endogenous *yqhD* gene coding for NADPH-dependent oxidoreductase (Nakamura and Whited, 2003).

E. coli expressing the *K. pneumoniae dhaB* and *gdrA-gdrB* genes, and the *A. brasilense KGSADH* gene maximally produced 38.7 g/L 3-HP from glycerol with 0.53 g/L-h 3-HP productivity and 35% yield in an aerobic fed-batch fermentation. As by-products, lactate and acetate were produced at 12% (mol/mol) yield each (Rathnasingh et al., 2009). Even though the maximum 3-HP concentration in the present study was lower than that reported by Rathnasingh et al. (2009), the two-step fed-batch fermentation separating the cell growth stage from the 3-HP production stage exhibited less by-product formation. It is possible that efficient 3-HP production can be achieved by modifying aldehyde dehydrogenase to become more specific for 3-HPA and by altering endogenous glycerol metabolism to glyceraldehyde-3-phosphate.

4. Conclusion

The *dhaB* and *dhaR* gene clusters coding for glycerol dehydratase (DhaB) and its reactivation factor (DhaR) were cloned from *L. brevis* KCTC 33069, characterized and expressed in *E. coli*. Active expression of DhaB, DhaR and AldH proteins allowed *E. coli* JHS00947 to convert glycerol to 3-HP. The two-step fed-batch fermentation using glycerol and glucose as co-substrate was developed to produce high concentrations of 3-HP with low by-product formation. More researches are in progress to clone the genes coding for aldehyde dehydrogenases highly specific for 3-HPA and develop host strains able to utilize glycerol efficiently.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2012.11.063.

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