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Enhanced co-production of hydrogen and poly-(*R*)-3-hydroxybutyrate by recombinant PHB producing *E. coli* over-expressing hydrogenase 3 and acetyl-CoA synthetase

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ABSTRACT

Recombinant *Escherichia coli* was constructed for co-production of hydrogen and polyhydroxybutyrate (PHB) due to its rapid growth and convenience of genetic manipulation. In particular, anaerobic metabolic pathways dedicated to co-production of hydrogen and PHB were established due to the advantages of directing fluxes away from toxic compounds such as formate and acetate to useful products. Here, recombinant *E. coli* expressing hydrogenase 3 and/or acetyl-CoA synthetase showed improved PHB and hydrogen production when grown with or without acetate as a carbon source. When hydrogenase 3 was over-expressed, hydrogen yield was increased from 14 to 153 mmol H_2/mol glucose in a mineral salt (MS) medium with glucose as carbon source, accompanied by an increased PHB yield from 0.55 to 5.34 mg PHB/g glucose in MS medium with glucose and acetate as carbon source.

1. Introduction

Hydrogen, as a renewable, efficient, and clean secondary energy source, has attracted increasing attentions (Maeda et al., 2011; Oh et al., 2011; Zhang, 2011). Biological hydrogen production is becoming important due to its flexible and eco-friendly nature compared with thermochemical methods (Das and Veziroglub, 2008). *Escherichia coli*, a well-characterized microorganism with a set of readily available tools for genetic manipulation and physiological regulation, has shown its potential and advantageous for fermentative hydrogen production (Clomburg and Gonzalez, 2010; Hallenbeck, 2005). Metabolic engineering has been used to enhance hydrogen production using more effective genes and deleting competing pathways in *E. coli* (Maeda et al., 2008a; Yoshida et al., 2006; Yoshida et al., 2007).

An optimized, engineered hydrogen producing *E. coli* was reported to produce extracellular acetate and ethanol as main by-products converted from acetyl-CoA (Yoshida et al., 2006). The accumulation of a high acetate concentration in microbial cultures is toxic for cell growth, as it decouples transmembrane pH gradients and negatively affects internal osmotic pressure, intracellular pH and amino acid synthesis (Lin et al., 2006). However, acetate could be utilized as a carbon source from secreted acetate or exogenous acetate by acetyl-CoA synthetase (AcsA) form acetyl-CoA (Lin et al., 2006).

Poly-(*R*)-3-hydroxybutyric acid (PHB) is produced from acetyl-CoA by many bacteria under different culture conditions, as intracellular reserved compounds for carbon and energy as well as resistance to adverse growth conditions (Anderson and Dawes, 1990; Chen, 2009; Chen and Patel, 2012; Hazer and Steinbüchel, 2007). Recombinant *E. coli* has been used for PHB production and PHB flux studies (Fidler and Dennis, 1992; Gao et al., 2011; Lee and Choi, 2001; Theodorou et al., 2012; Tyo et al., 2010). Effects of dissolved oxygen concentration on the growth of *E. coli* have been discussed (van Wegen et al., 2001; Wang and Lee, 1997). PHB accumulation was reported under anaerobic conditions as high NADH/NAD ratios favored a high NADPH/NADP ratio which promotes PHB production (Carlson et al., 2005). Metabolic flux in anaerobic *E. coli* shows that acetyl-CoA as the PHB substrate is

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produced in conjunction with hydrogen production via pyruvate formate lyase (PFL) activity (Maeda et al., 2011). It can be hypothesized that hydrogen production stimulates more conversion of pyruvate to acetyl-CoA, resulting in enhanced PHB accumulation. It was reported that acetate could be used as a carbon source (Lin et al., 2006), which benefited PHB accumulation accompanied by reduced extracellular acetate formation (Carlson et al., 2005). Acetate used partially as a carbon source converted to acetyl-CoA is theoretically ATP supply adequate (Carlson et al., 2005; Hasona et al., 2004; Wang et al., 2010), as one glucose yields 3 ATP including 2 ATP from converting glucose to pyruvate and another one from substrate-level phosphorylation produced from two molecules of pyruvate converted to one acetate and ethanol (Hasona et al., 2004). Therefore, co-production of hydrogen and PHB should be beneficial for cell growth.

Hydrogen is formed from the reversible conversion of two protons and two electrons catalysed by hydrogenase 3 (Hyd 3) (Bagramyan and Trchounian, 2003), which is one part of formate hydrogen lyase (FHL) complex. Heterologous expressions of a variety of hydrogenases from different microbial strains in *E. coli* facilitated hydrogen productivity (Böck et al., 2006; Eitinger et al., 2005). However, most of the hydrogenases consist of several genes, e.g., *E. coli* native hydrogenase 3 is encoded by an operon of 8 kb in size termed as *hycABCDEFGHI* (*hyc* operon). It is difficult to PCR amplify such a large operon. So far, only the large subunit of Hyd 3, namely, HycE, was over-expressed, leading to 2.8-fold increase in hydrogen production (Yoshida et al., 2005). In addition to the *hycE* gene, all the genes in the *hyc* operon (except *hycA* which is a repressor of FHL (Sauter et al., 1992)) provide full Hyd 3 activity. For example, *hycG* (encoding small subunit of Hyd 3) (Sauter et al., 1992) and *hycF* (encoding iron-sulfur proteins of Hyd 3) function to express as intermediate electron carrier proteins within the FHL complex. Moreover, HycC and HycD are membrane proteins performing as electron transfer proteins (Sauter et al., 1992). The expression of the *hycBCDEFGHI* operon could result in stronger hydrogen production. Simple genetic manipulation method instead of the traditional time-consuming step-by-step cloning methods is needed to obtain the large operon for genetic engineering.

In the present study, a recombinant *E. coli* capable of co-producing hydrogen and PHB was constructed either by over-expression of the *hycBCDEFGHI* operon or by overexpression of acetyl-CoA synthetase for utilization of acetate as a carbon source. PHB and hydrogen production by this recombinant *E. coli* strain was studied. We aimed to exploit the metabolic correlation of producing PHB and hydrogen under anaerobic condition and possibilities of positively coping with acetate as carbon source in *E. coli* fermentations.

Table 1
Bacterial strains and plasmids used in this study.

Stains/plasmids	Description	Reference/source
E. coli EC100D *pir*-116	F$^-$ *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80d*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ$^-$ *rps*L *nup*G *pir*-116(DHFR)	(Metcalf et al., 1994)
E. coli MG1655	F$^-$ λ$^-$ *ilv*G$^-$ *rfb*-50 *rph*-1 wild type	(Sauer et al., 2004)
E. coli JM109	*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 Δ(*lac-pro*AB)/F′[*tra*D36 *pro*AB+*lac*Iq *lac*ZΔM15]	TaKaRa Bio Inc
E. coli BW25113	*lac*Iq *rrn*BT14 Δ*lac*ZWJ16 *hsd*R514 Δ*ara*BADAH33 Δ*rha*BADLD78	(Baba et al., 2006)
E. coli BW25113PHB	*E. coli* BW25113 harboring plasmid pBHR68 Amp^R	This study
E. coli BW25113hyd3	*E. coli* BW25113 *hydrogenase 3* gene cluster integrated into genome $attB_{HK022}$ site	This study
E. coli BWRY8	*E. coli* BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*fdnG* Δ*ldhA* Δ*frdC* Δ*poxB* without Kan^R	(Maeda et al., 2007)
E. coli BWRY8PHB	*E. coli* BWRY8 harboring pBHR68 Amp^R	This study
E. coli BWRY8PHBacs	*E. coli* BWRY8PHB harboring pMCSPRACS Amp^R, Cm^R	This study
E. coli BWRY8hyd3	*E. coli* BWRY8 with *hydrogenase 3* gene cluster integrated into genome $attB_{HK022}$ site Kan^R	This study
E. coli BWRY8hyd3PHB	*E. coli* BWRY8hyd3 harboring pBHR68 Kan^R, Amp^R	This study
E. coli BWRY8hyd3PHBacs	*E. coli* BWRY8hyd3PHB harboring pMCSPRACS Kan^R, Amp^R and Cm^R	This study
pMD18-T	Cloning vector, Amp^R	TaKaRa Bio Inc
pCP20	FLP recombinase helper plasmid, *ts-rep*, Amp^R, Cm^R	(Datsenko and Wanner, 2000)
pBHR68	*phaCAB* expression plasmid, Amp^R	(Spiekermann et al., 1999)
pLZZH13	Expression plasmid, pBBR1MCS-2 derivative, Kan^R	(Li et al., 2009)
pET200PR	*PR* expression plasmid, pET200 derivative, Kan^R	(Walter et al., 2007)
pET200REPR	*RE* promoter inserted into pET200PR, Kan^R	This study
pMCSPRACS	*RE-PR-acsA* inserted into pBBR1MCS-1, Cm^R	This study
pAH70	Integration vector with $attP_{HK022}$, Kan^R	(Haldimann and Wanner, 2001)
pAHhyd3	pAH70 derivative containing hyd3 gene from *E. coli* MG1655, Kan^R	This study
pAH69	Helper plasmid expressing phage HK022 *Int,* Amp^R	(Haldimann and Wanner, 2001)
pINT-ts	Helper plasmid harboring phage λ *Int,* Amp^R	(Haldimann and Wanner, 2001)
pCAH63	Integration vector containing $attP_{\lambda}$ and Cm^R	(Haldimann and Wanner, 2001)

3. Results

3.1. Cloning of hydrogenase 3 gene cluster and its integration into the E. coli genome

A cloning method based on homologous recombination and plasmid rescue was developed for extracting the large gene fragment *hycBCDEFGHI* operon encoding hydrogenase 3 from *E. coli*. In summary: a plasmid harboring an identical gene fragment to a specific site of a genome was integrated into the genome by the homologous recombination. Through genome extraction and digestion by a pre-designed restriction enzyme, the plasmid harboring the specific gene fragment was rescued (Fig. 1A).

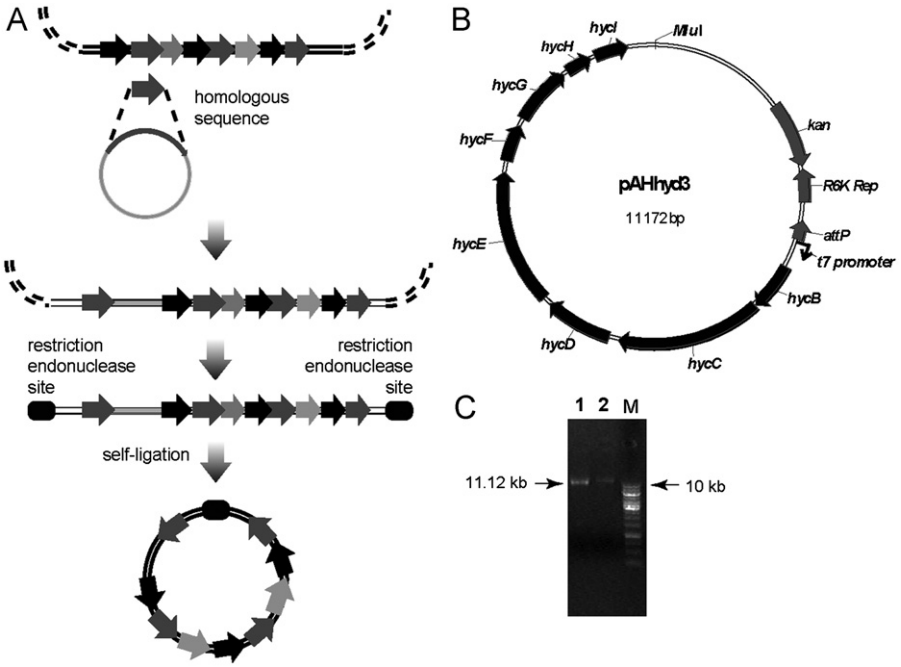

Fig. 1. Method developed for cloning a large gene cluster from a bacterial genome and the structure of plasmid pAHhyd3. (A) A large gene cluster cloning strategy based on homologous recombination and plasmid rescue. (More details see materials and methods.) (B) structure of the extracted plasmid pAHhyd3 harboring *hycBCDEFGHI* operon, (C) the extracted pAHhyd3 plasmid analysed by gel electrophoresis in 1% agarose (Left two lanes; 10 kb standard in right lane).

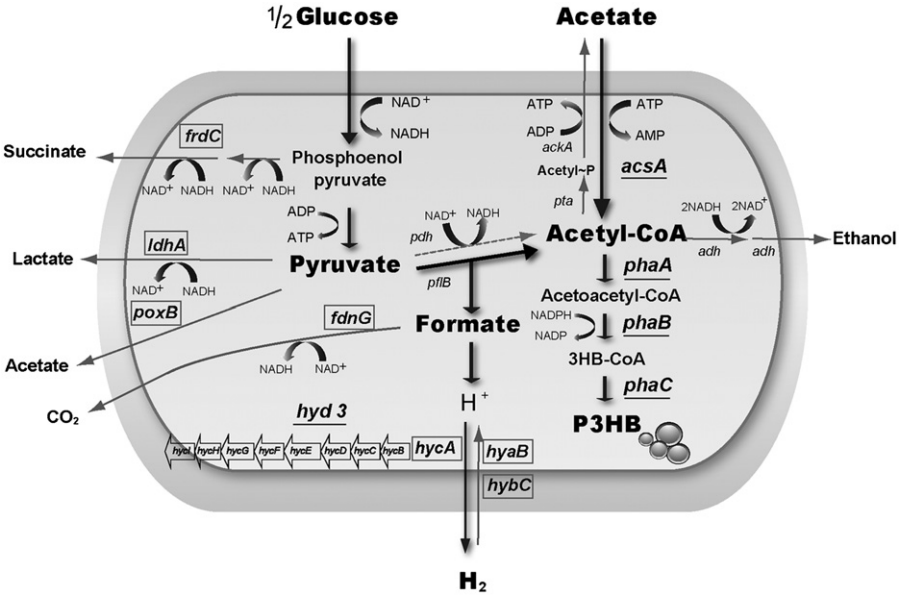

Fig. 2. Synthetic pathways for fermentative hydrogen and PHB production by *E. coli*. Glucose is metabolized to form phosphoenolpyruvate, pyruvate, formate and acetyl-CoA. Phosphoenolpyruvate is converted to succinate by fumarate reductase (*frdC*), and pyruvate is converted to either lactate by lactate dehydrogenase (*ldhA*), to acetate by pyruvate oxidase (*poxB*), or to formate and acetyl-CoA by pyruvate formate lyase (*pflB*). Formate is metabolized by formate dehydrogenase-*N* (*fdnG*). Acetate is used to form acetyl-CoA by acetyl-CoA synthetase (*acsA*). Hydrogen is produced from formate catalysed by a formate hydrogen lyase (FHL) system consisting of hydrogenase 3 (*hyd 3*). FHL is repressed by *hycA*. Hydrogen is consumed through the hydrogen uptake activity of hydrogenase 1 (*hyaB*) and hydrogenase 2 (*hybC*). PHB is accumulated from acetyl-CoA by joint actions of PHA synthase, beta-ketothiolase, and acetoacetyl-CoA reductase encoded by *phbC*, *phbA* and *phbB*, respectively. ATP and cofactor generated and consumed reactions were listed. Genes deleted in this study are shown in a frame and genes overexpressed are shown underlined.

Plasmid pAHhyc300 harboring a gene fragment *hyc300* homologous to a sequence between *hycA* and *hycB* was constructed and transformed into a wild type *E. coli* MG1655. Homologous recombination occurred, allowing genome integration upstream of the *hyc* gene cluster. Simultenuously, *hycA*, the inhibitor of FHL, was deleted. *T7* promoter was introduced to enhance the expression of *hycBCDEFGHI* operon. The kanamycin gene from pAHhyc300 integrated in the genome was used to screen for positive recombinants. Since the whole genome sequence of *E. coli* MG1655 has been well known, we were able to choose *Mlu*I for genome digestion. After genome extraction, *Mlu*I endonuclease digestion and self-ligation, DNA fragments were transformed into *E. coli* EC100D. Both PCR analysis and sequencing confirmed the success of cloning the 8 kb *hycBCDEFGHI* operon with a *T7* promoter upstream in plasmid pAHhyd3 (Fig. 1B and C). Site-specific recombination catalysed by Int_{HKO22} integrase inserted pAHhyd3 into the $attB_{HK022}$ site in *E. coli* genome (Haldimann and Wanner, 2001). The *T7* promoter was designed to strengthen the expression of *hycBCDEFGHI* operon as *T7* RNA polymerase was integrated into $attB_{\lambda}$ site in the genome of *E. coli* BWRY8 catalysed by Int_{λ} integrase (Section 2.2).

3.2. Construction of metabolic pathways in E. coli for co-production of hydrogen and PHB

Glucose is converted into organic acids such as lactate, acetate, succinate, formate and ethanol under anaerobic or fermentative respiration (Fig. 2). To enhance hydrogen production, it is important to increase the conversion of carbon sources to formate. This has been achieved by knocking out other glucose metabolic pathways (Maeda et al., 2007). Strain *E. coli* BWRY8 is a mutant of *E. coli* BW25113 (Δ*hyaB* Δ*hybC* Δ*hycA* Δ*fdnG* Δ*frdC* Δ*ldhA* Δ*poxB*) defective in large subunits of hydrogenase 1 and hydrogenase 2, repressor of FHL, α-subunit of formate dehydrogenase-*N*, lactate dehydrogenase, fumarate reductase, and pyruvate oxidase (Fig. 2). In *E. coli* BWRY8, metabolic flux from glucose to the pyruvate-formate pathway was increased by deletion of the branched pathways to form more formate and acetyl-CoA (Fig. 2). Formate formed hydrogen catalysed by formate hydrogen lyase (FHL) complex consisting of formate dehydrogenase-*H* (Axley et al., 1990) and hydrogenase 3 encoded by *hycABCDEFGHI* (Bagramyan and Trchounian, 2003) (Fig. 2). On the other hand, the PHB synthesis pathway was expressed by PHB synthase (PhbC), beta-ketothiolase (PhbA), and acetoacetyl-CoA reductase (PhbB) encoded by *phbC*, *phbA*, and *phbB*. Acetate as a carbon source could be used to form acetyl-CoA by acetyl-CoA synthetase (AcsA) (Lin et al., 2006).

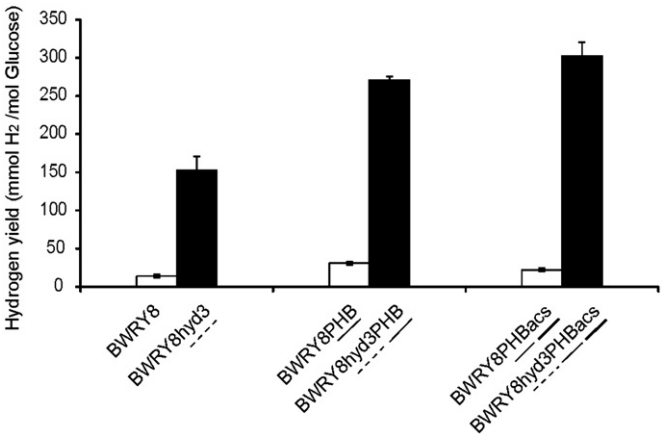

Fig. 3. Hydrogen production yield by various recombinants *E. coli* with or without hydrogenase 3 (Hyd 3). Bacteria were cultured for 24 h in 50 mL mineral salt medium supplemented with 20 g/L glucose under 37 °C and a rotation rate of 200 rpm in 125 mL anaerobic bottles. Data were expressed as average values and standard deviations (SD) of three parallel studies. BWRY8: *E. coli* BW25113 (Δ*hyaB* Δ*hybC* Δ*hycA* Δ*fdnG* Δ*frdC* Δ*ldhA* Δ*poxB*); BWRY8hyd3: Gene cluster *hycBCDEFGHI* was integrated into *E. coli* BWRY8 genome; BWRY8PHB: *E. coli* BWRY8 harboring pBHR68 for PHB accumulation; BWRY8hyd3PHB: *hycBCDEFGHI* was integrated into *E. coli* BWRY8PHB genome; BWRY8PHBacs: gene *acsA* expressed in *E. coli* BWRY8PHB; BWRY8hyd3PHBacs: *hycBCDEFGHI* was integrated into BWRY8PHBacs genome.

3.3. Overexpression of hydrogenase 3 enhances hydrogen production

The *hycBCDEFGHI* operon was cloned as stated above and integrated into *E. coli* BWRY8 genome. Hydrogen production yield by *E. coli* BWRY8hyd3 was improved to 153 mmol H_2/mol glucose in a mineral salt (MS) medium with glucose as carbon source compared with 14 mmol H_2/mol glucose in *E. coli* BWRY8 (Fig. 3). This enhanced hydrogen production phenomenon was also observed in PHB production strains *E. coli* BWRY8PHB and BWRY8hyd3PHB. *E. coli* BWRY8hyd3PHBacs overexpressing acetyl-CoA synthetase, resulted in 13-folds enhanced hydrogen production compared with *E. coli* BWRY8PHBacs. (Fig. 3) The isopropyl-β-D-thiogalactopyranoside (IPTG) induction for *T7* RNA polymerase expression improved hydrogen production further. However, the enhancement through T7 promoter was not significant, which agreed with our previous results (unpublished data). Interestingly, the presence of 10 mM sodium formate as a substrate for hydrogen production led to a marginal increase in the formation of hydrogen (data not shown).

The overexpression of *hycBCDEFGHI* operon in *E. coli* genome increased hydrogen production. Simultaneously, formate excretion was decreased (Table 3). The consumption of glucose was increased as a result of overexpression of *hycBCDEFGHI* operon. At the end, cell metabolism and growth were facilitated together with increased hydrogen formation (Table 3).

3.4. Effect of PHB synthesis and acetate utilization on hydrogen production

Pyruvate is degraded to form formate and acetyl-CoA which functions as a precursor for PHB, while formate is the precursor

Table 3
Cell growth, glucose consumption and by-products formation by all strains studied for hydrogen production.

E. coli strains	CDW (g/L)	Glucose consumed (g/L)	Acetate (g/L)	Ethanol (g/L)	Formate (g/L)
BWRY8	0.61 ± 0.03	5.04 ± 0.06	0.34 ± 0.01	0.32 ± 0.03	0.20 ± 0.01
BWRY8hyd3	0.60 ± 0.03	5.52 ± 0.32	0.40 ± 0.02	0.37 ± 0.02	0.14 ± 0.00
BWRY8PHB	0.54 ± 0.02	4.82 ± 0.40	0.34 ± 0.03	0.35 ± 0.01	0.19 ± 0.03
BWRY8hyd3PHB	0.61 ± 0.02	6.01 ± 0.26	0.44 ± 0.01	0.37 ± 0.03	0.10 ± 0.01
BWRY8PHBacs	0.57 ± 0.03	5.47 ± 0.07	0.29 ± 0.01	0.53 ± 0.04	0.21 ± 0.00
BWRYhyd3PHBacs	0.55 ± 0.04	6.20 ± 0.04	0.43 ± 0.02	0.65 ± 0.02	0.08 ± 0.01

Bacteria were cultured for 24 h in mineral salt medium supplemented with 20 g/L glucose at 37 °C at a rotation rate of 200 rpm under anaerobic conditions; data were expressed as average values and standard deviations (SD) of three parallel studies.

for hydrogen production (Fig. 2). Therefore, formation of acetyl-CoA is positively associated with hydrogen production. Hydrogen production in PHB synthesis strains was increased to almost double the amount of these without PHB formation (Fig. 3). It was obvious that consumption of acetyl-CoA by PHB synthesis accelerates pyruvate degradation, leading to more formate and thus more hydrogen production. With acetyl-CoA synthetase (AcsA) expressed, further improvement hydrogen production was observed although the positive change was small (Fig. 3). In this case, AcsA promoted acetate consumption that benefited formate and hydrogen synthesis (Lin et al., 2006).

3.5. Enhancement of PHB accumulation by Hyd 3

A high cell redox level under anaerobic condition favors PHB production as the one of the PHB synthesis enzymes β-ketothiolase (PhbA) consumes reduction equivalents (Carlson et al., 2005). A two-step shake flask cultivation process was used to test PHB production in strains capable of accumulating PHB. Not obvious PHB accumulation was observed in *E. coli* strains during the first 48 h of growth in LB medium without glucose as a carbon source. When glucose was added as a carbon source during the second step of the culture process, overexpression of *hycBCDEFGHI* operon resulted in about 2 and 3-fold increase in PHB production by *E. coli* BW25113hyd3PHB and BWRY8-hyd3PHB, respectively (Fig. 4).

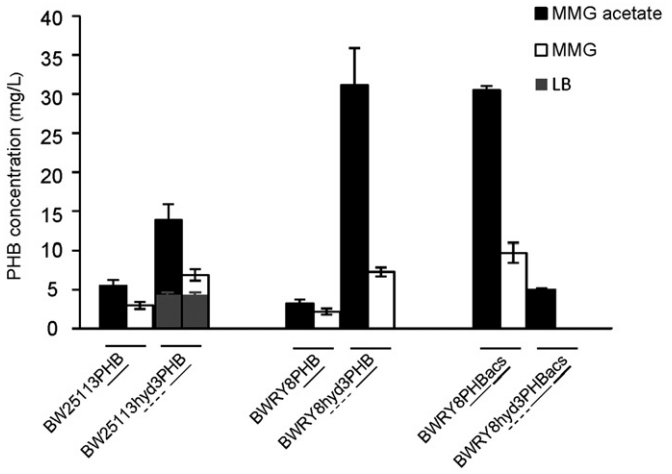

Fig. 4. PHB accumulation by *E. coli* BW25113, BWRY8 and *acsA* overexpressed strains grown in shake flasks, respectively. Bacterial strains were cultured for 48 h in LB medium under aerobic condition, followed by anaerobic cultivation in mineral salt medium supplemented with 20 g/L glucose and with or without 2 g/L acetate for another 48 h under 37 °C at a rotation rate of 200 rpm. Data were expressed as average values and standard deviations (SD) of three parallel studies. BW25113PHB: *E. coli* BW25113 harboring pBHR68 for PHB production; BW25113hyd3PHB: *hycBCDEFGHI* was integrated into *E. coli* BW25113PHB genome; BWRY8PHB: *E. coli* BWRY8 harboring pBHR68 for PHB production; BWRY8hyd3PHB: *hycBCDEFGHI* was integrated into *E. coli* BWRY8PHB genome; BWRY8PHBacs: *acsA* was expressed in *E. coli* BWRY8PHB; BWRY8hyd3PHBacs: *hycBCDEFGHI* was integrated into *E. coli* BWRY8PHBacs genome.

Overexpression of *hycBCDEFGHI* operon in PHB accumulation strains *E. coli* BWRY8hyd3PHB reduced acetate production (Table 4), as more acetyl-CoA was channelled into PHB production pathway.

3.6. The use of acetate as carbon source for enhanced PHB synthesis by E. coli harboring flux by AcsA

The addition of acetate to the culture increased PHB production (Fig. 4), as acetate could be a precursor for PHB. *E. coli* strain BW25113PHB and BW25113hyd3PHB doubled their PHB production when acetate was added to their culture medium compared to growth without acetate (Fig. 4). While PHB production was enhanced by almost 3.3-fold by *E. coli* BWRY8hyd3PHB in acetate culture medium compared with non-acetate medium. *E. coli* BWRY8hyd3PHB showed a 9.5-fold increase in PHB production compared with the amount by *E. coli* BWRY8PHB (Fig. 4). In *E. coli* BWRY8PHBacs, the PHB production was increased by 9.4-fold compared with *E. coli* BWRY8PHB which dedicated acetate used as carbon source for enhanced PHB accumulation. *E. coli* BWRY8-hyd3PHB and BWRY8PHBacs demonstrated the highest PHB accumulation among all strains studied (Fig. 4). The reaction of converting acetate to acetyl-CoA needs to consume one ATP, leading to poor growth of *E. coli* expressing AcsA (Table 5). It was surprising to find that co-expression of Hyd 3 and acetyl-CoA synthetase repressed PHB synthesis, and the mechanisms responsible for this phenomenon remained unclear (Fig. 4).

In the acetate containing medium, acetate was consumed by all *E. coli* BW25113 derived strains, whereas acetate accumulation was observed more active than assimilation by *E. coli* BWRY8 derived strains (Table 5). Obviously, deletions of the *ldhA* and *frdC* encoding lactate dehydrogenase and fumarate reductase increased acetate metabolic flux. At the same time, more ethanol was found accumulated by *E. coli* BWRY8PHBacs grown in acetate containing medium (Tables 4 and 5).

4. Discussion

Although a discussion was made linking PHB and hydrogen production (Carlson et al., 2005), no result was reported on co-production of PHB and hydrogen. For the first time, it was found that productions of PHB and hydrogen were mutually beneficial at least in the case of *E. coli* (Figs. 3 and 4).

E. coli BW25113 (Δ*hyaB* Δ*hybC* Δ*hycA* Δ*fdnG* Δ*frdC* Δ*ldhA* Δ*poxB*) is a metabolically engineered strain capable of production more hydrogen than wild type *E. coli* BW25113 does (Maeda et al., 2007). The strain produces significant amount of hydrogen even without the overexpression of hydrogenase 3 (Hyd3), a subunit of

Table 4
Cell growth, glucose consumption, PHB yield and by-products formation by all strains studied for PHB production in glucose mineral salt media.

E. coli strains	CDW (g/L)	Glucose consumed (g/L)	PHB yield (mg/g glucose)	Acetate (g/L)	Ethanol (g/L)	Formate (g/L)
BWRY8	0.98 ± 0.04	4.15 ± 0.07	ND	0.57 ± 0.02	0.59 ± 0.12	ND
BWRY8hyd3	1.01 ± 0.00	4.31 ± 0.01	ND	0.88 ± 0.26	0.85 ± 0.19	ND
BWRY8PHB	0.93 ± 0.06	5.18 ± 0.08	0.43 ± 0.08	0.84 ± 0.18	0.73 ± 0.13	ND
BWRY8hyd3PHB	1.00 ± 0.02	5.45 ± 1.00	1.34 ± 0.07	0.48 ± 0.02	0.63 ± 0.00	ND
BWRY8PHBacs	0.81 ± 0.01	3.21 ± 0.09	3.03 ± 0.46	0.36 ± 0.02	0.50 ± 0.09	0.25 ± 0.04
BWRY8hyd3PHBacs	0.90 ± 0.05	2.67 ± 0.04	ND	0.31 ± 0.05	0.76 ± 0.02	0.22 ± 0.03

Bacteria were cultured for 48 h in LB medium, then cultured in mineral salt medium supplemented with 20 g/L glucose for another 48 h under 37 °C at a rotation rate of 200 rpm under anaerobic condition; data were expressed as average values and standard deviations (SD) of three parallel studies.

Table 5
Cell growth, glucose consumption, PHB yield and by-products formation by all strains for PHB production in glucose mineral salt media supplemented with 2 g/L acetate.

E. coli strains	CDW (g/L)	Glucose consumed (g/L)	PHB yield (mg/g glucose)	Acetate consumed (g/L)	Ethanol (g/L)	Formate (g/L)
BW25113	1.00 ± 0.00	5.62 ± 0.23	ND	0.23 ± 0.03	0.21 ± 0.01	ND
BW25113hyd3	1.11 ± 0.01	5.91 ± 0.04	ND	0.07 ± 0.01	0.38 ± 0.01	ND
BW25113PHB	1.02 ± 0.04	5.02 ± 0.19	1.10 ± 0.11	0.15 ± 0.00	0.23 ± 0.03	ND
BW25113hyd3PHB	1.06 ± 0.03	4.95 ± 0.11	2.80 ± 0.46	0.02 ± 0.00	0.27 ± 0.05	0.09 ± 0.05
BWRY8	1.13 ± 0.02	7.79 ± 0.04	ND	−1.14 ± 0.01	1.31 ± 0.15	ND
BWRY8hyd3	1.23 ± 0.10	8.15 ± 0.11	ND	−1.44 ± 0.04	1.55 ± 0.08	ND
BWRY8PHB	1.00 ± 0.02	6.00 ± 0.34	0.55 ± 0.10	−0.84 ± 0.05	1.29 ± 0.04	0.08 ± 0.01
BWRY8hyd3PHB	1.13 ± 0.03	5.81 ± 0.23	5.34 ± 0.67	−0.68 ± 0.09	1.19 ± 0.15	0.07 ± 0.01
BWRY8PHBacs	0.89 ± 0.03	4.75 ± 0.26	6.42 ± 0.13	−0.15 ± 0.10	0.57 ± 0.00	0.45 ± 0.02
BWRYhyd3PHBacs	0.95 ± 0.01	4.76 ± 0.06	1.05 ± 0.05	−0.45 ± 0.02	1.09 ± 0.04	0.55 ± 0.04

Bacteria were cultured for 48 h in LB medium, then cultured in mineral salt medium supplemented with 20 g/L glucose and 2 g/L acetate for another 48 h under 37 °C at a rotation rate of 200 rpm under anaerobic condition; data were expressed as average values and standard deviations (SD) of three parallel studies. Negative acetate consumption values indicate more acetate produced than consumed.

formate dehydrogen lyase, which overexpression contributes to enhanced hydrogen production from formic acid (Yoshida et al., 2005). Hyd 3 encoded by *hycABCDEFGHI* operon consists of 9 genes. Except *hycA,* all genes encode their specific functional enzymes to catalyse formation of hydrogen from $2H^+$ and $2e^-$ (Maeda et al., 2008b). Cloning, molecular manipulation and overexpression of the whole *hycBCDEFGHI* operon should have important consequence for enhancing hydrogen production. Due to the large size of the operon, the cloning of the whole operon using traditional cloning methods is a challenge. New cloning technologies including sequence dependent or independent ones have continuously been invented and evolved to meet the challenge (Quan and Tian, 2009). Most of these methods are more suitable for gene assembly, which assemble or ligate smaller DNA fragments cloned from larger DNA fragments or gene clusters one by one (Ellis et al., 2011). The gene assembling methods are both labour intense and not cost efficient. To overcome these difficulties, a method of homologous recombination combined with plasmid rescue was developed (Fig. 1), allowing the successful cloning of the entire *hydrogenase 3* (*hycBCDEFGHI*) operon. Subsequently, *T7* promoter was inserted into the final *hycBCDEFGHI* plasmid and was integrated into the *E. coli* genome. The one step method for cloning large DNA fragments such as the 8 kb *hycBCDEFGHI* operon meets new demands in the rapidly developing synthetic biology field, which requires more accurate, efficient and convenient cloning and assembling technologies in order to quickly obtain multiple steps metabolic pathways.

When grown on glucose under anaerobic conditions, *E. coli* produces mixed acids including lactate, acetate, and formate as well as ethanol. Formate is the precursor for hydrogen (Fig. 2). The hydrogen production pathway can be strengthened via manipulating the fermentation pathways (Maeda et al., 2007). For example, acetate and formate are by-products from pyruvate, while acetate can be converted into acetyl-CoA (or vice versa) and be utilized as a carbon source (Lin et al., 2006), or be used as a precursor of PHB synthesis (Fig. 2). Therefore, PHB production in the absence of oxygen should have a significant effect on formations of extracellular by-products, co-production of hydrogen and PHB is beneficial for the cells as they avoid accumulation of toxic products such as formate, acetyl-CoA and acetate (Wlaschin et al., 2006).

Overexpression of *hycBCDEFGHI* operon results in strengthened metabolic flux from formate to hydrogen (Fig. 3), rapid consumption of formate should lead to increasing activation of PFL that catalyses pyruvate degradation to formate and accumulated more acetate and ethanol (Table 3). Simultaneously, more acetyl-CoA is available for the PHB synthesis pathway. As a result, enhanced production of hydrogen improved PHB production (Fig. 4). PHB production consumes acetyl-CoA from pyruvate, which strengthens carbon flux to formate and then to hydrogen production (Fig. 3). Meanwhile, the PHB synthesis pathway consumes NADPH, which is a redox sink for cell metabolic balance, and which also helps improve hydrogen production (Carlson et al., 2005). It is therefore suggested that co-production of bioenergy hydrogen and biomaterial PHB (or PHA) is an economic attractive option compared with hydrogen or PHA production only processes provided that PHA content can be increased in the recombinant.

When *E. coli* BWRY8 was cultured in LB medium under aerobic condition, PHB production was not observable compared with wild type *E. coli* BW25113, this was attributed to defects of seven genes *hyaB hybC hycA fdnG frdC ldhA poxB* in *E. coli* BWRY8 which affected PHB accumulation (Fig. 4). After testing different deletions of these seven genes, it was found that deletion of *fdnG* gene encoding formate dehydrogenase-*N*, a major enzyme of *Escherichia coli* nitrate respiration (Berg and Stewart, 1990), was the negative factor significantly reducing PHB production by *E. coli* BWRY8 (Δ*hyaB* Δ*hybC* Δ*hycA* Δ*fdnG* Δ*frdC* Δ*ldhA* Δ*poxB*) containing *phbCAB* (unpublished data).

Acetate can be utilized by bacteria as a carbon source either from secreted acetate or exogenous acetate by overexpression of acetyl-CoA sythetase (AcsA) (Lin et al., 2006). Hydrogen production achieved the highest level by *E. coli* BWRY8hyd3PHBacs, indicating that overexpression of acetyl-CoA synthetase is positive for the utilization of acetate during *E. coli* fermentative hydrogen production. When the PHB pathway was induced in cells, acetate production was decreased, as acetate can be used as part of carbon source for PHB production (Tables 4 and 5).

5. Conclusion

Recombinant PHB producing *E. coli* over-expressing entire *hydrogenase 3* (*hycBCDEFGHI*) operon in the genome was found to improve both hydrogen and PHB production as formation of both products avoided the accumulation of toxic compounds formate and acetate. When acetyl-CoA synthetase (AcsA) was also over-expressed, production of both hydrogen and PHB were further improved in the presence of acetate as a carbon source. Co-production of hydrogen and PHB under anaerobic conditions could be an attractive and economic option.

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