Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) Production in Recombinant *Aeromonas hydrophila* 4AK4 Harboring *phbA, phbB* and *vgb* Genes

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Summary: Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBHHx), a copolyester consisting of 3-hydroxybutyrate (3HB) and 3-hydroxyhexanoate (3HHx), can be synthesized by *Aeromonas hydrophila* strain 4AK4 using long chain fatty acids as the carbon source. The wild type *A. hydrophila* 4AK4 accumulated PHBHHx consisting of 12-15 mol% 3HHx regardless of growth conditions. When *phbA*, *phbB* and *vgb* genes encoding β-ketothiolase, acetoacetyl-CoA reductase and *vitreoscilla* hemoglobin, respectively, were introduced together into *A. hydrophila* 4AK4, the recombinant strain grew to over 20 g/L cell dry weight (CDW) after 48 h of shake flask cultivation in co-substrates of dodecanoate and gluconate (weight ratio 1:1), and the CDW contained 50% PHBHHx consisting of 9 mol% 3HHx. Under similar conditions, the wild type strain produced only 12 g/L CDW containing 32% PHBHHx with 15 mol% 3HHx. In comparison, recombinant harboring *phbA* and *phbB* produced 35% PHBHHx with 9 mol% 3HHx in 15 g/L CDW under the same conditions. The obvious differences in terms of the cell growth and PHBHHx production were attributed to the expression of *vgb* in *A. hydrophila* 4AK4, which was clearly observed in carbon monoxide difference spectra. The expression of *vgb* in the recombinant not only improved cell growth and PHBHHx accumulation, but also increased the plasmid stability during cell growth, especially under low dissolved oxygen tension in fermentors. PHBHHx production could be further increased to over 60% of the CDW by the over expression of *phaC* and *phaJ* from *Aeromonas caviae* encoding PHBHHx synthase and (R)-specific enoyl-CoA hydratase, respectively. Over expression of *phaC*, *phaJ* and *phaP*, alone or in various combinations, also increased the 3HHx content of PHBHHx from 14-34%. The above results showed that *A. hydrophila* was amenable to genetic manipulation, and that these modifications could be exploited to produce compounds with different properties for commercial and research applications.

Keywords: *Aeromonas hydrophila*; hemoglobin; PHB; polyhydroxyalkanoates; *vgb*

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Introduction

Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) or PHBHHx, a copolyester consisting of 3-hydroxybutyrate (3HB) and 3-hydroxyhexanoate (3HHx), is a biodegradable and biocompatible material that recently began receiving considerable attention [1-5]. In comparison with polyhydroxybutyrate (PHB) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV), PHBHHx has better physical properties [6] and biocompatibility [7, 8]. The material properties are strongly influenced by the 3HHx fraction of PHBHHx. As 3HHx content increases, the crystallinity [9] and melting point of PHBHHx decreases, while the flexibility and tractility increase [10]. PHBHHx containing various 3HHx contents can supply a wide range of materials with different properties for commercial and research applications.

Aeromonas hydrophila are the mostly studied PHBHHx-producing strains. Recent studies have focused on optimization of culture media and conditions for low-cost and efficient fermentative production of this material [3, 11]. Aliphatic substrates such as long-chain fatty acids including soybean oil or lauric acid have been used in fermentations as the sole carbon and energy sources for PHBHHx production. A two-stage fermentation process was developed to improve PHBHHx production efficiency [3]. In the first stage of the culture, biomass was produced from non-related carbon sources such as glucose, sucrose, or sodium gluconate; in the second stage, PHBHHx was synthesized by adding related carbon sources such as fatty acids into the culture media. In spite of these efforts, PHBHHx production efficiency was still much lower than that of the PHB. Previous studies using *Aeromonas hydrophila* revealed that the monomer composition of PHBHHx could not be easily regulated by changing substrates or fermentation conditions because all precursors for the synthesis were from the β-oxidation pathway [2, 12]. Furthermore, PHBHHx synthesized by *Aeromonas hydrophila* 4AK4 contained 12-15 mol% of 3HHx regardless of growth conditions [3]. This narrow range of monomer composition restricts the potential applications for PHBHHx.

In this study, foreign genes of *phbA*, *phbB* and *vgb* genes were introduced into *A. hydrophila* 4AK4. The *phbA* and *phbB* genes encoding β-ketothiolase and acetoacetyl-CoA reductase, respectively, provided an additional metabolic pathway to synthesize 3HB precursors from acetyl-CoA [13], while the *vgb* gene encoding *vitreoscilla* hemoglobin increased the rate of oxygen uptake, especially in a high density cell culture when oxygen

concentration is very low [14-16]. As a result, enhanced cell growth and product yield is observed [17]. We found that co-expression of these three genes in *A. hydrophila* 4AK4 resulted in a highly-efficient recombinant strain for which the monomer composition of PHBHHx was reliably controlled. In this paper, attempts were also made to over express PHBHHx synthesis genes from *Aeromonas caviae* including *phaC*, *phaJ* or *phaP* encoding PHBHHx synthase, (R)-specific enoyl-CoA hydratase or phasin, respectively, for possible enhanced production of PHBHHx by recombinant *A. hydrophila* 4AK4.

Materials and Methods

Strains and plasmids. Wild type strain *Aeromonas hydrophila* 4AK4 was stored in the Microbiology Lab of Tsinghua University. Recombinant *Aeromonas hydrophila* 4AK4 (pTG01) harboring *phbA* and *phbB* genes, and recombinant *Aeromonas hydrophila* 4AK4 (pVGAB) harboring *phbA*, *phbB* and *vgb* genes were used for this study [5]. Plasmids pTG01 and pVGAB were introduced into *Aeromonas hydrophila* 4AK4 by conjugation. These plasmids are illustrated in Figure 1. The *phbA* and *phbB* genes were from plasmid pUC-AB, which was described by Zhao et al. in 2003 [18]. The *vgb* operon consisting of the *vgb* oxygen-responsive promoter, the *vgb* gene and terminator were cloned from plasmid pBBR322-*vgb*, which was kindly provided by Dr. HM Yu of the Dept of Chemical Engineering, Tsinghua University [19]. PHBHHx synthesis genes *phaC*, *phaP* and *phaJ* were kindly donated by Prof Y Doi of RIKEN, Japan. They were used to construct the plasmids listed in Table 1 using pBBR1MCS-2 as a starting plasmid. The plasmid construction processes are illustrated in Figure 2.

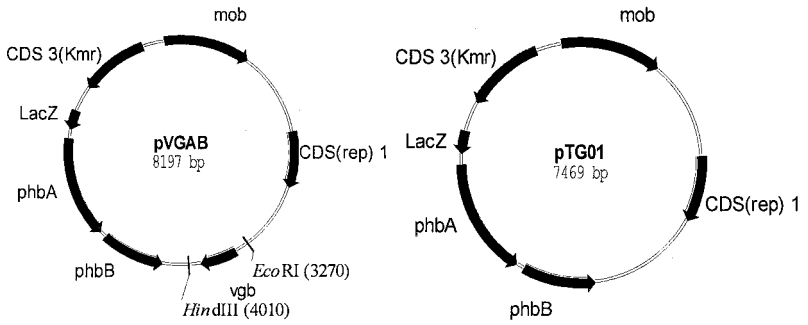

Figure 1. Plasmids pTG01 and pVGAB.

Flask culture media and culture conditions. LB medium for seed culture contained (g/L) yeast extract (5), tryptone (10), NaCl (10) and kanamycin (0.005). The medium for the flask culture consisted of dodecanoate (0.30), sodium gluconate (0.45), $(NH_4)_2SO_4$ (3.9), $MgSO_4 \cdot 7H_2O$ (0.5), $Na_2HPO_4 \cdot 12H_2O$ (3.5), KH_2PO_4 (2.5), Yeast Extract (1), and 1 ml L^{-1} trace element solution. Trace element solution consisted of $FeCl_3 \cdot 6H_2O$ (20), $CaCl_2 \cdot H_2O$ (10), $CuSO_4 \cdot 5H_2O$ (0.03), $MnCl_2 \cdot 4H_2O$ (0.05), $ZnSO_4 \cdot 7H_2O$ (0.1) and 0.5 mol L^{-1} HCl.
The seed and flask cultures were incubated at 30°C for 48 hours at 200 rpm on a rotary shaker. (NBS Series 25D, New Brunswick, USA)
The following medium conditions were employed to study the effect of various ratios of dodecanoate to sodium gluconate on cell growth and PHBHHx composition and production.

Culture medium and conditions for a 6 L fermentor. The fermentation medium consisted of (g/L) dodecanoate (10), $(NH_4)_2SO_4$ (4), $MgSO_4 \cdot 7H_2O$ (0.5), $Na_2HPO_4 \cdot 12H_2O$ (2), KH_2PO_4 (1.55), yeast extract (1), and a 1 mL/L trace element solution. A 6 L automatic fermentor (NBS 3000, New Brunswick, USA) with a working volume of 3 L was inoculated with the above PHBHHx-producing microorganisms at 30°C
and pH 6.5. Dissolved oxygen (DO) was provided by injecting filtered air at a flow rate of

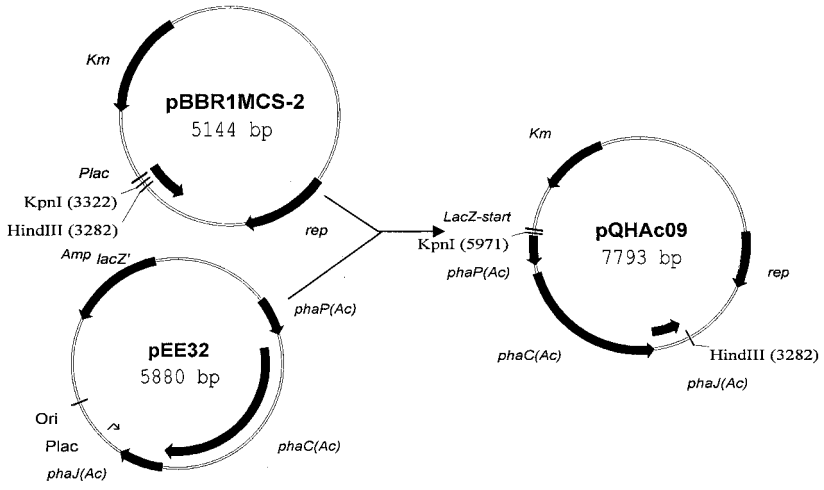

Figure 2. Construction of pQHAc09 containing phaPCJ from pBBR1MCS-2 and pEE32.

Table 1. PHBHHx synthesis genes were constructed in plasmids in combinations.

Plasmids[a]	Gene Combination	Promoter
pQHAc01	$phaP_{Ac}$	*lac*
pQHAc03	$phaJ_{Ac}$	*lac*
pQHAc04	$phaPC_{Ac}$	*lac*
pQHAc05	$phaCJ_{Ac}$	*lac*
pQHAc06	$phaPJ_{Ac}$	*lac*
pQHAc09	$phaPCJ_{Ac}$	*lac*
pQHAc19	$phaPC'J_{Ac}$ [b]	*lac*

[a:] Derivatives of pBBR1MCS-2; [b:] *phaC'* is *phaC* negative mutant

5 L min^{-1}, and was maintained at 15% of air saturation by automatically adjusting the agitation rate from 200 to 800 rpm. The pH was controlled automatically by the addition of an aqueous solution of NaOH.

Inoculants were obtained using a 12 h growth of the strains in seed media. The inoculation volume was 5% (V/V). A fed-batch process was used for fermentation in order to avoid dodecanoate foaming. When only dodecanoate was used as the carbon source, 30 g dodecanoate and 6 g $(NH_4)_2SO_4$ were added every 4 hours in the growth log phase; when co-substrates were used, 30 g dodecanoate, 30 g sodium gluconate and 9 g $(NH_4)_2SO_4$ were added every 6 hours. The fermentation process was stopped when the agitation rate dropped from 800 rpm to 500 rpm.

Analysis methods. Cells were harvested by centrifugation, washed sequentially with ethanol and distilled water, and then lyophilized. PHBHHx content and composition were determined using gas chromatography (GC) after methanolysis of lyophilized cells in chloroform [20]. GC analysis was performed using a Hewlett-Packard 6890 equipped with a 30 m HP-5 capillary column.

Plasmid stability during the flask and fermentation culture was determined as follows: samples were diluted 10^7-10^8 fold using sterile phosphate buffered saline (PBS), and two aliquots (0.2 mL) were cultured on LB plates with 50 μg/mL kanamycin (selective) or without antibiotics (non-selective) for 16 hours. The plasmid stability was determined using the following formula:

$$\text{Plasmid stability (\%)} = \frac{\text{Number of single colonies in selective LB plate}}{\text{Number of single colonies in nonselective LB plate}} \times 100\%$$

Vitreoscilla hemoglobin protein (VHb) was determined following the carbon monoxide

(CO) difference spectra method described by Yu et al. in 2000 [21].

All the data presented in the tables and figures, with the exception of some fermentation results, were the average of three parallel samples.

Result and Discussion

Expression of phbA and phbB in Aeromonas hydrophila 4AK4 (pVGAB) facilitates PHBHHx production and controllable monomer composition. When different ratios of the co-substrates dodecanoate and sodium gluconate (Table 2) were used as carbon sources, both cell growth (pVGAB) and PHBHHx production in flask cultures were effected (Fig. 3). As the proportion of sodium gluconate in carbon sources increased, the mole fraction of 3HHx in PHBHHx synthesized by *A. hydrophila* 4AK4 (pVGAB) decreased, which indicated that sodium gluconate could be used to adjust the monomer composition. This result also indicated that the expression of *phbA* and *phbB* genes were successful, thereby providing an additional pathway of 3HB synthesis from other carbon sources. This result was similar to our previous result [5]. Although increasing the proportion of sodium gluconate in carbon sources lowered the PHBHHx yield, it was the best and most convenient way to control PHBHHx monomer composition. When co-substrates of 1:1 dodecanoate to sodium gluconate were used, the PHBHHx contained 7.4 mol% 3HHx. The properties of this polymer are the most similar to traditional plastics[6] An interesting phenomenon is shown in Figures 3 and 4. When a 1:1 ratio of dodecanoate to sodium gluconate was used, CDW yield increased to 20 gL^{-1} in shake flasks. From an initial pH of 6.5, the final pH in the flask broth was 5.5 when dodecanoate was used as a substrate (Fig. 4). As the proportion of sodium gluconate in the carbon source increased, the final pH in the flask broth also increased, reaching 8.5 when dodecanoate was completely consumed. Thus, the high CDW achieved using a 1:1 ratio of the co-substrates may have resulted from the stabilization of the pH during the flask culturing process. This effect would not be observed in large scale cultures because pH control is automatic in most fermentors, nevertheless, this high CDW could make flask results comparable to those obtained in fermentor vessels.

Final pH differences observed following the flask culture experiment likely resulted from the metabolic pathways processing the alternate substrates. Acetyl-CoA is produced from sodium gluconate via glycolysis and the pyruvate dehydrogenase complex, while this precursor is generated by beta-oxidation of dodecanoate. Excluding reduced cofactors

Table 2. Ratios of dodecanoate to gluconate used as co-substrate for flask culture.

Ratios of DD to Gn (wt:wt)	DD (g/L)	Gn (g/L)	$(NH_4)_2SO_4$ (g/L)
1:0	10	0	3
2:1	30	15	9
1:1	30	30	9
1:2	15	30	9
1:3	15	45	9
0:1	0	45	6

DD: dodecanoate; Gn: sodium gluconate.

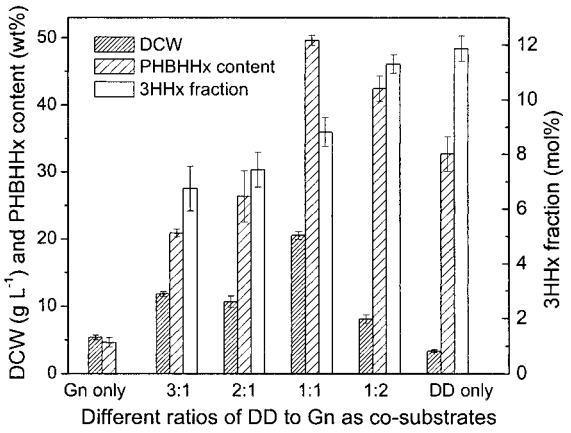

Figure 3. Cell growth and PHBHHx accumulation by *Aeromonas hydrophila* 4AK4 (pVGAB) in 48 h flask cultures using co-substrates containing different ratios of dodecanoate (DD) to sodium gluconate (Gn).

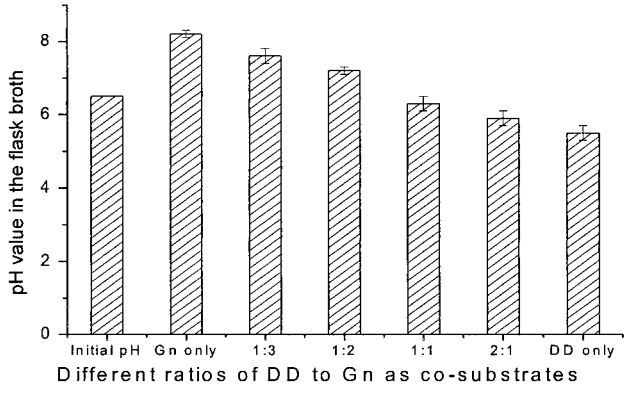

Figure 4. Effect of ratios of dodecanoate to Na-Gluconate on pH in the 48 h flask culture of *A. hydrophila* 4AK4 (pVGAB).

and ATP, one round of glycolysis will produce two moles of acetyl-CoA, two moles of H^+, and two moles of CO_2. Two rounds of β–oxidation produce two moles of acetyl-CoA and two moles of H^+. The carbon dioxide generated by glycolysis will exist as the bicarbonate ion in solution, and contribute to the rise in pH. In *Pseudomonas putida*, 3-hydroxydecanoyl-ACP:CoA transacylase, encoded by the *phaG* gene, has a critical role in channeling the intermediates of fatty acid biosynthesis pathway to PHA production from sugars [22]. A similar pathway could explain our observations, with an intermediate of fatty acid biosynthesis used by the cells when grown on sodium gluconate. Fatty acid biosynthesis consumes H^+, which would further elevate the pH.

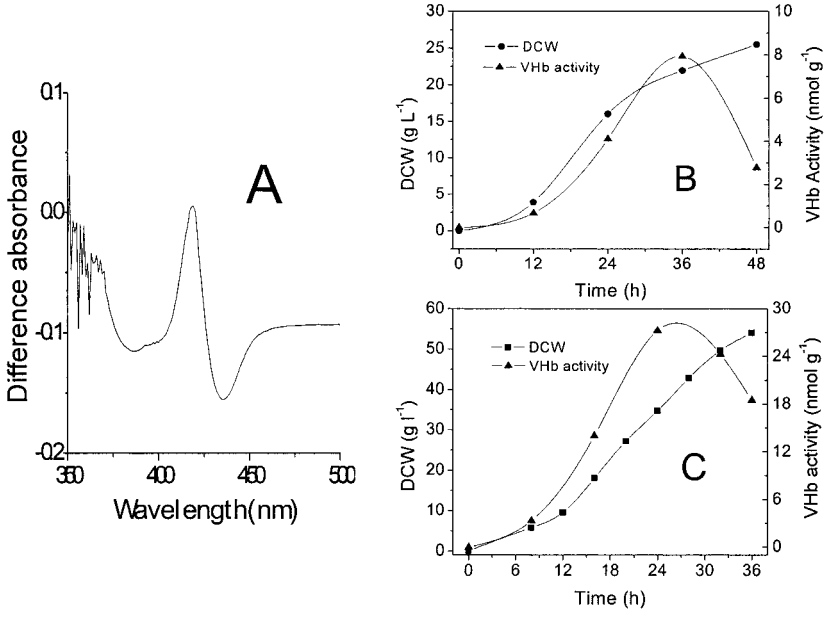

Figure 5. Characteristic peaks of *Vitreoscilla* hemoglobin protein (VHb) in extracts of Aeromonas hydrophila 4AK4 (pVGAB). (A) Carbon monoxide difference spectra; (B) Time profile of VHb generated during flask culturing; (C) Time profile of VHb generated during fermentation in fermentor.

Vitreoscilla hemoglobin gene (vgb) was successfully expressed in Aeromonas hydrophila 4AK4 (pVGAB). A functional assay was employed to monitor *vitreoscilla* hemoglobin (VHb) protein, expressed as nmol protein per gram wet cell (nmol g^{-1}), using the method of CO difference spectra. Figure 5A demonstrates that the *vgb* gene was successfully expressed in *A. hydrophila* 4AK4 (pVGAB), as indicated by the characteristic absorbance of VHb protein at 419 and 436 nm [19].

The time profile of VHb activity during flask (Fig. 5B) and fermentation cultures (Fig. 5C) indicated that cell growth was closely associated with the expression of VHb. This was attributed to the regulation by the *vgb* native promoter which was triggered at a low dissolved oxygen concentration. The maximal VHb activity in the fermentor culture was about twice as high as that observed for the flask culture. Although it is difficult to compare the oxygen concentration in the flask culture and fermentation culture, this result indicated that the expression of the *vgb* gene was promoted in a high cell density culture in which dissolved oxygen was rapidly consumed.

Expression of Vitreoscilla hemoglobin gene (vgb) in Aeromonas hydrophila 4AK4 (pVGAB) enhanced cell growth and PHBHHx production. PHBHHx production by the wild type *A. hydrophila* strain 4AK4, and the recombinant strains 4AK4 (pTG01) and 4AK4 (pVGAB), are shown in Figure 6. In 48 hours of flask culture using 30 g L^{-1} dodecanoate and 30 g L^{-1} sodium gluconate as co-substrates, recombinant *A. hydrophila*

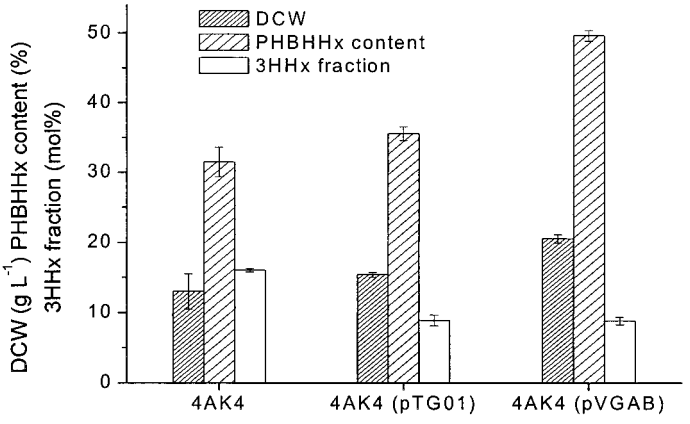

Figure 6. Cell growth and PHBHHx accumulation by wild type *Aeromonas hydrophila* strain 4AK4 (wild-type), and recombinant *A. hydrophila* strains 4AK4 (pTG01) and 4AK4 (pVGAB) using 30 gL^{-1} dodecanoate and 30 gL^{-1} sodium gluconate as carbon sources.

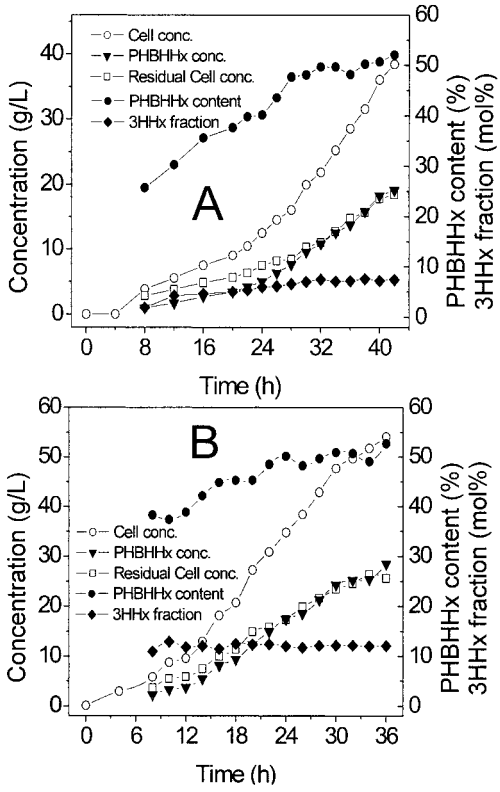

Figure 7. Time profile of cell and residual cell concentration, PHBHHx content, PHBHHx concentration and 3HHx mol faction during culture of *A. hydrophila* 4AK4 (pVGAB). Strains were cultured in a 6 L fermentor using either (A) 1:1 dodecanoate to sodium gluconate or (B) dodecanoate as carbon sources.

4AK4 harboring *phbA*, *phbB* and *vgb* genes [4AK4 (pVGAB)] could produce 20.5 g L^{-1} CDW containing 49.6% PHBHHx, while recombinant *A. hydrophila* 4AK4 harboring *phbA* and *phbB* genes produced 15.4 g L^{-1} CDW containing 35.6% PHBHHx, and the wild type only produced 13.0 g L^{-1} CDW containing 31.5% PHBHHx. These results indicated that the *vgb* gene had a strong positive effect on cell growth and PHBHHx accumulation.

Figure 7 shows the growth and PHBHHx production by recombinant *A. hydrophila* 4AK4 (pVGAB) using different carbon sources in a 6 L fermentor vessel. When a 1:1 ratio of dodecanoate to sodium gluconate was used as the carbon source, 38.4 g L^{-1} CDW containing 52 wt% PHBHHx consisting of 7.4 mol% 3HHx was obtained in 42 hours,

while using dodecanoate as the sole carbon source, 54.0 L^{-1} CDW containing 52.7 wt% PHBHHx with 12.4 mol% 3HHx was produced in 36 hours. These results demonstrated that the introduction of *phaA* and *phaB* genes provided more 3HB for PHBHHx synthesis and that the PHBHHx monomer composition in recombinant *A. hydrophila* 4AK4 (pVGAB) was controllable.

Comparisons between wild type and recombinant *A. hydrophila* 4AK4 are summarized in Table 3. When dodecanoate was used as the sole carbon source, the PHBHHx yield was 0.791 g L^{-1} h^{-1}, the highest achieved in *A. hydrophila* 4AK4 (pVGAB). This result indicated that the new recombinant could efficiently produce PHBHHx with a controllable monomer composition.

Table 3. Comparison of cell growth and PHBHHx production between wild type *A. hydrophila* 4AK4, recombinant *A. hydrophila* 4AK4 (pTG01) and *A. hydrophila* 4AK4 (pVGAB) in a 6 L fermentor vessel.

Strain 4AK4	CDW (g L^{-1})	PHBHHx/CDW (wt%)	3HHx /PHBHHx (mol%)	Productivity (g L^{-1} h^{-1})	Carbon sources (Culture time)
Wild Type	40.4	54.6	14.4	0.525	DD only (42h)
pTG01	32.8	52.0	6.7	0.355	DD:Gn=1:1 (48h)
pTG01	51.5	62.0	9.7	0.570	DD only (56h)
pVGAB	38.4	52.0	7.4	0.475	DD:Gn=1:1 (42 h)
pVGAB	54.0	52.7	12.0	0.791	DD only (36h)

DD: dodecanoate; Gn: sodium gluconate

Vitreoscilla hemoglobin (vgb) expression in Aeromonas hydrophila 4AK4 (pVGAB) enhanced plasmid stability during high cell density culture. An interesting and important feature of the recombinant strain harboring the *vgb* gene was the high plasmid stability observed during cell culture in the absence of antibiotics (Fig. 8). During the first 24 hours, the plasmid stability observed for the two recombinant *A. hydrophila* 4AK4 strains was similar; both gradually dropped from nearly 100% to about 50%. In the next 24 hours, the plasmid stability in the recombinant strain without *vgb* gene continued decreasing to about 38% (Fig. 8A); while in *A. hydrophila* 4AK4 (pVGAB), plasmid stability slightly increased to just over 60% (Fig. 8B).

The expression of *vgb* not only improved the cell growth and PHBHHx production, but also helped maintain the plasmid stability. The expression of the *vgb* gene promoted cell

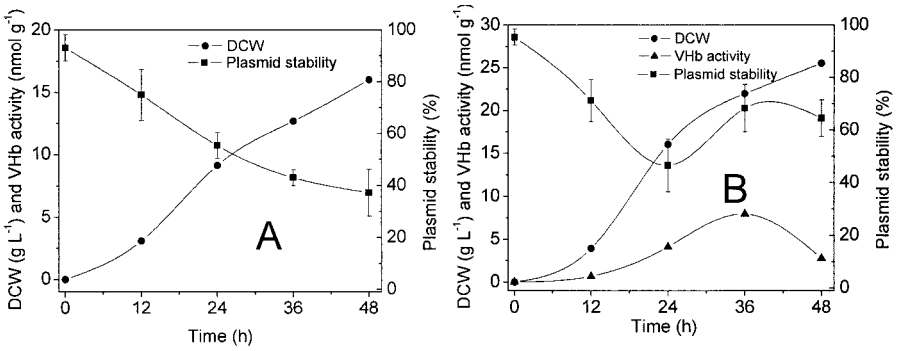

Figure 8. Cell growth, VHb activity and plasmid stability during flask culture using co-substrates of dodecanoate and sodium gluconate as the carbon sources (A) *Aeromonas hydrophila* 4AK4 (pTG01) and (B) *Aeromonas hydrophila* 4AK4 (pVGAB).

growth under oxygen limiting conditions. Thus, as the cell density increased to a high level and oxygen became limiting, recombinant strains harboring *vgb* gene had an advantage over the wild type. This would account for the increased plasmid stability observed for *A. hydrophila* 4AK4 (pVGAB) but not for *A. hydrophila* 4AK4 (pTG01) in the late 24 hours of culturing (Figs. 8-9).

Recombinant strains harboring foreign plasmids tend to lose their plasmids and revert to wild type strains during growth in the absence of selection pressure, such as antibiotics. In industrial scale fermentation using recombinant strains, plasmid stability is always an important factor to consider. In this regard, recombinant *A. hydrophila* 4AK4 (pVGAB) has an advantage. As shown in Figure 9, this recombinant strain had satisfactory plasmid stability (over 90%) in rapid growth culture, which indicates that it has potential use for fermentation process development.

Effect of over-expression of phaC, phaJ or phaP on cell growth and PHBHHx production. PHBHHx production could be further increased to over 60% of CDW by the over expression of *phaC* and *phaJ* from *Aeromonas caviae* encoding PHBHHx synthase and (R)- specific enoyl-CoA hydratase, respectively. Over expression of *phaC, phaJ* or *phaP* alone or in various combinations also resulted in increased 3HHx content from around 14% to over 34% in PHBHHx (Table 4). Expression of phaCJ resulted in the best yield of PHBHHx in the cells. The above results indicated that *A. hydrophila* is a convenient organism for genetic manipulation.

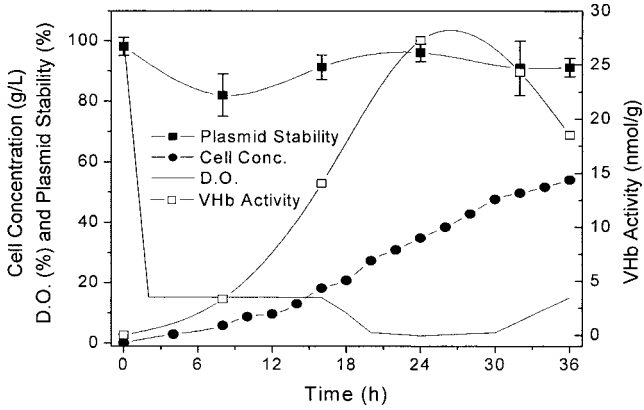

Figure 9. Time profiles of cell growth, DO, plasmid stability and VHb activity during the fermentation culture of *A. hydrophila* 4AK4 (pVGAB) using dodecanoate as the carbon source in a 6 L fermentor vessel.

Table 4. Effect of $phaP_{Ac}$, $phaC_{Ac}$ and $phaJ_{Ac}$ on PHBHHx production by *A. hydrophila* 4AK4 grown in dodecanoate for 48 h.

Strains	PHBHHx (%)	3HHx (mol%)	PHA (g/l)	CDW (g/l)
4AK4	52.57±3.47	14.26±0.29	2.06±0.17	3.91±0.09
($phaP_{Ac}$)	57.81±1.69	17.77±0.28	2.53±0.06	4.38±0.16
($phaPC_{Ac}$)	58.21±1.65	17.59±1.12	2.53±0.07	4.35±0.01
($phaCJ_{Ac}$)	63.48±0.92	22.70±0.62	2.74±0.10	4.31±0.20
($phaPJ_{Ac}$)	60.32±2.34	16.76±0.14	2.66±0.04	4.42±0.10
($phaPCJ_{Ac}$)	62.01±3.85	25.57±0.52	2.85±0.13	4.60±0.13
($phaPC'J_{Ac}$)	63.91±2.44	21.28±0.58	2.75±0.07	4.31±0.11

Conclusion

Aeromonas hydrophila 4AK4 harboring *phbA*, *phbB* and *vgb* showed a remarkable ability to grow and produce PHBHHx. The obvious differences in terms of cell growth and PHBHHx production were attributed to the expression of *vgb* in *A. hydrophila* 4AK4. The expression of *vgb* in the recombinant also increased the plasmid stability during cell growth, especially under low dissolved oxygen tension in fermentors. Over expression of *phaC*, *phaJ* and *phaP*, alone or in various combinations, further increased cell growth, PHBHHx contents and 3HHx content. The above results indicated that *A. hydrophila* is a convenient organism for genetic manipulation and for process development applications.

Acknowledgments

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