J Ind Microbiol Biotechnol (2014) 41:1039–1050
DOI 10.1007/s10295-014-1451-2

BIOENERGY/BIOFUELS/BIOCHEMICALS

SIMB
Society for Industrial Microbiology and Biotechnology

Production of 3-hydroxypropionic acid from glycerol by acid tolerant *Escherichia coli*

Mugesh Sankaranarayanan · Somasundar Ashok · Sunghoon Park

Received: 11 February 2014 / Accepted: 18 April 2014 / Published online: 1 May 2014

Abstract The biological production of 3-hydroxypropionic acid (3-HP) has attracted significant attention because of its industrial importance. The low titer, yield and productivity, all of which are related directly or indirectly to the toxicity of 3-HP, have limited the commercial production of 3-HP. The aim of this study was to identify and select a 3-HP tolerant *Escherichia coli* strain among nine strains reported to produce various organic acids efficiently at high titer. When transformed with heterologous glycerol dehydratase, reactivase and aldehyde dehydrogenase, all nine *E. coli* strains produced 3-HP from glycerol but the level of 3-HP production, protein expression and activities of the important enzymes differed significantly according to the strain. Two *E. coli* strains, W3110 and W, showed higher levels of growth than the others in the presence of 25 g/L 3-HP. In the glycerol fed-batch bioreactor experiments, the recombinant *E. coli* W produced a high level of 3-HP at 460 ± 10 mM (41.5 ± 1.1 g/L) in 48 h with a yield of 31 % and a productivity of 0.86 ± 0.05 g/L h. In contrast, the recombinant *E. coli* W3110 produced only 180 ± 8.5 mM 3-HP (15.3 ± 0.8 g/L) in 48 h with a yield and productivity of 26 % and 0.36 ± 0.02 g/L h, respectively. This shows that the tolerance to and the production of 3-HP differ significantly among the well-known, similar strains of *E. coli*. The titer and productivity obtained with *E. coli* W were the highest reported thus far for the biological production of 3-HP from glycerol by *E. coli*.

M. Sankaranarayanan and S. Ashok have contributed equally to this work.

M. Sankaranarayanan · S. Ashok · S. Park (✉)
School of Chemical and Biomolecular Engineering, Pusan National University, San 30, Jangjeon-dong, Geumjeong-gu, Busan 609-735, South Korea
e-mail: parksh@pusan.ac.kr

Keywords *Escherichia coli* · 3-Hydroxypropionic acid · Acid tolerance · Glycerol metabolism

Introduction

3-Hydroxypropionic acid (3-HP) is a non-chiral carboxylic acid containing a hydroxyl group on its third carbon atom. The compound is used in the synthesis of a range of industrially important chemicals, such as acrylic acid, 1,3-propanediol, methyl acrylate, propiolactone, malonic acid and acrylamide [6, 9]. 3-HP is also used as a cross-linking agent for polymer coatings, metal lubricants and antistatic agents. This important platform chemical can be produced either by chemical or biological processes, but the biological method is considered to be more feasible from technological and environmental aspects [10].

A range of microorganisms, such as *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas denitrificans*, have been studied for the biological production of 3-HP from glycerol [1–3, 12, 14] or glucose [22] as the carbon source. When glycerol is used as carbon source, two enzymes, glycerol dehydratase (DhaB) and aldehyde dehydrogenase (KGSADH), are required. DhaB converts glycerol to 3-hydroxypropionaldehyde (3-HPA) in the presence of coenzyme B_{12} and KGSADH catalyzes the conversion of 3-HPA to 3-HP with $NAD(P)^+$ as electron acceptor. During 3-HP production, DhaB often loses its activity due to the damage in coenzyme B_{12} which is bound to DhaB. Then, the enzyme glycerol dehydratase reactivase (GdrAB) replaces the damaged coenzyme B_{12} with a new coenzyme B_{12} to reactivate DhaB (Fig. 1) [13]. Rathnasingh et al. [21] reported that the recombinant *E. coli* SHBGK (*E. coli* BL21 DE3) could produce 39 g/L 3-HP from 111 g/L glycerol with a productivity of 0.54 g/L h. Ashok et al. [3, 4]

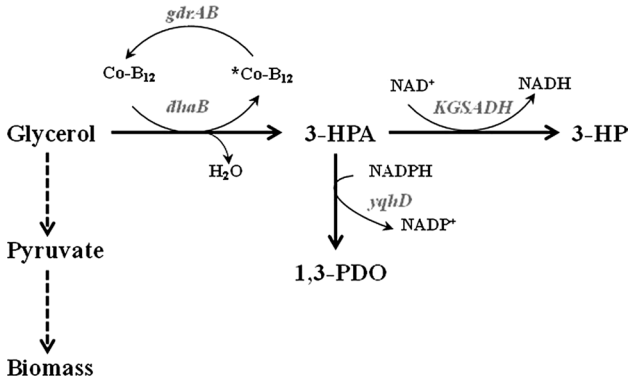

Fig. 1 3-Hydroxypropionic acid production Pathway from glycerol in recombinant *E. coli* overexpressing glycerol dehydratase (*dhaB*), glycerol dehydratase reactivation factors (*gdrAB*) and aldehyde dehydrogenase (*KGSADH*). *Co-B_{12} represents the damaged coenzyme-B_{12} due to the catalytic activity of DhaB. The *Co-B_{12} is replaced with new undamaged Co-B_{12} by the enzymes GdrAB

and Huang et al. [9] reported that recombinant *K. pneumoniae*, which overexpresses an aldehyde dehydrogenase, could produce 28 g/L and 48 g/L 3-HP, respectively, [3, 9]. Zhou et al. [34] reported that recombinant *P. denitrificans* can also produce 3-HP from glycerol, even though the 3-HP produced was degraded and re-assimilated for cell growth [34]. With glucose as the carbon source, several pathways have been suggested. However, only one pathway involving malonyl-CoA as an intermediate proved to be successful thus far [16, 22].

Despite the considerable progress, many problems in the biological production of 3-HP have been encountered [10]. When glycerol is used as substrate, the accumulation of the toxic intermediate, 3-hydroxypropionaldehyde (3-HPA), is a common problem. The requirement of expensive coenzyme B_{12}, which is needed for the catalytic activity of DhaB, has also been indicated as a serious problem, particularly when *E. coli* is used as a host. However, the most common and serious problem is considered the toxic effects of 3-HP on cell growth and 3-HP production. Generally, many organic acids including 3-HP exhibit toxic effects before their concentrations reach commercially important levels [32]. In the case of 3-HP, toxicity started at 200 mM, which is too low for commercial production [31, 33]. The level of tolerance of many organic acids is species- and/or strain-specific [25, 30]. For example, some *E. coli* strains, such as W3110, W, K12, and B, can produce larger amounts of lactate than other strains [8, 17, 28, 35]. In addition, *E. coli* DF40 and *E. coli* Mach1 were reported to have a higher tolerance to 3-HP [29, 33].

The aim of this study is to examine the tolerance of various *E. coli* strains to 3-HP and identify a suitable strain as a host for the production of 3-HP from glycerol. To this end, nine strains of *E. coli*, which have been reported to produce large amounts of carboxylic acids, were evaluated for their growth in the presence of 25 g/L 3-HP. In addition, the *E. coli* strains were transformed with glycerol dehydratase, glycerol dehydratase reactivase and aldehyde dehydrogenase, and assessed for their capability to produce 3-HP from glycerol in the presence or absence of 3-HP. Finally, for the best two *E. coli* strains, W3110 and W, bioreactor fed-batch experiments were carried out to evaluate their potential for the high titer production of 3-HP from glycerol.

Materials and methods

Strains, plasmids, genetic methods and materials

Escherichia coli XL1 blue was purchased from the Korean Culture Center of Microorganisms (KCCM). *Escherichia coli* BL21 (DE3) was obtained from Novagen (Darmstadt, Germany). *Escherichia coli* Mach1 was supplied by Invitrogen (Carlsbad, CA, USA). *Escherichia coli* W, *E. coli* K12 MG1655 and *E. coli* LE392 were acquired from Korean Collection for Type Cultures (KCTC). Unless indicated otherwise, all other *E. coli* strains used in this study were procured from the American Type Culture Collection (ATCC). The genomic DNA isolation kit and pGEM-T vector were purchased from Promega (Madison, WI, USA). The high fidelity *pfx* polymerase was acquired from Invitrogen (Seoul, Korea). The restriction and DNA-modifying enzymes and the pUC19 plasmid were obtained from New England Bio-Labs (Beverly, MA, USA). Miniprep and DNA gel extraction kits were purchased from Qiagen (Mannheim, Germany). The primers were synthesized by Cosmotech Co. Ltd. (Seoul, Korea). 3-HP was purchased from Tokyo Kasei Kogyo Co. Ltd, Tokyo, Japan (TCI America). Because 3-HPA is unavailable commercially, it was synthesized chemically from acrolein using the method reported by Hall and Stern (1950). The yeast extract (Cat. 212750) was obtained from Difco (Becton–Dickinson; Franklin Lakes, NJ, USA). Unless stated otherwise, glycerol and all other chemicals and enzymes were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cloning of *dhaB*, *gdrAB* and *KGSADH*

Lysogen broth (LB) medium was used for routine genetic engineering, protein expression and culture maintenance. Kanamycin (Kmr) at 50 mg/L and chloramphenicol (Cm) at 25 mg/L were added to the culture medium where indicated. The standard recombinant DNA procedures were used for gene cloning, plasmid isolation and electroporation [18, 23]. The plasmid pDK7 (p15A)/*dhaB123*, *gdrAB* was developed to overexpress glycerol dehydratase (DhaB; Gene bank # YP_001337149.1, YP_001337150.1, YP_001337151.1)

Table 1 Bacterial strains and plasmids used in this study

Strains and plasmids	Description	Source/references
Strains		
E. coli XL-1 blue	Cloning host	KCCM, Korea
E. coli W3110*	D-lactic acid production (45 g/L)	ATCC27325/ [35]
E. coli W*	D-lactic acid (85 g/L)	KCTC1039/ [28]
E. coli K12*	D-lactic acid production (32 g/L)	KCTC1116/ [17]
E. coli BL21 (DE3)*	3-HP production (39 g/L)	Novagen/ [21]
E. coli B*	L-lactic acid production (63 g/L)	ATCC11303/ [8]
E. coli C*	Expression host	ATCC8739/This study
E. coli LE392*	Poly-3-hydroxy butyrate production (0.71 g/L)	KCTC1042/ [25]
E. coli Mach1*	3-HP MIC studies	Invitrogen/ [33]
E. coli DF40*	3-HP MIC studies	ATCC53918/ [29]
E. coli W3110 DUBGK	Recombinant *E. coli* W3110 harboring pUC19/*KGSADH* and pDK7 (p15A)/*dhaB*123, *gdrAB*	This study
E. coli W DUBGK	Recombinant *E. coli* W harboring pUC19/*KGSADH* and pDK7 (p15A)/*dhaB*123, *gdrAB*	This study
E. coli K12 DUBGK	Recombinant *E. coli* K12 harboring pUC19/*KGSADH* and pDK7 (p15A)/*dhaB*123, *gdrAB*	This study
E. coli BL21(DE3) DUBGK	Recombinant *E. coli* BL21(DE3) harboring pUC19/*KGSADH* and pDK7 (p15A)/*dhaB*123, *gdrAB*	This study
E. coli B DUBGK	Recombinant *E. coli* B harboring pUC19/*KGSADH* and pDK7 (p15A)/*dhaB*123, *gdrAB*	This study
E. coli C DUBGK	Recombinant *E. coli* C harboring pUC19/*KGSADH* and pDK7 (p15A)/*dhaB*123, *gdrAB*	This study
E. coli LE392 DUBGK	Recombinant *E. coli* LE392 harboring pUC19/*KGSADH* and pDK7 (p15A)/*dhaB*123, *gdrAB*	This study
E. coli Mach1 DUBGK	Recombinant *E. coli* Mach1 harboring pUC19/*KGSADH* and pDK7 (p15A)/*dhaB*123, *gdrAB*	This study
E. coli DF40 DUBGK	Recombinant *E. coli* DF40 harboring pUC19/*KGSADH* and pDK7 (p15A)/*dhaB*123, *gdrAB*	This study
Plasmids		
pDK7 (p15A)/*dhaB123*, *gdrAB*	*dhaB123*, *gdrAB* in pDK7 plasmid with p15A; Cm^r	[4]
pRKS1	*KGSADH* in pRSFDuet; Km^r	[21]
pUC19/*KGSADH*	*KGSADH* in pUC19; Km^r	[11]

Final titre achieved in respective studies is mentioned in parenthesis

* Strains used as expression hosts in various acid productions

and glycerol dehydratase reactivase (GdrAB; Gene bank # YP_001337148.1) using the *tac* promoter [4]. For overexpression of aldehyde dehydrogenase, the *KGSADH* gene (Gene bank # AB241137) was amplified from the pRKS1 plasmid and cloned in the pUC19 plasmid under the *lac*P promoter by replacing the β-galactosidase (*lacZ*α) gene [11]. The expression plasmids, pDK7 (p15A)/*dhaB123*, *gdrAB* and pUC19/*KGSADH*, were introduced to various *E. coli* strains for 3-HP production from glycerol (Table 1).

Protein expression and gel electrophoresis

To confirm that the recombinant strains had been constructed properly, the recombinant *E. coli* strains (*E. coli* W3110 DUBGK, *E. coli* W DUBGK, *E. coli* K12 DUBGK, *E. coli* BL21 (DE3)DUBGK, *E. coli* B DUBGK, *E. coli* C DUBGK, *E. coli* LE392 DUBGK, *E. coli* Mach1 DUBGK and *E. coli* DF40 DUBGK) were grown in modified M9 medium containing the following components (per liter of deionized water): $MgSO_4 \cdot 7H_2O$, 0.25 g; NaCl, 1.0 g; NH_4Cl, 1.0 g; yeast extract, 0.2 g; and glycerol, 100 mmol. The medium was supplemented with 100 mM potassium phosphate buffer (pH 7.0). The cultures were induced with 0.1 mM IPTG at ~0.6 OD_{600}. The cells were harvested after induction, and centrifuged at 10,000 *g* at 4 °C for 15 min. The cell pellets were washed twice with 100 mM potassium phosphate buffer (pH 7.0) and resuspended in the same buffer. The cells were lysed using a French Pressure

Cell (FA-078A, Thermo Electron Co., Waltham, MA) at 1,250 psi. The cell lysate was centrifuged at 25,000 *g* for 30 min and the supernatants were used for SDS-PAGE analysis. Protein expression was examined on 12 % SDS-PAGE under denaturing conditions [15]. The proteins were stained with Coomassie Brilliant Blue R-250. The intensity of the protein bands in SDS-PAGE was quantified and compared using ImageJ software provided by the National Institute of Health (NIH) [24].

Determination of enzyme activities

The DhaB activity in the crude cell extract was measured using the method reported by Raj et al. [19]. The KGSADH activity was determined by measuring the level of NAD^+ reduction to NADH at 340 nm using the method reported by Raj et al. [20]. Briefly, the reaction mixture containing 100 mM potassium phosphate buffer (pH 8.0), 1 mM DTT and the enzyme extract was incubated at 37 °C for 5 min, and the reaction was initiated by the addition of 2.0 mM 3-HPA and 2.0 mM NAD^+. The amount of NADH formed was determined using a molar extinction coefficient ($\Delta\varepsilon_{340}$) of 6.22×10^3 M^{-1} cm^{-1}. One unit of KGSADH activity was defined as the amount of enzyme required to reduce 1 μmol of NAD^+ to NADH in 1 min. All enzyme activities were measured using the crude cell extracts.

Culture medium and cultivation conditions

Unless stated otherwise, shake flask cultivation was carried out with a working volume of 50 mL in a 250-mL non-baffled Erlenmeyer flask at 37 °C on an orbital incubator shaker at 250 rpm. The modified M9 medium was used, which contained (per liter of deionized water): $MgSO_4 \cdot 7H_2O$, 0.5 g; NaCl, 2.0 g; NH_4Cl, 2.0 g; yeast extract, 1 g; glycerol, 100 mmol; 50 mg kanamycin and 25 mg chloramphenicol. The medium was supplemented with 100 mM potassium phosphate buffer (pH 7.0). The flasks were plugged with oxygen-permeable cotton plugs. The cultures were induced at 0.6 ± 0.05 OD_{600} with 0.1 mM IPTG and supplemented with coenzyme B_{12} (final concentration of 2 μM) at 3, 6, 9, and 12 h of cultivation. When examining the 3-HP tolerance of the various strains, the modified M9 medium was supplemented with 25 g/L of 3-HP after neutralization with NaOH. The samples were withdrawn periodically to determine the cell mass, residual substrate and metabolites. All shake flask experiments were carried out in triplicate; the standard deviations of the measurements of biomass and metabolites were <10 %.

Bioreactor experiments were carried out at 37 °C and pH 7.0 in a 1.5-L capacity Biotron-LiFlus GM bioreactor (Biotron, Seoul, Korea) with a 1-L initial working volume. The same modified M9 medium as in the flask experiments was employed except that glycerol concentration was increased to 200 mM. IPTG at 0.1 mM was added to induce 3-HP production when the cell OD_{600} reached 1.5 ± 0.1. Coenzyme B_{12} (2 μM) was added at 3-h intervals until 48 h of cultivation. Air flow rate was 0.5 vvm and agitation speed was 650 rpm. Initially, the reactor was run as a batch mode, and then switched to a fed-batch mode when glycerol concentration was lowered to 25 mM. Concentrated glycerol solution (10 M) was added continuously at 3.5 mL/h. Samples were withdrawn periodically to determine the cell mass, residual substrate concentration and metabolite concentration. The reported measurements of the biomass and metabolites are the means of technical replicates (multiple samples) from a single experiment, and the standard deviation was <10 %.

Analytical methods

The cell concentration was measured in a 10-mm-path-length cuvette using a double-beam spectrophotometer (Lambda 20, Perkin Elmer; Norwalk, CT, USA). One unit of absorbance at 600 nm (OD_{600}) corresponded to 0.3 g dried cell mass per liter. The protein concentrations in the cell-free extract were determined on a microtiter plate reader (1420, Wallac Victor 2; Perkin Elmer) using the Bradford method [5] with bovine serum albumin as the standard. The concentrations of glycerol, 3-HP and other metabolites were determined by HPLC using a slight modification of the method reported by Raj et al. [19]. Briefly, the supernatants, which were obtained by centrifugation of the culture samples at 10,000×*g* for 10 min, were filtered through a Tuffryn membrane (Acrodisc, Pall Life Sciences) and eluted through a 300 × 7.8 mm Aminex HPX-87H (Bio-Rad, USA) column at 65 °C using 2.5 mM H_2SO_4 as the mobile phase.

Results

Effect of 3-hydroxypropionic acid on the growth and glycerol metabolism of different *E. coli* strains

The effects of 3-HP on the cell growth and glycerol assimilation of nine *E. coli* strains were examined. The specific cell growth rate (μ_{max}) and glycerol consumption rate of all the nine strains decreased when 3-HP (25 g/L) was added to the culture medium. Typically, μ_{max} decreased by 50 % in the presence of ~25 g/L 3-HP. The decrease in μ_{max} and the glycerol consumption rate varied substantially depending on the strain, indicating a different sensitivity of the strains to 3-HP. In the presence of 3-HP, *E. coli* C, K12 and W showed a higher μ_{max} of 0.58, 0.56 and 0.51 h^{-1}, respectively, than the other strains. *E. coli* DF40 showed the

Table 2 Maximum specific growth rate and final cell densities of various *E. coli* strains cultivated with and without 3-HP

Strains	μ_{max} (h^{-1})		Final cell density (g cdw/L)	
	Without 3-HP	With 3-HP*	Without 3-HP	With 3-HP*
E. coli W3110	0.51	0.31	0.89	0.95
E. coli W	0.77	0.51	0.85	1.19
E. coli K12	0.82	0.56	0.95	1.17
E. coli BL21(DE3)	0.72	0.47	0.94	0.94
E. coli B	0.71	0.42	0.77	0.83
E. coli C	0.76	0.58	0.98	1.38
E. coli LE392	0.77	0.24	0.88	0.56
E. coli Mach1	0.65	0.44	0.91	0.85
E. coli DF40	0.53	0.16	0.93	0.69

The standard deviation of the measurements was <10 %

* 25 g/L 3-HP

lowest μ_{max} of 0.16 h^{-1} (Table 2). Interestingly, three fast-growing *E. coli* strains, C, K12 and W, showed ~1.4 times higher final cell densities at 24 h in the presence of 3-HP (Table 2). The concentrations of 3-HP were also determined before and after cultivation. At 24 h, >97 % of 3-HP was retained in the samples of all *E. coli* strains, indicating that they do not degrade or assimilate 3-HP.

Expression of DhaB, GdrAB and KGSADH in various *E. coli* strains

To establish the ability to produce 3-HP from glycerol, three enzymes, DhaB, GdrAB and KGSADH, were introduced to the nine *E. coli* strains using two compatible plasmids: pUC19* (ColE1; P_{lac}) and pDK7 (P15A; P_{tac}). The expression of these enzymes in the cells grown in the modified M9 medium was examined by SDS-PAGE (Fig. 2) and enzyme activity measurements (Table 3). All recombinant *E. coli* strains showed prominent protein bands at 62 kDa (corresponding to DhaB1 and/or GdrA), 53 kDa (KGSADH) and 21 kDa (DhaB2) in the cell-free soluble fraction. The protein bands for DhaB3 (16 kDa) and GdrB (12 kDa) were not visible, possibly because of their small sizes. Although the same plasmids were used, the expression levels of these proteins when estimated by the band intensity varied significantly in these *E. coli* strains (Table 3). For example, *E. coli* W3110 DUBGK and W DUBGK showed similar 62 and 53 kDa protein expression: ~7.2 and ~3.1 %, respectively, of the total soluble proteins. The recombinant *E. coli* K12 showed a low level of 62 kDa protein expression (~2.3 %), whereas the level of KGSADH (53 kDa) expression was ~11 % of the total soluble protein. *E. coli* BL21(DE3), B, C, and Mach1 showed relatively high levels of 62 kDa protein expression: 7.5, 4.7, 2.7, and 8.2 %, compared to that of KGSADH. In particular, the BL21(DE3) and B strains, used for 3-HP production previously [19, 21], showed high levels of 62 kDa protein expression (7.5 and 4.7 %) but very low levels of 53 kDa protein expression (~0.8 and 0.4 %).

The enzyme activities of DhaB and KGSADH were determined using the crude cell extracts (Table 3). Generally, the enzyme activities correlated well with the level of expression of the corresponding proteins estimated from SDS-PAGE (Table 3). The recombinant *E. coli* W3110 and W showed similar activities for DhaB and KGSADH at 1.6 ± 0.1 U/mg protein and 1.8 ± 0.2 U/mg protein, respectively. Recombinant *E. coli* K12, which showed the highest level of KGSADH expression but very low

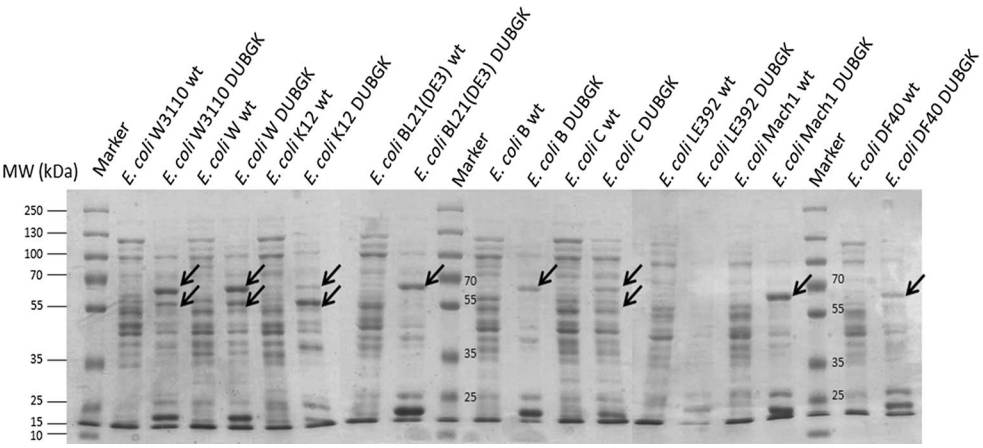

Fig. 2 SDS-PAGE analysis of the cell-free soluble extract of various recombinant *E. coli* strains overexpressing DhaB, GdrAB and KGSADH. Correspondingly, cell-free soluble extract of various *E. coli* wild-type strains was used as the reference samples. The *upper arrow* indicates DhaB and GdrA (~62 kDa) and the *lower arrow* represents KGSADH (~53 kDa)

Table 3 Quantity and activities of the DhaB and KGSADH protein in crude cell extracts of various recombinant *E. coli* strains

Strain	Amount of soluble protein (%)*		Activity (U/mg protein)	
	DhaB	KGSADH	DhaB	KGSADH
E. coli W3110 DUBGK	7.1	3.1	1.60	2.00
E. coli W DUBGK	7.3	3.2	1.58	1.66
E. coli K12 DUBGK	2.3	10.9	0.06	3.40
E. coli BL21(DE3) DUBGK	7.5	0.8	1.28	0.08
E. coli B DUBGK	4.7	0.4	0.06	0.04
E. coli C DUBGK	2.7	1.1	0.26	0.08
E. coli Mach1 DUBGK	8.2	0.9	1.74	0.13
E. coli DF40 DUBGK	2.8	1.6	0.08	0.90

The standard deviation of the measurements was <10 %

Amount of soluble protein on SDS-PAGE was calculated using imageJ (NIH) software

* The relative expression of target protein in comparison to the amount of total protein

levels of 62 kDa protein(s) expression, exhibited the highest KGSADH activity of 3.4 ± 0.3 U/mg protein but a very low DhaB activity of 0.06 U/mg protein. *E. coli* Mach1 DUBGK showed the highest DhaB activity (1.7 ± 0.1 U/mg protein) and the lowest KGSADH activity (0.13 ± 0.01 U/mg protein), which are similar to the expression levels of the corresponding proteins. Both enzyme activities were low in recombinant *E. coli* B although this strain produced the highest amount of 3-HP in the shake flask (see the "Production of 3-HP from glycerol by the recombinant *E. coli* strains on a flask scale"). In the case of recombinant *E. coli* LE392 DUBGK, cell growth was too low and the enzyme activity could not be measured.

Production of 3-HP from glycerol by the recombinant *E. coli* strains on a flask scale

The recombinant *E. coli* strains overexpressing DhaB, GdrAB and KGSADH were evaluated for their ability to produce 3-HP from glycerol in M9 media (Fig. 3). In most cultures, a small amount of 3-HPA accumulated immediately after adding coenzyme B_{12} at 3 h. In particular, *E. coli* Mach1 DUBGK accumulated relatively large amounts of 3-HPA at ~1 mM and cell growth stopped completely after the accumulation of 3-HPA (data not shown). 3-HPA is toxic to cell growth and 3-HP production, even though it is the immediate precursor of 3-HP [21]. The μ_{max} of all recombinant *E. coli* strains decreased by approximately 50 % compared to their corresponding hosts (Table 4). Nevertheless, among the nine recombinants, two recombinants, *E. coli* W3110 DUBGK and W DUBGK, exhibited a higher μ_{max} than the others. For 3-HP production, five recombinant strains, W3110, W, K12, B, and DF40, showed better results than the others (Table 4). *Escherichia coli* B DUBGK produced the highest 3-HP at 35.6 ± 2.5 mM, followed by W DUBGK and W3110 DUBGK at 29.2 ± 2.5 and 28.7 ± 2.3 mM, respectively. 3-HP production in these three strains was initiated earlier than the others (Fig. 3).

In the next study, the flask experiments were performed using the culture medium supplemented with 25 g/L of 3-HP to determine the toxic effects of 3-HP on its production. The experiment was conducted for five recombinants only (*E. coli* W3110, W, K12, B and DF40), which produced higher levels of 3-HP in the previous experiment (Fig. 3). The recombinants of the K12, B and DF40 strains did not show any growth. Therefore, only the results for W3110 and W are shown (Fig. 4). In the presence of 25 g/L 3-HP, both W3110 DUBGK and W DUBGK exhibited a significant decrease in μ_{max}, glycerol consumption and 3-HP production. Glycerol consumption decreased by ~40 % for both strains, and 3-HP production also decreased by 68 and 52 % for W3110 and W, respectively (Table 4). Between the two strains, *E. coli* W DUBGK (14.1 ± 1.1 mM 3-HP) produced much higher 3-HP levels than W3110 DUBGK (9.3 ± 0.8 mM 3-HP). These results, along with previous experiments, indicate that the ability to grow and produce 3-HP varies considerably among the nine *E. coli* strains tested. Moreover, the two strains, *E. coli* W3110 and W, have greater potential to produce 3-HP than the others at a higher 3-HP titer.

Bioreactor cultivation of recombinant *E. coli* W3110 and *E. coli* W overexpressing DhaB, GdrAB and KGSADH

Fed-batch bioreactor experiments were carried out with the two best *E. coli* recombinants, W3110 DUBGK and W DUBGK (Fig. 5). *Escherichia coli* W3110 DUBGK showed a μ_{max} of 0.53 h^{-1} and a final cell density of 5.52 ± 0.32 g cdw/L, respectively (Fig. 5a; Table 5). Up to the initial 30 h, rapid glycerol consumption (431 ± 10 mM) and 3-HP production (155 ± 10 mM) were observed. In the next 18 h between 30 and 48 h, however, the amount of 3-HP production and glycerol consumption decreased to 25 ± 2 and 241 ± 10 mM, respectively. After culturing for 48 h, the estimated 3-HP molar yield, titer and productivity of recombinant *E. coli* W3110 DUBGK were 0.26 ± 0.02, 16.2 ± 0.5 g/L and 0.36 ± 0.03 g/L h, respectively (Table 5). The enzyme activities of DhaB and KGSADH were determined. The DhaB activity was the highest at 12 h (6.63 ± 0.33 U/mg protein) and decreased continuously with time, reaching less than 0.65 ± 0.05 U/mg protein at 36 h (Fig. 5c). KGSADH also showed the highest activity at 12 h (2.57 ± 0.22 U/mg protein), which then decreased to 0.61 ± 0.06 U/mg protein at 48 h.

Using the *E. coli* W strain as the host, a higher μ_{max} of 0.74 h^{-1} and higher final cell density of 9.76 ± 0.88 g

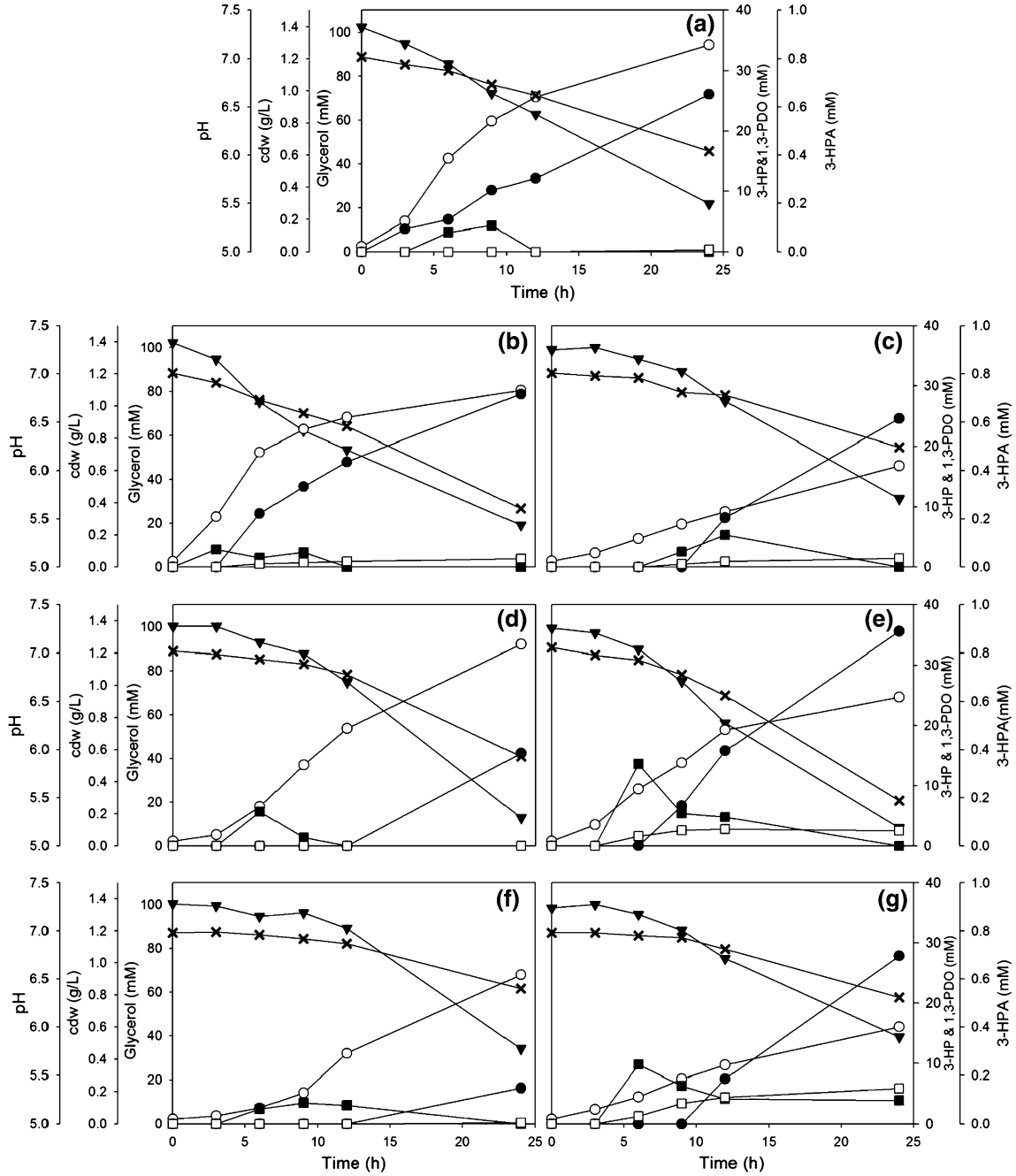

Fig. 3 Time-course profile of cell growth, glycerol consumption, 3-HPA, 3-HP and 1,3-PDO production from glycerol by various recombinant *E. coli* strains overexpressing DhaB, GdrAB and KGSADH. All the flasks were cultivated at 37 °C and 250 rpm by supplementing 2 μM coenzyme B_{12} at 3, 6, 9 and 12 h of cultivation. The standard deviations of the biomass and metabolite measurements were <10 %. **a** *E. coli* W3110, **b** *E. coli* W, **c** *E. coli* K12, **d** *E. coli* BL21(DE3), **e** *E. coli* B, **f** *E. coli* C and **g** *E. coli* DF40. Symbols: *closed circle* (3-HP), *closed triangle* (Glycerol), *open square* (1,3-PDO), *closed square* (3-HPA), *open circle* (Biomass) and *cross mark* (pH)

cdw/L were obtained. 3-HP production was rapid in the initial 15 h but the rate decreased afterward. Compared to W3110, the initial 3-HP production rate and final titer at 48 h were much higher. At 48 h, the 3-HP molar yield, titer and productivity were estimated to be 0.31 ± 0.02, 41.5 ± 1 g/L and 0.86 g/L h, respectively (Table 5). On the other hand, *E. coli* W DUBGK diverted large amounts of carbon towards two major byproducts, acetate (97.9 ± 5.7 mM) and 1,3-PDO (76.7 ± 5.2 mM) during 3-HP production. The enzyme activities of DhaB and

Table 4 Cell growth and metabolites profile of the recombinant *E. coli* strains cultivated in the shake flask

Strains and conditions	Specific growth rate (h^{-1})	Final biomass (g/L)	Glycerol consumed (mM)	1,3-PDO (mM)	3-HP (mM)	Acetate (mM)	Lactate (mM)	Ethanol (mM)	Carbon recovery[a] (%)
E. coli W3110 DUBGK	0.48	1.28	80.90	0.34	28.71	13.72	ND	7.12	57.71
E. coli W DUBGK	0.49	1.09	93.24	1.25	29.27	20.93	ND	4.77	62.32
E. coli K12 DUBGK	0.21	0.63	68.80	1.48	24.59	3.05	ND	7.34	52.24
E. coli BL21(DE3) DUBGK	0.32	1.26	87.30	ND	15.44	8.47	ND	6.83	44.01
E. coli B DUBGK	0.24	0.93	95.72	2.92	35.63	10.61	ND	6.13	59.76
E. coli C DUBGK	0.30	0.93	65.83	0.21	5.94	6.33	ND	6.76	33.33
E. coli DF40 DUBGK	0.22	0.60	58.90	5.92	27.83	3.60	7.18	6.40	86.23
With 25 g/L 3-HP									
E. coli W3110 DUBGK	0.27	0.81	47.10	2.10	9.31	11.09	ND	7.40	53.14
E. coli W DUBGK	0.33	0.78	57.30	2.29	14.08	12.58	ND	5.73	61.52

Results were calculated after 24 h of cell cultivation in shake flask. The standard deviation of the measurements was <10 %

ND Not detected

[a] Glycerol carbon recovered in the form of metabolites and biomass. Carbon dioxide was not included in the calculations

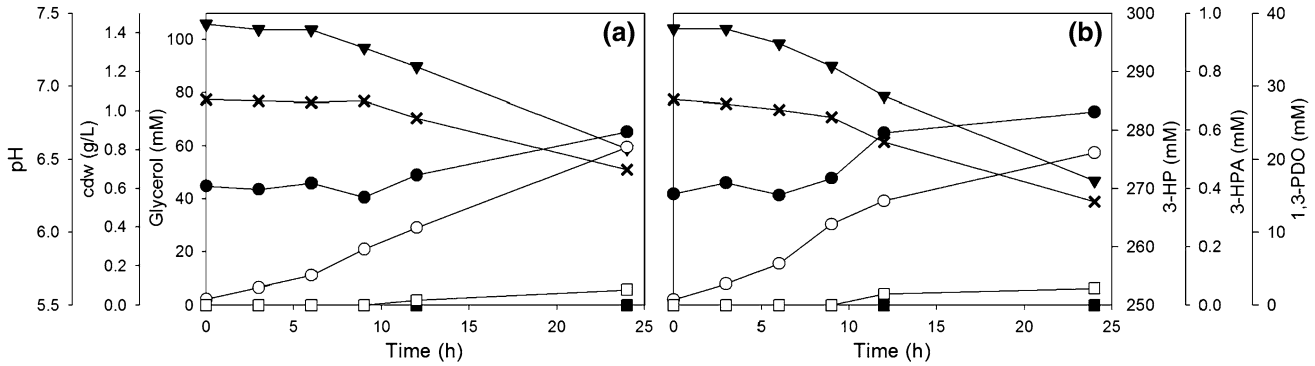

Fig. 4 Time-course profile of cell growth, glycerol consumption, production of 3-HP and 1,3-PDO from glycerol by two recombinant *E. coli* (*E. coli* W3110 and *E. coli* W) strains overexpressing DhaB, GdrAB and KGSADH in the presence of 25 g/L of 3-HP supplemented in the culture medium. 2 μM coenzyme B_{12} was added at 3, 6, 9, and 12 h of cultivation. The standard deviations of the biomass and metabolite measurements were <10 %. **a** *E. coli* W3110 and **b** *E. coli* W. Symbols: *closed circle* (3-HP), *closed triangle* (Glycerol), *open square* (1,3-PDO), *closed square* (3-HPA), *open circle* (Biomass) and *cross mark* (pH)

KGSADH were also measured for *E. coli* W (Fig. 5f). The DhaB activity was the highest at 12 h (11.62 ± 0.74 U/mg protein) and decreased continuously with time, reaching less than 0.8 U/mg protein at 37 h. KGSADH showed the highest activity at 6 h (1.4 U/mg protein), remained stable at above 0.8 U/mg protein for up to 28 h, and decreased rapidly to 0.2 U/mg protein in the next 9 h. Unlike the flask studies, W DUBGK exhibited significantly different DhaB and KGSADH activities than W3110 DUBGK. The reason for this is unclear, but the different activities might have affected the performance of both strains in the bioreactor experiments. Overall, these results suggest that among the nine *E. coli* strains tested, *E. coli* W is the most suitable strain for 3-HP production from glycerol.

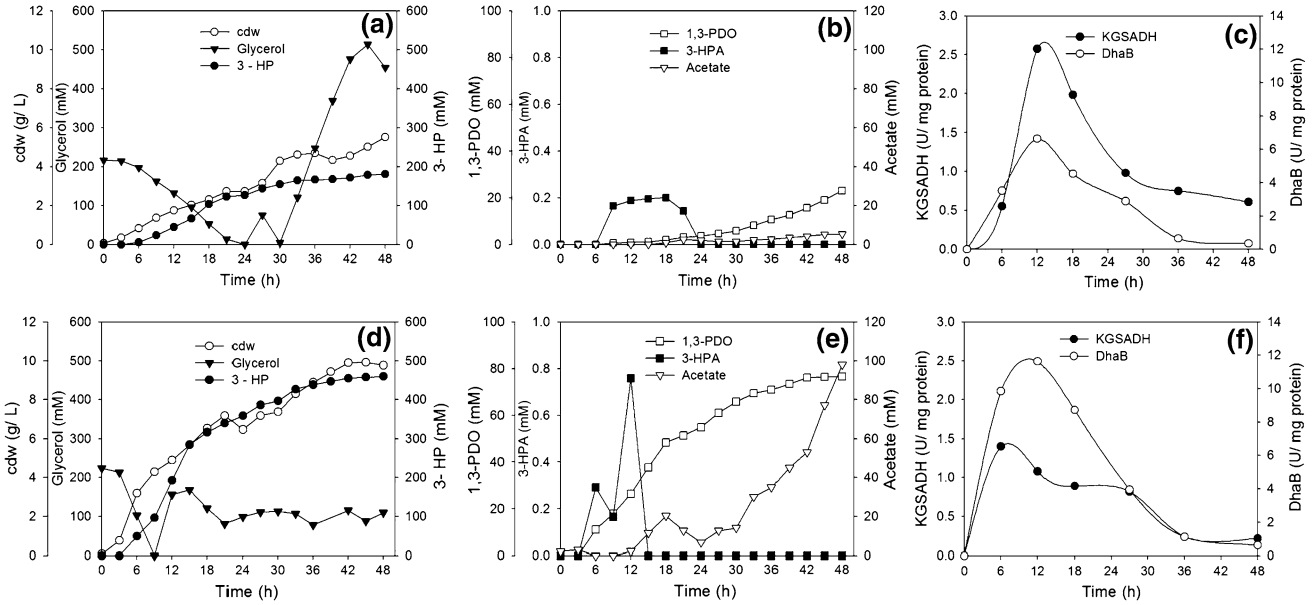

Fig. 5 Glycerol fed-batch bioreactor experiments with recombinant *E. coli* W3110 and *E. coli* W overexpressing DhaB, GdrAB and KGSADH and the time course profile of enzyme activities from its cell free extract. **a** Time course profile of cell growth, glycerol consumption and 3-HP production of *E. coli* W3110 under aerobic conditions. **b** Time course profile of 1,3-PDO, 3-HPA and acetate production of *E. coli* W3110. **c** Enzyme activity analysis of DhaB and KGSADH of *E. coli* W3110. **d** Time course profile of cell growth, glycerol consumption and 3-HP production of *E. coli* W under aerobic conditions. **e** Time course profile of 1,3-PDO, 3-HPA and acetate production of *E. coli* W. **f** Enzyme activity analysis of DhaB and KGSADH of *E. coli* W. The standard deviations of the measurements of the biomass, metabolites and enzyme activities were <10 %

Table 5 Glycerol consumption, cell growth, 3-HP and metabolite production by recombinant *E. coli* W3110 DUBGK and *E. coli* W DUBGK in the bioreactor

	E. coli W3110 DUBGK	*E. coli* W DUBGK
X_{final} (g cdw/L)	5.52	9.76
μ_{max} (h^{-1})	0.53	0.74
Glycerol consumed (mM)	672	1,465
3-HP (mM)	180.15	459.30
1,3-PDO (mM)	23.00	76.75
Acetate (mM)	5.31	97.90
3-HP molar yield	0.26	0.31
3-HP productivity (g/L h)	0.36	0.86

Results were calculated after 48 h of cell cultivation in bioreactor
The standard deviation of the measurements was <10 %

Discussion

The aim of this study was to identify a suitable 3-HP tolerant *E. coli* host that can produce high levels of 3-HP from glycerol. Nine *E. coli* strains, which have been reported to produce organic acids efficiently, were examined for cell growth, glycerol consumption, enzyme activities of DhaB and KGSADH, and 3-HP production on the flask and bioreactor scales (Table 6). When glycerol dehydratase (DhaB) and aldehyde dehydrogenase (KGSADH) were introduced, all nine strains produced 3-HP from glycerol but the level differed significantly according to the strain. When the strains were challenged with toxic 3-HP by adding 3-HP (25 g/L) in the culture medium, only two recombinant strains, *E. coli* W3110 DUBGK and W DUBGK, showed cell growth and 3-HP production. As summarized in Table 6, the evaluation and screening of *E. coli* strains in the present study, which was conducted on a flask scale, were quite efficient. An examination of the growth of the host strains in the presence of 3-HP did not eliminate many strains, but an assessment by the enzyme activities and 3-HP production, particularly in the presence of 25 g/L 3-HP, could eliminate the least promising strains. On the other hand, the difference between the two best strains, *E. coli* W and W3110, could be revealed partially by flask culture experiments in the presence of 25 g/L 3-HP and be fully appreciated only by a bioreactor experiment. In addition, this study suggests that the characteristics of the *E. coli* strains are significantly different, and the tolerance of each strain to organic acids varies considerably. Therefore, careful selection of the appropriate host strain is essential for the development of a recombinant strain in the production of organic acids, such as 3-HP.

Table 6 Summary of screening of *E. coli* strains for 3-HP production

	Growth (with 3-HP)[a]	Enzyme activity[b]	3-HP Production[c]		
			Flask culture (without 3-HP)	Flask culture (with 3-HP)	Bioreactor
E. coli W3110	○	○	○	○	x
E. coli W	○	○	○	○	○
E. coli K12	○	x	○	ND	ND
E. coli BL21 (DE3)	○	x	x	ND	ND
E. coli B	○	x	○	ND	ND
E. coli C	○	x	x	ND	ND
E. coli LE392	x	x	ND	ND	ND
E. coli Mach1	○	x	ND	ND	ND
E. coli DF40	x	x	○	ND	ND

Flask cultures (second and fifth columns) were conducted in the medium supplemented with 25 g/L 3-HP

ND Not determined

[a] (○) represents a good growth (growth rate in the presence of 3-HP is >60 % that in the absence of 3-HP), while (x) a poor growth (growth rate in the presence of 3-HP was <35 % that in the absence of 3-HP)

[b] (○) represents high and balanced activities between DhaB and KGSADH, while (x) low and imbalanced activities between the two enzymes

[c] (○) represents a high 3-HP production, while (x) a poor 3-HP production (<40 % the highest 3-HP production)

Upon exposure to high concentrations of acids, bacteria turn on the acid resistance metabolisms, which are often related to the decarboxylation of amino acids, expression of the stress-related proteins or proton pumps, etc. [30]. For different *E. coli* strains, comprehensive studies on the expression or efficiency of diverse acid tolerance responses have not been conducted. However, it has been reported that *E. coli* K12 and W3110 enhance the gene expression and/or metabolisms for ATP production, glutamate decarboxylation to γ-aminobutyric acid (GABA) and proton pump when challenged with high acid concentrations or low medium pH [26]. Similarly, the *E. coli* W and B strains were reported to overexpress the acid and stress tolerance proteins at high acid concentrations or low medium pH. It will be interesting if the better performance of *E. coli* W and W3110 can be explained based on the differential expression and/or efficiency of these acid tolerance metabolisms.

In addition to the high acid tolerance, the better performance of the recombinant *E. coli* W and W3110 in 3-HP production appears to result from the high and balanced expression of glycerol dehydratase (DhaB) and 3-HPA dehydrogenase (KGSADH) (Table 3). A lower KGSADH activity than the DhaB activity can result in the accumulation of the highly toxic aldehyde, 3-HPA, which is detrimental to cell growth and 3-HP production [21]. The unsuccessful 3-HP production in the recombinants, *E. coli* BL21(DE3), Mach1 and C, which exhibit a serious imbalance between the DhaB and KGSADH activities (Table 3), confirmed the importance of the balance of these enzyme activities. The decrease in the 3-HP production rate and glycerol consumption rate in bioreactor experiments also appears to be related to the activity of these enzymes (Fig. 5). Stable maintenance of the 3-HP pathway enzymes at the late stages should extend the period of the active production of 3-HP and improve its titer. Interestingly, acetate and 1,3-PDO are the only byproducts in a bioreactor culture with *E. coli* W, which is unlike the recombinant *E. coli* BL21(DE3) studied previously [21]. Acetate is as toxic to *E. coli* (unpublished data) as 3-HP and a decrease in acetate production should alleviate the toxic effects of the acids to some extent. On the other hand, acetate production under aerobic conditions is indicative of an overflow metabolism, which is caused by the improper regeneration of NAD^+ [7, 27]. Therefore, the continuous regeneration of NAD^+ in recombinant *E. coli* W DUBGK should be examined.

In conclusion, carboxylic acids, such as 3-HP, seriously impede the performance of the host strains as they accumulate in the culture broth. In the present study, various *E. coli* strains were evaluated and screened based on cell growth, glycerol consumption, enzyme activities of DhaB and KGSADH, and 3-HP production. Among the nine strains tested, *E. coli* W DUBGK showed the highest 3-HP production. In the glycerol fed-batch bioreactor experiment, *E. coli* W DUBGK produced a high titer (41.5 ± 1.1 g/L), molar yield (0.31 ± 0.02) and productivity (0.86 ± 0.05 g/L h). Further studies to enhance the 3-HP tolerance of the *E. coli* W strain and improve its titer and productivity are currently under way.

Acknowledgement This study was supported financially by the Korean Ministry of Education, Science, and Technology through the

Advanced Biomass R&D Center (ABC; Grant No. 2010-0029799), KAIST, Korea. The authors are grateful for the financial assistance provided by the BK21 Plus Program for Advanced Chemical Technology at Pusan National University. In addition, Dr. Ashok Somasundar is grateful for the financial support provided by "2014 Post-Doc. Development Program of Pusan National University".

References

1. Arasu MV, Kumar V, Ashok S, Song H, Rathnasingh C, Lee HJ, Seung D, Park S (2011) Isolation and characterization of the new *Klebsiella pneumoniae* J2B strain showing improved growth characteristics with reduced lipopolysaccharide formation. Biotechnol Bioprocess Eng 16:1134–1143
2. Arasu MV, Sarkar R, Sekar BS, Kumar V, Rathnasingh C, Choi J, Song H, Seung D, Park S (2013) Isolation of a novel *Pseudomonas species* SP2 producing vitamin B_{12} under aerobic condition. Biotechnol Bioprocess Eng 18(1):43–51
3. Ashok S, Mohan Raj S, Ko Y, Sankaranarayanan M, Zhou S, Kumar V, Park S (2013) Effect of *puuC* overexpression and nitrate addition on glycerol metabolism and anaerobic 3-hydroxypropionic acid production in recombinant *Klebsiella pneumoniae* Δ*glpK* Δ*dhaT*. Metab Eng 15:10–24. doi:10.1016/j.ymben.2012.09.004
4. Ashok S, Sankaranarayanan M, Ko Y, Jae KE, Ainala SK, Kumar V, Park S (2013) Production of 3-hydroxypropionic acid from glycerol by recombinant *Klebsiella pneumoniae* Δ*dhaT* Δ*yqhD* which can produce vitamin B_{12} naturally. Biotechnol Bioeng 110(2):511–524. doi:10.1002/bit.24726
5. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 72(1):248–254
6. Datta R, Tsai SP, Bonsignore P, Moon SH, Frank JR (1995) Technological and economic potential of poly (lactic acid) and lactic acid derivatives. FEMS Microbiol Rev 16(2–3):221–231
7. De Mey M, De Maeseneire S, Soetaert W, Vandamme E (2007) Minimizing acetate formation in *E. coli* fermentations. J Ind Microbiol Biotechnol 34(11):689–700
8. Dien B, Nichols N, Bothast R (2001) Recombinant *Escherichia coli* engineered for production of L-lactic acid from hexose and pentose sugars. J Ind Microbiol Biotechnol 27(4):259–264
9. Huang Y, Li Z, Shimizu K, Ye Q (2013) Co-production of 3-hydroxypropionic acid and 1,3-propanediol by *Klebseilla pneumoniae* expressing *aldH* under microaerobic conditions. Bioresour Technol 128:505–512. doi:10.1016/j.biortech.2012.10.143
10. Kirk RE, Othmer DF (1951) Encyclopedia of chemical technology, vol 6. Interscience, New York
11. Ko Y, Ashok S, Zhou S, Kumar V, Park S (2012) Aldehyde dehydrogenase activity is important to the production of 3-hydroxypropionic acid from glycerol by recombinant *K. pneumoniae*. Process Biochem 47(7):1135–1143. doi:10.1016/j.procbio.2012.04.007
12. Kumar V, Sankaranarayanan M, Durgapal M, Zhou S, Ko Y, Ashok S, Sarkar R, Park S (2013) Simultaneous production of 3-hydroxypropionic acid and 1,3-propanediol from glycerol using resting cells of the lactate dehydrogenase-deficient recombinant *K. pneumoniae* overexpressing an aldehyde dehydrogenase. Bioresour Technol 135:555–563. doi:10.1016/j.biortech.2012.11.018
13. Kumar V, Ashok S, Park S (2013) Recent advances in biological production of 3-hydroxypropionic acid. Biotechnol Adv 31(6):945–961
14. Kumar V, Sankaranarayanan M, Jae KE, Durgapal M, Ashok S, Ko Y, Sarkar R, Park S (2012) Co-production of 3-hydroxypropionic acid and 1,3-propanediol from glycerol using resting cells of recombinant *K. pneumoniae* J2B strain overexpressing aldehyde dehydrogenase. Appl Microbiol Biotechnol 96(2):373–383. doi:10.1007/s00253-012-4187-9
15. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227(5259): 680–685
16. Lynch M, Gill R, Warnecke-Lipscomb T (2011) Method for producing 3-hydroxypropionic acid and other products. WO Patent 2,011,038,364
17. Mazumdar S, Clomburg JM, Gonzalez R (2010) *Escherichia coli* strains engineered for homofermentative production of D-lactic acid from glycerol. Appl Environ Microbiol 76(13):4327–4336
18. Miller JH (1972) Experiments in molecular genetics, vol 60. Cold Spring Harbor Laboratory Cold Spring Harbor, New York
19. Raj SM, Rathnasingh C, Jo J-E, Park S (2008) Production of 3-hydroxypropionic acid from glycerol by a novel recombinant *E. coli* BL21 strain. Process Biochem 43(12):1440–1446. doi:10.1016/j.procbio.2008.04.027
20. Raj SM, Rathnasingh C, Jung W-C, Selvakumar E, Park S (2010) A novel NAD+ -dependent aldehyde dehydrogenase encoded by the *puuC* gene of *K. pneumoniae* DSM 2026 that utilizes 3-hydroxypropionaldehyde as a substrate. Biotechnol Bioprocess Eng 15(1):131–138. doi:10.1007/s12257-010-0030-2
21. Rathnasingh C, Raj SM, Jo JE, Park S (2009) Development and evaluation of efficient recombinant *E. coli* strains for the production of 3-hydroxypropionic acid from glycerol. Biotechnol Bioeng 104(4):729–739. doi:10.1002/bit.22429
22. Rathnasingh C, Raj SM, Lee Y, Catherine C, Ashok S, Park S (2012) Production of 3-hydroxypropionic acid via malonyl-CoA pathway using recombinant *E. coli* strains. J Biotechnol 157(4):633–640. doi:10.1016/j.jbiotec.2011.06.008
23. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual (3-volume set)
24. Schneider CA, Rasband WS, Eliceiri KW (2012) NIH image to ImageJ: 25 years of image analysis. Nat Methods 9(7):671–675
25. Slater SC, Voige W, Dennis D (1988) Cloning and expression in *E. coli* of the *Alcaligenes eutrophus* H16 poly-β-hydroxybutyrate biosynthetic pathway. J Bacteriol 170(10):4431–4436
26. Stincone A, Daudi N, Rahman AS, Antczak P, Henderson I, Cole J, Johnson MD, Lund P, Falciani F (2011) A systems biology approach sheds new light on *E. coli* acid resistance. Nucl Acids Res 39(17):7512–7528
27. Vemuri G, Altman E, Sangurdekar D, Khodursky A, Eiteman M (2006) Overflow metabolism in *E. coli* during steady-state growth: transcriptional regulation and effect of the redox ratio. Appl Environ Microbiol 72(5):3653–3661
28. Wang Y, Tian T, Zhao J, Wang J, Yan T, Xu L, Liu Z, Garza E, Iverson A, Manow R (2012) Homofermentative production of D-lactic acid from sucrose by a metabolically engineered *E. coli*. Biotechnol Lett 34(11):2069–2075
29. Warnecke-Lipscomb T, Lynch M, Gill R (2010) Methods, systems and compositions for increased microorganism tolerance to and production of 3-hydroxypropionic acid (3-HP). WO Patent 2,010,011,874
30. Warnecke T, Gill RT (2005) Organic acid toxicity, tolerance, and production in *E. coli* biorefining applications. Microb Cell Fact 4:25. doi:10.1186/1475-2859-4-25
31. Warnecke TE, Lynch MD, Karimpour-Fard A, Lipscomb ML, Handke P, Mills T, Ramey CJ, Hoang T, Gill RT (2010) Rapid dissection of a complex phenotype through genomic-scale mapping of fitness altering genes. Metab Eng 12(3):241–250. doi:10.1016/j.ymben.2009.12.002
32. Warnecke TE, Lynch MD, Karimpour-Fard A, Sandoval N, Gill RT (2008) A genomics approach to improve the analysis and design of strain selections. Metab Eng 10(3–4):154–165. doi:10.1016/j.ymben.2008.04.004

33. Warnecke TE, Lynch MD, Lipscomb ML, Gill RT (2012) Identification of a 21 amino acid peptide conferring 3-hydroxypropionic acid stress-tolerance to *E. coli*. Biotechnol Bioeng 109(5):1347–1352. doi:10.1002/bit.24398
34. Zhou S, Catherine C, Rathnasingh C, Somasundar A, Park S (2013) Production of 3-hydroxypropionic acid from glycerol by recombinant *P. denitrificans*. Biotechnol Bioeng 110(12):3177–3187. doi:10.1002/bit.24980
35. Zhou S, Causey T, Hasona A, Shanmugam K, Ingram L (2003) Production of optically pure D-lactic acid in mineral salts medium by metabolically engineered *E. coli* W3110. Appl Environ Microbiol 69(1):399–407