Appl Microbiol Biotechnol (2009) 82:703–712
DOI 10.1007/s00253-008-1816-4

Effect of anaerobic promoters on the microaerobic production of polyhydroxybutyrate (PHB) in recombinant *Escherichia coli*

Xiao-Xing Wei · Zhen-Yu Shi · Mei-Qing Yuan · Guo-Qiang Chen

Received: 23 October 2008 / Revised: 5 December 2008 / Accepted: 7 December 2008 / Published online: 24 December 2008

Abstract Nine anaerobic promoters were cloned and constructed upstream of PHB synthesis genes *phbCAB* from *Ralstonia eutropha* for the micro- or anaerobic PHB production in recombinant *Escherichia coli*. Among the promoters, the one for alcohol dehydrogenase (P_{adhE}) was found most effective. Recombinant *E. coli* JM 109 (pWCY09) harboring P_{adhE} and *phbCAB* achieved a 48% PHB accumulation in the cell dry weight after 48 h of static culture compared with only 30% PHB production under its native promoter. Sixty-seven percent PHB was produced in the dry weight (CDW) of an acetate pathway deleted (*Δpta* deletion) *E. coli* JW2294 harboring the vector pWCY09. In a batch process conducted in a 5.5-l NBS fermentor containing 3 l glucose LB medium, *E. coli* JW2294 (pWCY09) grew to 7.8 g/l CDW containing 64% PHB after 24 h of microaerobic incubation. In addition, molecular weight of PHB was observed to be much higher under microaerobic culture conditions. The high activity of P_{adhE} appeared to be the reason for improved micro- or anaerobic cell growth and PHB production while high molecular weight contributed to the static culture condition.

X.-X. Wei · Z.-Y. Shi · M.-Q. Yuan · G.-Q. Chen (✉)
MOE Laboratory for Protein Sciences,
Department of Biological Sciences and Biotechnology,
Tsinghua University,
Beijing 100084, China
e-mail: chengq@mail.tsinghua.edu.cn

M.-Q. Yuan
College of Biology Science, China Agricultural University,
Beijing 100094, China

G.-Q. Chen
Multidisciplinary Research Center, Shantou University,
Guangdong 515063, China

Keywords PHB · Polyhydroxyalkanoates · Microaerobiosis · Anaerobic promoter · *phbCAB* · Alcohol dehydrogenase

Introduction

Polyhydroxyalkanoates (PHA) are natural polyesters produced by many bacteria as carbon and energy storage materials (Anderson and Dawes 1990). These biopolyesters have attracted increasing attentions due to their biodegradability, thermo-processibility, and sustainability (Chen and Wu 2005). However, the high production cost prevents PHA from large-scale application as biodegradable plastics. Efforts to lower the PHA production cost have always been made since their first discovery in *Bacillus megaterium* (Lemoigne 1926).

To reduce the PHA production cost, cheap carbon sources such as sucrose (Quillaguaman et al. 2007), whey (Ahn et al. 2000; Wong and Lee 1998), starch (Rhu et al. 2003), and soy waste (Hong et al. 2000) were used as raw materials for PHA production. On the other hand, many attempts have been directed toward the development of high productive strains and more efficient fermentation processes (Hartmann et al. 2006; Manna and Paul 2003; Poirier et al. 1995; Rhu et al. 2003). So far, all successful processes on PHB production are aerobic ones requiring energy consuming aerations (Chen 2002; Chen et al. 1991; Chen and Page 1997; Chen et al. 2001). In addition, high cell density fermentation is mostly dependent on oxygen uptake and dissolved oxygen concentration; the oxygen supply problems also add more difficulties in process scale up (Byrom 1992; Lee et al. 2000).

Harding carried out an integrated assessment for PHA production from the cradle-to-factory gate compared with

the petroleum-based plastics, especially polypropylene (PP) and polyethylene (PE) (Harding et al. 2007). The study has pointed out that high energy consumption in PHA production dramatically weakened the advantage of PHA as environmentally friendly materials.

PHA production with low level of oxygen demand has been considered as environmentally and economically sustainable, as micro- or anaerobic growth typically permits simpler reactor design, easier control strategies, and convenient operation conditions (Carlson et al. 2005). Poly(R-3-hydroxybutyrate) (PHB), the best known member of the PHA, and as also the model PHB product for process development, was anaerobically produced by recombinant *Escherichia coli* in either growth or non-growth medium (Carlson et al. 2005). *E. coli* possesses both aerobic and anaerobic metabolic regulation mechanisms. The expression levels of corresponding genes encoding metabolic enzymes vary along with the alteration of dissolved oxygen concentration (Lin and Iuchi 1991). The fumarate nitrate reduction (Fnr) protein and the aerobic respiration control (ArcAB) system are two global transcriptional regulators of *E. coli*, which sense the presence of oxygen and elicit a cascade of metabolic changes within the cell by regulating the transcription of many oxygen-regulated genes (Compan and Touati 1994; Spiro and Guest 1991). Nikel et al. synthesized PHB in recombinant *E. coli arcA* mutant strain under microaerobic environment, where enhanced PHB content and cell growth were obtained when glucose was replaced with glycerol as the carbon source (Nikel et al. 2006, 2008).

The key problem for microaerobic PHB synthesis is the relatively low PHB content in its cell dry weight (CDW). We assume that the downregulation of the PHB relative genes at low level of dissolved oxygen concentration may lead to the low PHB level in the cells. In many studies, PHA synthesis operons were directly cloned and expressed in recombinant *E. coli* for PHA production under aerobic conditions (Choi et al. 1998; Pettinari et al. 2001; Slater et al. 1988). Although these operons were from different microorganisms, these strains were almost exclusively aerobic organisms, and they accumulated PHA at high aeration conditions. These PHA operons were constructed and expressed on high copy number vectors, yet the native promoters regulating PHA operons hardly produced high PHB content under non-aerobic conditions (Carlson et al. 2005).

Therefore, in this study, for the first time, we intended to use anaerobic promoters for achieving PHB production under micro- or anaerobic conditions. For this purpose, a suitable promoter should be selected to control PHA operon in *E. coli* for high PHA accumulation under low or non-oxygen supply.

Materials and methods

Cloning the anaerobic promoters

The preparation of genomic DNA of *E. coli* was carried out according to the standard protocol (Bergthorsson and Ochman 1995). Primers used for amplifying the anaerobic promoters from the genomic DNA were designed while *Xho*I and *Nde*I sites were added at 5′-end of the forward and reverse primers, respectively (Table 1).

In this study, a series of promoters from *E. coli* genome were cloned according to the alteration of the expression levels detected by microarray shown in *E. coli* Gene Expression Database (http://chase.ou.edu/oubcf/). From the database, we can find that the activities of these promoters were at least 100 times enhanced when the culture condition was exchanged from aerobic to anaerobic condition. The PHB synthesis operon *phbCAB* from *Ralstonia eutropha* was constructed downstream these promoters to detect the effect on PHB synthesis under low-oxygen conditions, respectively.

Bacterial strains and plasmid construction

All bacterial strains and plasmids used in this study are described in Table 1. Molecular manipulations were carried out according to standard procedures (Sambrook and Russell 2001). Wild type *E. coli* strain BW25113 and two mutant strains with single deleted genes *Δpta*::kan$^+$ and *Δack*::kan$^+$ were obtained from the single-gene knockout library of the Keio collection in Japan (Baba et al. 2006). They were constructed using the Wanner's method of one-step disruption on chromosomal genes (Datsenko and Wanner 2000). Plasmid pBHR68 containing *R. eutropha* PHB synthesis operon was kindly donated by Professor Alexander Steinbüchel of Münster University in Germany (Spiekermann et al. 1999). This operon can be efficiently expressed in *E. coli* using the *R. eutropha* native promoter, in which PHA synthase, beta-ketothiolase, and acetoacetyl-CoA reductase were encoded by *phbC*, *phbA*, and *phbB*, respectively (Antonio et al. 2000).

The *phbCAB* operon was PCR amplified from pBHR68 using the primers of 5′-TCATCATATGATGGCGACCGG CAAAGGCGCG-3′ and 5′-ATGCGGATCCTCAGCCCA CATGCAGGCCGC-3′. *Nde*I and *Bam*HI digestion sites were added in the 5′- and 3′-end of the PCR product shown underlined. The purified target PCR fragment was digested by *Nde*I/*Bam*HI and was inserted into vector pBluescript II SK$^-$ (Invitrogen) with *Nde*I/*Bam*HI digestion to generate pCAB. The amplified anaerobic promoters were digested and inserted into *Xho*I/*Nde*I site in pCAB, respectively, to generate serial plasmids pWYC1–9 (Tables 1 and 2), in

Table 1 Strains of *Escherichia coli*, plasmids, and primers used in this study

Strains, plasmids	Relevant phenotype	References or sources
E. coli strains		
JM109		From Invitrogen Inc.
BW25113	Wild type strain	Baba et al. (2006)
JW2293	As BW25113 plus *ΔackA*::kan$^+$	Baba et al. (2006)
JW2294	As BW25113 plus *Δpta*::kan$^+$	Baba et al. (2006)
Plasmids		
pBluescript II SK$^-$	A high copy number cloning vector, Amp^R	From Invitrogen Inc.
pBHR68	A pBluescript II SK$^-$ derivative containing *phbCAB* operon from *R. eutropha* H16 with native promoter, Amp^R	Spiekermann et al. (1999)
pPR9TT	Containing promoterless *lacZ*, Amp^R Cm^R	Santos et al. (2001)
pLzzc01	*gfp* gene containing plasmid	Conserved in our lab
pCAB	A pBluescript II SK$^-$ derivative containing promoterless *phbCAB*, Amp^R	This study
pWYC1–9	pBluescript II SK$^-$ derivatives containing *phbCAB* from *R. eutropha* with a series of promoters, Amp^R	This study
pW09-gfp	P_{adhE}::gfp for promoter activity assay	This study
pW09-lacZ	P_{adhE}::lacZ for promoter activity assay	This study
Primers[a,b]		
For P_{hdeA} (212 bp)	5′-CTGACTCGAGAATGCAGTCGATTTAATAAA-3′	5′-ATTCCATATGCGTAATATCCTCAACTATAA-3′
For P_{hdeD} (196 bp)	5′-CTGACTCGAGTCATGTTGATGGAAAATATC-3′	5′-ATTCCATATGAGAACCACCCTATAAAATTA-3′
For P_{gadE} (249 bp)	5′-CTGACTCGAGTTGTTTGCTATTTACAAGCT-3′	5′-ATTCCATATGAACTTGCTCCTTAGCCGTTA-3′
For P_{gadA} (405 bp)	5′-CTGACTCGAGGTTTACGTAACGTTGCTTTA-3′	5′-ATTC_CATATGGAACTCCTTAAATT-3′
For P_{ftnA} (194 bp)	5′-CTGACTCGAGATATTTTAATCAGCGAGGGG-3′	5′-ATTCCATATGGATAGTGCTCCACAAAGGTT-3′
For P_{pflA} (191 bp)	5′-CTGACTCGAGTTAGATTTGACTGAAATC-3′	5′-ATTCCATATGGGTGTTTCTCCAGA-3′
For P_{ldhA} (207 bp)	5′-CTGACTCGAGGGGTAGTTAATATCCTGATT-3′	5′-ATTCCATATGAAGACTTTCTCCAGTGATGT-3′
For P_{arcA} (423 bp)	5′-CTGACTCGAGATTGCCACTCTTCTTGATCA-3′	5′-ATTCCATATGCAGGCTAAGAGGGGCCGGAC-3′
For P_{adhE} (473 bp)	5′-AAAACTCGAGGGTTAGCTCCGAAGCAAAAG-3′	5′-CACCATATGGCTCTCCTGATAATGTTAAAC-3′

[a] Forward primers are listed in left column and reverse primers are listed in right column; the restriction sites are underlined
[b] The numbers in parentheses represent the length of nucleotides upstream each gene (see "Materials and methods" section) when the promoters were cloned

which *phbCAB* was under the control of each anaerobic promoter.

Culture medium and growth conditions

All strains were cultured in Luria–Bertani (LB) medium supplemented with 100 μg/ml ampicillin. One percent seed culture was inoculated into the medium. The temperature was maintained at 37 °C for all the cultures. Glucose used for shake flask and fermentor studies were 10 and 30 g/l, respectively. Micro-aerobic growth was conducted using 250-ml sealed tubes completely filled with growth medium as static cultures, while aerobic growth was carried out using a 500-ml shake flask containing 100 ml culture under vigorous agitation at 200 rpm (NBS, Series 25D, New Brunswick, NJ, USA).

A 5.5-l NBS fermentor (Bioflo 3000, New Brunswick Scientific Co. Inc., NJ, USA) with an operation volume of 3 l was employed to study the microaerobic growth and PHB production by recombinant *E. coli*. Seed culture (50 ml) was grown in a 500-ml shake flask and then transferred to the NBS fermentor containing 3 l LB medium. The pH in the fermentor was maintained at 7.0 via automatic addition of 5 mol/l sodium hydroxide solution. Batch cultures were operated without air supply; a gentle agitation of 75 rpm was applied throughout the cell growth to prevent biomass sedimentation and to promote heat and substrate exchanges.

Study of cell growth, by product formation, and PHB production

To determine cell dry weight (CDW) and metabolic concentrations, 30 ml of a culture was withdrawn from the NBS fermentor every 2 h and stored under 4 °C if the sample was not used immediately. The sample was centrifuged at 5,000 ×*g* and 4 °C for 10 min. The supernatant was stored at −20 °C for late analysis and the cell pellet was washed in 20 ml distilled water and weighted after lyophilization for at least 24 h. Lyophilized cells were subjected to methanolysis and assayed with a gas chromatograph to determine the intracellular PHB content (Brandl et al. 1988). PHB molecular weight was analyzed

Table 2 Effect of different promoters on PHB accumulation in recombinant *E. coli* JM109

Plasmids	Promoters	CDW (g/l)	PHB content (%)
pBHR68	Native	1.73±0.13	29.7±3.40
pWYC01	P_{hdeA}	1.57±0.16	<4
pWYC02	P_{hdeD}	1.15±0.09	<4
pWYC03	P_{gadE}	1.53±0.20	<4
pWYC04	P_{gadA}	1.58±0.06	<4
pWYC05	P_{ftnA}	1.63±0.18	14.3±1.55
pWYC06	P_{pflA}	1.37±0.04	<4
pWYC07	P_{ldhA}	1.59±0.08	9.92±1.35
pWYC08	P_{arcA}	1.80±1.06	28.4±3.57
pWYC09	P_{adhE}	1.65±0.18	48.2±4.48

The recombinant harboring different plasmids, respectively, were cultivated at 37 °C for 48 h as described in "Materials and methods" section. Each value represents the average and standard deviation of three parallel experiments

CDW: cellular dry weight; PHB content: the amount of PHB is given as a weight percentage of the CDW; *hdeA*: stress response protein acid-resistance protein; *hdeD*: acid-resistance membrane protein; *gadE*: DNA-binding transcriptional activator; *gadA*: glutamate decarboxylase A; *ftnA*: ferritin iron storage protein; *pflA*: pyruvate formate lyase; *ldhA*: fermentative D-lactate dehydrogenase; *arcA*: DNA-binding response regulator in two-component regulatory system with ArcB or CpxA; *adhE*: iron-dependent alcohol dehydrogenase

by gel permeation chromatography using a Spectra System P2000 (Thermo Separation, USA) equipped with a TOSOH GMHxl C0085 column (Sim et al. 1997).

The concentrations of glucose and byproducts lactate, succinate, acetate, formate, and ethanol were determined by high performance liquid chromatography (SpectraSYSTEM, SCM1000) equipped with an organic acid analysis column (Aminex HPX-87H ion column, 300×7.8 mm), an automatic sampler (SYSTEM AS3000), and a refractive index detector (SpectraSYSTEM, RI-150). Samples from culture supernatant were first filtered through a 0.22-μm filter unit. Then, 10 μl of samples was loaded into the column operated at 35 °C. A 50 mM H_2SO_4 solution was used as the mobile phase with a flow rate of 0.5 ml/min.

Promoter activity assay

The promoter activity of P_{adhE} was detected in JW2294 (*pta*), JW2293 (*ack*), and BW25113 under microaerobic condition. The *phbCAB* genes were substituted by two report genes, *lacZ* and *gfp*, to generate two new reporter plasmids, pW09-lacZ and pW09-gfp. The plasmids with the reporter gene constructs were introduced into *E. coli* strains (*Δpta*$^-$ and *Δack*$^-$ and wild type) by electroporation. Transformants were selected using ampicillin; they were inoculated into the LB medium. After 24 h of microaerobic cultivation, OD_{600} of cell cultures were measured by spectrophotometer (Biowave DNA, WPA Company, UK). Beta-galactosidase activity and green fluorescent protein (GFP) were measured to observe the relative expression levels of P_{adhE} in *Δack* or *Δpta* mutant strains (Griffith and Wolf 2002; Aoki et al. 2004).

Results

Effect of promoters on microaerobic PHB production by recombinant *E. coli* JM109

Nine anaerobic promoters were cloned from *E. coli* genome DNA and used for inducing micro- or anaerobic PHB production by recombinant *E. coli* (Tables 1 and 2). The sequences of all these promoters were analyzed and found to be identical to the data of NCBI. These promoters were constructed upstream the *phbCAB* genes from *R. eutropha* to generate a series of independent plasmids named pWYC01 to pWYC09. Each of the nine plasmids was introduced into *E. coli* JM109 with pBHR68 harboring *E. coli* as a control. The recombinants harboring the above plasmids were grown for 48 h in LB medium containing 10 g/l glucose and 100 μg/ml ampicillin under static flask culture condition, respectively (Table 2). The highest PHB content was 48% in cell dry weight obtained from *E. coli* JM109 (pWYC09) harboring *phbCAB* under the control of anaerobic promoter P_{adhE} cloned from alcohol dehydrogenase (ADH) gene in *E. coli*. P_{adhE} produced 60% more PHB in dry weight compared with the native promoter under the static microaerobic culture condition in *E. coli* JM109. All other recombinants carrying other anaerobic promoters produced less PHB compared with the control strain harboring plasmid pBHR68. Meanwhile, no significant difference was observed for dry weight of these strains (Table 2). Therefore, more studies were conducted with P_{adhE} and its recombinant strains harboring pWYC09.

PHB production by acetate pathway mutated *E. coli* BW25113

It was reported that the expression level of *adhE* gene could be enhanced 15-fold in acetate pathway mutated *E. coli* under anaerobic condition (Singh et al. 2006). The increased expression level could be attributed to the increased activity of the P_{adhE} in such a mutant strain. P_{adhE} with enhanced activity could increase PHB production in the acetate pathway mutated *E. coli*.

Two mutant strains with single-gene deletion in *E. coli* BW25113 named JW2293 (*ΔackA*::kan$^+$) and JW2294 (*Δpta*::kan$^+$), respectively (Baba et al. 2006) were both introduced with the plasmid pWYC09 containing P_{adhE} as promoter for *phbCAB* genes. PHB production was observed in all recombinants grown for 48 h under static culture condition (Table 3). The highest PHB content reached 67%,

Table 3 PHB production by recombinant *E. coli* BW25113 and its acetate pathway deleted recombinant *E. coli* strains harboring *phaCAB* grown under micro- or anaerobic conditions

Plasmids	Strains	CDW (g/l)	PHB (g/l)	PHB content (wt.%)
pWYC09	JW2294	1.76±0.10	1.19±0.09	67.4±2.4
pWYC09	JW2293	1.65±0.08	0.97±0.10	58.6±3.7
pWYC09	BW25113	1.61±0.15	0.81±0.13	50.2±3.8
pBHR68	JW2294	1.68±0.07	0.52±0.07	30.9±3.4
pBHR68	JW2293	1.63±0.02	0.61±0.07	37.2±3.8
pBHR68	BW25113	1.84±0.04	0.61±0.09	33.0±4.3

The above *E. coli* harboring pWYC09 containing P_{adhE} promoter and *phaCAB* were cultivated in shake flasks at 37 °C for 48 h. *E. coli* strains harboring pBHR68 were used as the control. Each value represents the average and standard deviation of three parallel experiments
CDW: cellular dry weight; PHB content: the amount of PHB is given as a weight percentage of the CDW

which was observed in acetate pathway mutated *E. coli* JW2294 (pWYC09) containing anaerobic promoter. All native promoter recombinants (pBHR68) including the acetate pathway mutants produced less PHB compared with all those equipped with anaerobic P_{adhE}. Similarly, growth difference was not obvious among all recombinants (Table 3). The two acetate pathway mutated strains harboring pWYC09 produced more PHB than its wild type strain BW25113 did, while no obvious difference was found with the strains harboring pBHR68 containing a native promoter.

In all cases studied above, one can easily find that anaerobic promoter P_{adhE} was very useful for initiating PHB synthesis under static micro- or anaerobic culture condition. Acetate pathway mutated strains helped significantly to enhance the PHB production under P_{adhE} promoter.

Study of P_{adhE} activity in *E. coli* BW25113 and its acetate pathway mutated strains

To investigate the relationship between PHB production and transcriptional level of *phbCAB* operon, activities of promoter P_{adhE} in recombinant *E. coli* BW25113, JW2294 (*pta*), and JW2293 (*ack*) were studied under microaerobic conditions. The *phbCAB* genes were replaced by *lacZ* and *gfp*, respectively, resulting in two new reporter plasmids pW09-lacZ and pW09-gfp. Compared with the native recombinant *E. coli* BW25113, activities of β-galactosidase and green fluorescent protein (GFP) were observed to be more than 3- to 5-fold higher in the *Δack* or *Δpta* acetate pathway mutated strains, suggesting more activity for P_{adhE} expressed in the acetate pathway mutated strains compared with its native strain *E. coli* BW25113 (Fig. 1).

Growth and PHB production by recombinant *E. coli* BW25113 (pWYC09) and its *Δpta* mutant JW2294 (pWYC09)

Incubated in fermentors, acetate pathway mutated *Δpta* mutant *E. coli* strain JW2294 (pWYC09) produced 64% PHB in its 7.8 g/l cell dry weight (CDW) after 24 h incubation in a 5.5-l fermentor without air supply. By contrast, the original strain *E. coli* BW25113 containing pWYC09 grew to only 6.7 g/l CDW containing 45% PHB (Table 4). Both strains produced lactate and acetate, with *Δpta* mutant JW2294 (pWYC09) producing less acetate and more lactate compared to the original BW25113 under the same conditions.

Cell growth behaved similarly for both strains (Fig. 2a), yet PHB production by *Δpta* mutant JW2294 (pWYC09) accelerated much faster than the original BW25113 strain did (Fig. 2b), and so were glucose consumptions (Fig. 2c). On the other hand, residual biomass were accumulated more and faster by the original BW25113 (pWYC09) strain compared with the *Δpta* mutant JW2294 (pWYC09) (Fig. 2d), indicating that the wild type had more cells while the *Δpta* strain had more PHB. Obviously, acetate deletion is harmful for cell growth although it promotes PHB synthesis (Table 4).

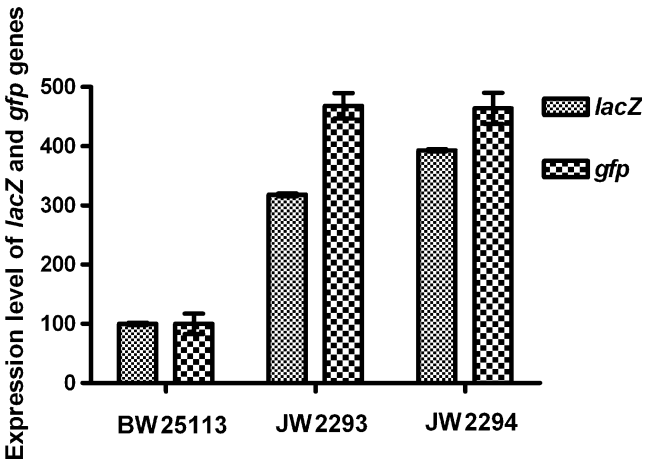

Fig. 1 Expression of *gfp* and *lacZ* genes under P_{adhE} in *E. coli* JW2294 (*pta*) and JW2293 (*ack*) mutants relative to wild type BW25113 after 24 h microaerobic culture. *E. coli* strains harboring plasmids pW09-gfp or pW09-lacZ were cultivated in LB medium, respectively. Value 100 corresponds to equal gene expression relative to wild type BW25113 and values above 100 correspond to fold increase. Data are the means of triplicate experiments

Table 4 The highest yields of the fermentation products produced by recombinant *E. coli* BW25113 and the acetate deleted mutant *E. coli* JW2294 (*pta*) derived from BW15113 grown for 24 h under microaerobic conditions in a 5.5-l NBS fermentor, respectively

Strains	CDW (g/l)	PHB (g/l)	PHB content (% of CDW)	Lactate (g/l)	Acetate (g/l)
JW2294 (pWYC09)	7.75±1.76	5.0±1.45	64.3±7.4	14.3±3.12	2.3±0.31
BW25113 (pWYC09)	6.72±1.59	3.0±1.28	45.5±3.9	8.3±2.66	5.3±0.83

Each value represents the average and standard deviation of at least two parallel experiments
CDW: cellular dry weight; PHB content: the amount of PHB is given as a weight percentage of the CDW

Interestingly, the *Δpta* strain produced less acetate but more lactate compared with that of the wild type (Table 4). The production of both lactate and acetate is a waste of substrates under microaerobic conditions; the *Δpta* strain accumulated totally 16.6 g/l lactate and acetate together, more than the 13.6 g/l accumulated by the wild type. In this case, the *Δpta* strain has lower substrate utilization efficiency compared with the wild type although the mutant produced more PHB.

Molecular weights of PHB produced under microaerobic conditions

Static cultures of recombinants *E. coli* JW2293 (pWCY09), JW2294 (pWCY09), and BW25113 (pWCY09) produced PHB with higher molecular weights and similar polydispersities compared with that of their growth under shake flask aerobic conditions (Table 5). The highest PHB weight

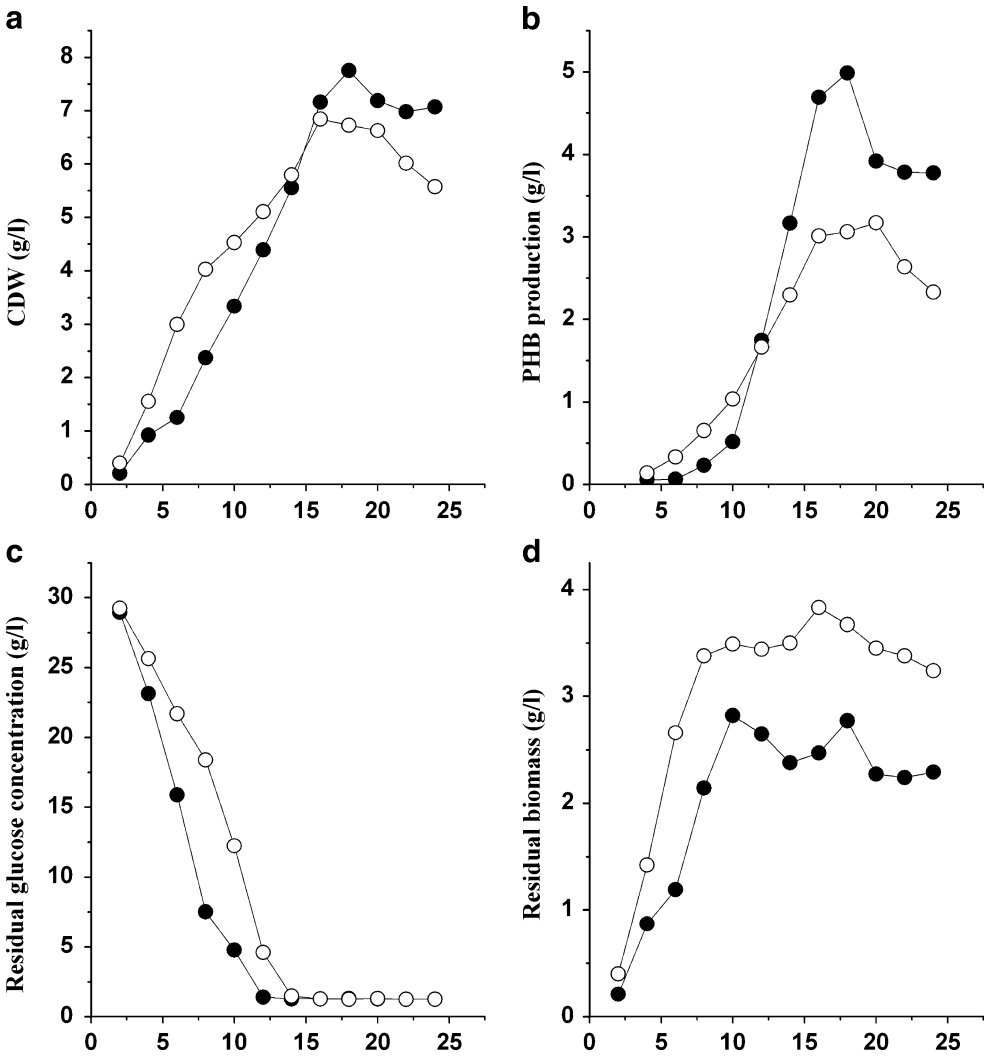

Fig. 2 Kinetics of CDW, PHB production, residual glucose concentration, and residual biomass of recombinant *E. coli* harboring P_{adhE} and *phbCAB* genes cultivated in a 5.5-l fermentor for 24 h. No air was supplied during cultivation, and a 75 rpm agitation was applied to maintain homogeneous conditions: *X-axis* represents the fermentation time (h); *Y-axis* represents the concentration of the broth. **a** CDW (g/l); **b** PHB production (g/l); **c** residual glucose concentration (g/l); **d** residual biomass (g/l). *Solid symbols*: *pta* mutant *E. coli* strain JW2294 (pWYC09); *open symbols*: wild type *E. coli* strain BW25113 (pWYC09)

Table 5 Molecular weight of PHB under different culture conditions

Strains	Growth condition	Molecular weight		
		Mw ($\times 10^4$ Da)	Mn ($\times 10^4$ Da)	Mw/Mn
JW2293 (pWYC09)	Static culture	781	340	2.29
JW2294 (pWYC09)	Static culture	1,449	808	1.79
BW25113 (pWYC09)	Static culture	1,117	507	2.20
JW2293 (pWYC09)	Shake culture	465	273	1.70
JW2294 (pWYC09)	Shake culture	393	221	1.78
BW25113 (pWYC09)	Shake culture	276	140	1.97

Various recombinant *E. coli* strains harboring pWYC09 were cultivated at 37 °C for 48 h, respectively, as described in "Materials and methods" section

Mw: weight average molecular weight; *Mn*: number average molecular weight; *Mw/Mn*: polydispersity

average molecular weight (Mw) of 14.5×10^6 was found in *E. coli* JW2294 (pWCY09) grown under microaerobic static culture conditions, compared with the lowest Mw of PHB of only 2.76×10^6 produced by *E. coli* BW25113 (pWCY09) incubated under aerobic shake flask conditions. Micro-aerobic growth may be a convenient way to obtain PHB with ultrahigh Mw.

Discussion

The production of chemicals including PHA under micro-aerobic conditions has become increasingly attractive due to the low energy cost of the process (Carlson et al. 2005). Unlike the traditional definition (Nordkvist et al. 2003; Sabra et al. 2002; Tsai et al. 1996; Zeng and Deckwer 1996), microaerobic culture studied here includes static cultivation or agitated fermentation without the use of nitrogen to completely remove oxygen. Preliminary experiments designed to compare the strictly anaerobic and microaerobic culture indicated that there was no significant difference on PHB production (data not shown). *E. coli* is a suitable facultative bacterium used for testing micro- or anaerobic PHA production when PHB synthesis genes *phabCAB* are introduced into the cells (Carlson et al. 2005). In addition, *E. coli* incubated under micro- or anaerobic conditions can maintain a high NADPH/NADP ratio which is beneficial to PHB production (Dawes and Senior 1973; de Graef et al. 1999).

A series of anaerobic promoters were used to express *phbCAB* operon of *R. eutropha* for PHB production in recombinant *E. coli* under micro- or anaerobic conditions (Table 1). When recombinant *E. coli* (pWYC09) strains grew under static conditions, the anaerobic promoter P_{adhE}, which is the promoter for alcohol dehydrogenase, helped to achieve a 60% increase on PHB production compared with that of recombinant *E. coli* harboring control plasmid pBHR68 with its native promoter (Tables 2 and 3). Nine promoters were selected for micro- or anaerobic PHB production based on transcriptome analysis using the *E. coli* Gene Expression Database of the University of Oklahoma (http://chase.ou.edu/oubcf/). Among the genes downstream of the nine promoters, four of them including *gadA*, *pflA*, *ldhA*, and *adhE* are enzymes coding metabolism genes; three of them including *gadE*, *ftnA*, and *arcA*, are regulator genes; the other two including *hdeA* and *hdeD* are open reading frames with unknown functions. Gene *adhE* codes alcohol dehydrogenase (ADH) which catalyzes reactions converting acetyl-CoA to acetaldehyde and then to ethanol (Goodlove et al. 1989). In *E. coli*, expression of *adhE* was reported to be 10-fold higher during anaerobic growth than that under aerobic conditions, and the regulation is both at transcriptional and post-transcriptional levels (Chen and Lin 1991; Leonardo et al. 1993; Mikulskis et al. 1997). The regulation of *adhE* is independent of Fnr and ArcA although there is a Fnr consensus sequence (TTGAT-N_4-ATCAA) located at −42.5 bp from the −188 transcriptional start site in its promoter P_{adhE} (Membrillo-Hernandez et al. 1999).

Some carbon flux is converted into acetate from acetyl-CoA *via* Pta–Ack pathway involving phosphotransacetylase (EC 2.3.1.8) (Pta) and acetate kinase (EC 2.7.2.1) (Ack) (Kakuda et al. 1994). PHB was produced in Pta–Ack pathway mutated *E. coli* for reducing byproducts and enhancing PHB accumulation (Miyake and Miyamoto 2000; Shi et al. 1999). Chang et al. studied the PHB production in *pta* mutant under aerobic conditions. Their results suggested that *pta* mutant had reduced growth on glucose due to the perturbation of acetyl-CoA flux; this defect was restored by the introduction of PHB synthesis pathway (Chang et al. 1999). In addition, Singh et al. found that the *adh* gene expression level revealed by real-time PCR was notably higher in *ackA-pta* mutant than that of its wild type *E. coli* under anaerobic conditions (Singh et al. 2006). In the present work, two single deletion mutants, JW2293 (*ack*) and JW2294 (*pta*), were introduced with plasmid pWYC09 encoding anaerobic promoter P_{adhE} for the micro- or anaerobic PHB production. In the static

culture using glucose as a sole carbon source, PHB content increased from 48% of cell dry weight (CDW) in the wild type to 67% in *pta* mutant while the cells showed a weak growth (Table 3). When cultured in a 5.5-l computer-controlled fermentor, the CDW of *E. coli* JW2294 (pWYC09) achieved 7.75 g/l at 18 h with 64% PHB content (Table 4; Fig. 2). Lower PHB content accompanied by higher residual biomass was observed in wild type *E. coli* BW25113 harboring pWYC09. A 4-fold increase of P_{adhE} activity was observed in *E. coli* strains JW2294 (*pta*) or JW2293 (*ack*) compared to that in *E. coli* BW25113, as was detected by two reporter systems (Fig. 1). The enhanced activity in acetate mutations was similar to what was reported by Singh et al. (2006). It was clearly revealed that the PHB content in CDW was correlated with the promoter activity.

Carlson et al. reported a 50% PHB production in CDW of recombinant *E. coli* DH5α harboring *phbCAB* from *R. eutropha* under anaerobic conditions when grown in rich medium (Carlson et al. 2005). Nikel et al. produced the PHB in a microaerobic bioreactor using an *E. coli arcA* mutant as a host. The PHB synthesized by *arcA2* mutant carrying *phbBAC* genes of *Azotobacter* sp. FA8 achieved 35% of its CDW when grown on glucose (Nikel et al. 2006; Pettinari et al. 2001). Furthermore, when glycerol was used to replace glucose, PHB increased to 51% after a 60-h fed-batch cultivation (Nikel et al. 2008). It appeared that anaerobic promoter P_{adhE} applied for the first time in this study yielded more PHB under microaerobic conditions than all of the reported data. When grown in a fermentor, the cell growth and PHB productivity reached their maximum at approximately 20 h. The final CDW of recombinant *E. coli* JW2294 (pWYC09) reached only about 8 g/l under microaerobic conditions. More process development and optimization are needed to achieve high PHB productivity. In this paper, the PHB synthetic operon was proven as a good model for gene expression and product accumulation in recombinant *E. coli* under low levels of dissolved oxygen. From the above results, promoter P_{adhE} and acetate mutant strain *E. coli* JW2294 show a potential on microbial PHB production without high aeration demand.

The weight average molecular weights (Mw) of PHB produced by wild type *R. eutropha* are usually in the range of $2–4\times10^6$ (Normi et al. 2005). However, once the *phbCAB* operon from *R. eutropha* was expressed in *E. coli*, PHB with Mw exceeding 3×10^6 were synthesized in many cases (Choi and Lee 2004). So far, the highest Mw of PHB was 2.0×10^7 obtained from recombinant *E. coli* (Kusaka et al. 1997). Previous studies suggested that PHB Mw was dependent on the PHA synthase properties (Agus et al. 2006b); PHB Mw decreased with increasing synthase activity when *phbCAB* operon from *R. eutropha* was overexpressed in recombinant *E. coli* (Agus et al. 2006a). In this study, we have found that microaerobic conditions could effectively enhance the PHB Mw.

Acknowledgment We are grateful to Professor Alexander Steinbüchel of the University of Münster in Germany for the generous donation of plasmid pBHR68 and to Keio collection, Japan, for kindly providing us with *E. coli* mutant strains. This research was supported by Natural Sciences Foundation of China Grant No. 30570024. Thanks must also be extended to National High Tech 863 Grant (Project Nos. 2006AA02Z242 and 2006AA020104), and National Basic Research Program of China (973 Program) No. 2007CB707804.

References

Agus J, Kahar P, Abe H, Doi Y, Tsuge T (2006a) Altered expression of polyhydroxyalkanoate synthase gene and its effect on poly [(R)-3-hydroxybutyrate] synthesis in recombinant *Escherichia coli*. Polym Degrad Stab 91:1645–1650

Agus J, Kahar P, Abe H, Doi Y, Tsuge T (2006b) Molecular weight characterization of poly[(R)-3-hydroxybutyrate] synthesized by genetically engineered strains of *Escherichia coli*. Polym Degrad Stab 91:1138–1146

Ahn WS, Park SJ, Lee SY (2000) Production of Poly(3-hydroxybutyrate) by fed-batch culture of recombinant *Escherichia coli* with a highly concentrated whey solution. Appl Environ Microbiol 66:3624–3627

Anderson AJ, Dawes EA (1990) Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol Rev 54:450–472

Antonio RV, Steinbüchel A, Rehm BH (2000) Analysis of in vivo substrate specificity of the PHA synthase from *Ralstonia eutropha*: formation of novel copolyesters in recombinant *Escherichia coli*. FEMS Microbiol Lett 182:111–117

Aoki T, Satoh K, Imamura T, Watabe H (2004) A new method for detecting single nucleotide polymorphism using GFP-display. J Biochem Biophys Meth 60:61–67

Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol 2:0008

Bergthorsson U, Ochman H (1995) Heterogeneity of genome sizes among natural isolates of *Escherichia coli*. J Bacteriol 177:5784–5789

Brandl H, Gross RA, Lenz RW, Fuller RC (1988) *Pseudomonas oleovorans* as a source of poly(beta-hydroxyalkanoates) for potential applications as biodegradable polyesters. Appl Environ Microbiol 54:1977–1982

Byrom D (1992) Production of poly-β-hydroxybutyrate: poly-β-hydroxyvalerate copolymers. FEMS Microbiol Rev 103:247–250

Carlson R, Wlaschin A, Srienc F (2005) Kinetic studies and biochemical pathway analysis of anaerobic poly-(R)-3-hydroxybutyric acid synthesis in *Escherichia coli*. Appl Environ Microbiol 71:713–720

Chang DE, Shin S, Rhee JS, Pan JG (1999) Acetate metabolism in a pta mutant of *Escherichia coli* W3110: importance of maintaining acetyl coenzyme a flux for growth and survival. J Bacteriol 181:6656–6663

Chen GQ (2002) Production and application of microbial polyhydroxyalkanoates. Kluwer/Plenum, New York, pp 155–166.

Chen YM, Lin EC (1991) Regulation of the *adhE* gene, which encodes ethanol dehydrogenase in *Escherichia coli*. J Bacteriol 173:8009–8013

Chen GQ, Page WJ (1997) Production of poly-beta-hydroxybutyrate by *Azotobacter vinelandii* in a two-stage fermentation process. Biotechnol Tech 11:347–350

Chen GQ, Wu Q (2005) The application of polyhydroxyalkanoates as tissue engineering materials. Biomaterials 26:6565–6578

Chen GQ, Konig KH, Lafferty RM (1991) Production of poly-D(−)-3-hydroxybutyrate and poly-D(−)-3-hydroxyvalerate by strains of *Alcaligenes latus*. Antonie Van Leeuwenhoek 60:61–66

Chen GQ, Zhang G, Park SJ, Lee SY (2001) Industrial scale production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate). Appl Microbiol Biotechnol 57:50–55

Choi JI, Lee SY (2004) High level production of supra molecular weight poly(3-hydroxybutyrate) by metabolically engineered *Escherichia coli*. Biotechnol Bioprocess Eng 9:196–200

Choi JI, Lee SY, Han K (1998) Cloning of the *Alcaligenes latus* polyhydroxyalkanoate biosynthesis genes and use of these genes for enhanced production of Poly(3-hydroxybutyrate) in *Escherichia coli*. Appl Environ Microbiol 64:4897–4903

Compan I, Touati D (1994) Anaerobic activation of arcA transcription in *Escherichia coli*: roles of Fnr and ArcA. Mol Microbiol 11:955–964

Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A 97:6640–6645

Dawes EA, Senior PJ (1973) The role and regulation of energy reserve polymers in micro-organisms. Adv Microb Physiol 10:135–266

de Graef MR, Alexeeva S, Snoep JL, Teixeira de Mattos MJ (1999) The steady-state internal redox state (NADH/NAD) reflects the external redox state and is correlated with catabolic adaptation in *Escherichia coli*. J Bacteriol 181:2351–2357

Goodlove PE, Cunningham PR, Parker J, Clark DP (1989) Cloning and sequence analysis of the fermentative alcohol-dehydrogenase-encoding gene of *Escherichia coli*. Gene 85:209–214

Griffith KL, Wolf RE (2002) Measuring beta-galactosidase activity in bacteria: cell growth, permeabilization, and enzyme assays in 96-well arrays. Biochem Biophys Res Commun 290:397–402

Harding KG, Dennis JS, von Blottnitz H, Harrison ST (2007) Environmental analysis of plastic production processes: comparing petroleum-based polypropylene and polyethylene with biologically-based poly-beta-hydroxybutyric acid using life cycle analysis. J Biotechnol 130:57–66

Hartmann R, Hany R, Pletscher E, Ritter A, Witholt B, Zinn M (2006) Tailor-made olefinic medium-chain-length poly[(R)-3-hydroxyalkanoates] by *Pseudomonas putida* GPo1: batch versus chemostat production. Biotechnol Bioeng 93:737–746

Hong K, Leung YC, Kwok SY, Law KH, Lo WH, Chua H, Yu PH (2000) Construction of recombinant *Escherichia coli* strains for polyhydroxybutyrate production using soy waste as nutrient. Appl Biochem Biotechnol 84–86:381–390

Kakuda H, Shiroishi K, Hosono K, Ichihara S (1994) Construction of Pta–Ack pathway deletion mutants of *Escherichia coli* and characteristic growth profiles of the mutants in a rich medium. Biosci, Biotechnol, Biochem 58:2232–2235

Kusaka S, Abe H, Lee SY, Doi Y (1997) Molecular mass of poly[(R)-3-hydroxybutyric acid] produced in a recombinant *Escherichia coli*. Appl Microbiol Biotechnol 47:140–143

Lee SH, Oh DH, Ahn WS, Lee Y, Choi J, Lee SY (2000) Production of poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) by high-cell-density cultivation of *Aeromonas hydrophila*. Biotechnol Bioeng 67:240–244

Lemoigne M (1926) Produits de deshydration et depolymerisation de l'acide β-oxybytyrique. Bull Soc Chim Biol (Paris) 8:770–782

Leonardo MR, Cunningham PR, Clark DP (1993) Anaerobic regulation of the *adhE* gene, encoding the fermentative alcohol dehydrogenase of *Escherichia coli*. J Bacteriol 175:870–878

Lin EC, Iuchi S (1991) Regulation of gene expression in fermentative and respiratory systems in *Escherichia coli* and related bacteria. Annu Rev Genet 25:361–387

Manna A, Paul AK (2003) Growth-associated production and characterization of poly (3-hydroxybutyric acid) of *Azotobacter beijerinckii* DAR-102. Indian J Exp Biol 41:129–134

Membrillo-Hernandez J, Kwon O, De Wulf P, Finkel SE, Lin ECC (1999) Regulation of adhE (encoding ethanol oxidoreductase) by the Fis protein in *Escherichia coli*. J Bacteriol 181:7390–7393

Mikulskis A, Aristarkhov A, Lin EC (1997) Regulation of expression of the ethanol dehydrogenase gene (adhE) in *Escherichia coli* by catabolite repressor activator protein Cra. J Bacteriol 179:7129–7134

Miyake M, Miyamoto C (2000) Phosphotransacetylase as a key factor in biological production of polyhydroxybutyrate. Appl Biochem Biotechnol 84–6:1039–1044

Nikel PI, Pettinari MJ, Galvagno MA, Mendez BS (2006) Poly(3-hydroxybutyrate) synthesis by recombinant *Escherichia coli* arcA mutants in microaerobiosis. Appl Environ Microbiol 72:2614–2620

Nikel PI, Pettinari MJ, Galvagno MA, Mendez BS (2008) Poly(3-hydroxybutyrate) synthesis from glycerol by a recombinant *Escherichia coli* arcA mutant in fed-batch microaerobic cultures. Appl Microbiol Biotechnol 77:1337–1343

Nordkvist M, Jensen NBS, Villadsen J (2003) Glucose metabolism in *Lactococcus lactis* MG1363 under different aeration conditions: requirement of acetate to sustain growth under microaerobic conditions. Appl Environ Microbiol 69:3462–3468

Normi YM, Hiraishi T, Taguchi S, Sudesh K, Najimudin N, Doi Y (2005) Site-directed saturation mutagenesis at residue F420 and recombination with another beneficial mutation of *Ralstonia eutropha* polyhydroxyalkanoate synthase. Biotechnol Lett 27:705–712

Pettinari MJ, Vazquez GJ, Silberschmidt D, Rehm B, Steinbüchel A, Mendez BS (2001) Poly(3-hydroxybutyrate) synthesis genes in *Azotobacter* sp. strain FA8. Appl Environ Microbiol 67(11):5331–5334

Poirier Y, Nawrath C, Somerville C (1995) Production of polyhydroxyalkanoates, a family of biodegradable plastics and elastomers, in bacteria and plants. Bio-Technol 13:142–150

Quillaguaman J, Munoz M, Mattiasson B, Hatti-Kaul R (2007) Optimizing conditions for poly(beta-hydroxybutyrate) production by *Halomonas boliviensis* LC1 in batch culture with sucrose as carbon source. Appl Microbiol Biotechnol 74:981–986

Rhu DH, Lee WH, Kim JY, Choi E (2003) Polyhydroxyalkanoate (PHA) production from waste. Water Sci Technol 48:221–228

Sabra W, Kim EJ, Zeng AP (2002) Physiological responses of *Pseudomonas aeruginosa* PAO1 to oxidative stress in controlled microaerobic and aerobic cultures. Microbiology-SGM 148: 3195–3202

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Santos PM, Di Bartolo I, Blatny JM, Zennaro E, Valla S (2001) New broad-host-range promoter probe vectors based on the plasmid RK2 replicon. FEMS Microbiol Lett 195:91–96

Shi H, Nikawa J, Shimizu K (1999) Effect of modifying metabolic network on poly-3-hydroxybutyrate biosynthesis in recombinant *Escherichia coli*. J Biosci Bioeng 87:666–677

Sim SJ, Snell KD, Hogan SA, Stubbe J, Rha CK, Sinskey AJ (1997) PHA synthase activity controls the molecular weight and polydispersity of polyhydroxybutyrate in vivo. Nat Biotechnol 15:63–67

Singh R, Yang YT, Lu BQ, Bennett GN, San KY (2006) Expression of the *pfl* gene and resulting metabolite flux distribution in nuo and ackA-pta *E. coli* mutant strains. Biotechnol Prog 22:898–902

Slater SC, Voige WH, Dennis DE (1988) Cloning and expression in *Escherichia coli* of the *Alcaligenes eutrophus* H16 poly-beta-hydroxybutyrate biosynthetic pathway. J Bacteriol 170:4431–4436

Spiekermann P, Rehm BH, Kalscheuer R, Baumeister D, Steinbuchel A (1999) A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. Arch Microbiol 171:73–80

Spiro S, Guest JR (1991) Adaptive responses to oxygen limitation in *Escherichia coli*. Trends Biochem Sci 16:310–314

Tsai PS, Nageli M, Bailey JE (1996) Intracellular expression of Vitreoscilla hemoglobin modifies microaerobic *Escherichia coli* metabolism through elevated concentration and specific activity of cytochrome o. Biotechnol Bioeng 49:151–160

Wong HH, Lee SY (1998) Poly-(3-hydroxybutyrate) production from whey by high-density cultivation of recombinant *Escherichia coli*. Appl Microbiol Biotechnol 50:30–33

Zeng AP, Deckwer WD (1996) Bioreaction techniques under microaerobic conditions: from molecular level to pilot plant reactors. Chem Eng Sci 51:2305–2314