0273-2289/00/84–86/0381/$12.50

Construction of Recombinant *Escherichia coli* Strains for Polyhydroxybutyrate Production Using Soy Waste as Nutrient

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Abstract

Construction and comparison of recombinant *Escherichia coli* strains harboring the polyhydroxybutyrate (PHB) operon from *Ralstonia eutropha* using vectors possessing different promotors, as well as the production of PHB from soy waste by the recombinant strain, are reported. The lac promotor was the most efficient on expression of the *phb* operon among the three promotors studied: i.e., lac promotor, T7 promotor and the normal σ^{70} promotor. The pKS/PHB was the most efficient plasmid for phb operon expression among the three plasmids used: i.e., pKS^{-}, pAED4, and pJM9131. It was observed that isopropyl-β-D-thiogalactopyranoside was not required for the induction of the expression of *phb* operon. The cell dry wt and polyhydroxyalkanoate content by *E. coli* XL-1 Blue (pKS/PHB) were 3.025 g/L and 27.83%, respectively.

Index Entries: *Escherichia coli*; polyhydroxybutyrates; lac promotor; T7 promotor; Fourier-transform infrared; soy waste.

Introduction

Polyhydroxyalkanoates (PHAs) are polymers, which are synthesized by many bacteria, and are considered as carbon and energy storage in response to nutrient limitation. They are increasingly attracting public attention for their properties of biodegradability, biocompatibility, and

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optical activity *(1)*. PHAs can be produced from renewable resources and completely mineralized to carbon dioxide and water by a wide variety of microorganisms *(2)*. Polyhydroxybutyrate (PHB) is the most common member of PHAs. It can be synthesized by most of the PHA-producing strains. Copolymer of poly(3-hydroxybutyrate-co-3-hydroxyvalarate) has been developed because it possesses more applicable properties compared to PHB, and it has been introduced into the market as BIOPOL® *(3)*.

Comparing the price of BIOPOL ($16/kg [US$]) to that of polypropylene ($1/kg [US$]), the conventional plastics, the cost of PHAs is much higher. Current advances in fermentation and purification technology as well as the development of superior bacterial strains by recombinant DNA techniques are likely to lower the price of PHA to approx $4/kg (US$) *(3)*. PHB production using recombinant *Escherichia coli* has several advantages, such as a faster growth rate, a larger amount of PHB accumulation, and the availability of a well-established high cell density culture technique *(3)*.

Recombinant *E. coli* strains harboring the PHA synthase gene of *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) in a stable high copy number plasmid have been developed to produce PHB with high PHA content and high productivity *(3)*. Inexpensive carbon substrates, such as CO_2 *(4)*, molasses *(5)*, and xylose *(6)*, have been used to produce PHA by recombinant strains to lower the cost of PHA production.

In our previous work, we successfully produced PHAs from food wastes of malt, soy, ice cream, and confectionery by both pure strains of *Alcaligenes* spp. and activated sludge *(7,8)*. In this article, we report on the construction and comparison of recombinant *E. coli* strains harboring the *R. eutropha* PHB operon using vectors possessing different promotors, as well as the production of PHB from soy waste by the recombinant strain.

Materials and Methods

Bacterial Strains and DNA of Plasmids

Table 1 gives the *E. coli* strains and plasmids used in this study. Cells were maintained in 15% (v/v) glycerol stock at –80°C after growing in Luria-Bertani medium at 37°C overnight. Eighty micrograms per milliliter of ampicillin was added for *E. coli* XL-1 Blue harboring plasmid pKS-PHB and pAED4-PHB, and 50 μg/L of kanamycin was added for *E. coli* HMS174 harboring pJM9131.

Construction of Recombinant E. coli *Strains*

Plasmids were isolated using the Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI). Isolated plasmids and digested DNA fragments were analyzed by electrophoresis in horizontal slab gels containing 0.8% (w/v) agarose, and 1-kb DNA ladder (Promega, Madison, WI) was used as marker. DNA restriction fragments were isolated from agarose gel using the QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA). Restriction enzymes and T4 ligase (Promega) were used

Table 1
E. coli Strains and Plasmids Used

Strains/plasmids	Relevant characteristics	Source
E. coli		
XL-1 Blue	*endA1, hsdR17 (rk^-, mk^+), supE44, thi^-, λ^-, recA1, gyrA96, relAl, (lac), [F', proAB, lacIq, lacAΔM15,* Tn*10*Tc*]*	Stratagene, Cambridge, UK
HMS174	F^-, *met*B, *hsd*S, *sup*E, *sup*F	Ref. *14*
BL21(DE3)	F^-, *hsd*R, *rec*A, *Rif* R	Ref. *13*
Plasmid		
pKS–	2.96 kb, Amp^r (80 μg/mL), lac promotor	Stratagene
pAED4	3.32 kb, Amp^r (80 μg/mL), T7 promotor	Dr. Paul Matsudaira
pKS/PHB	8.2 kb, phb operon, Amp^r (80 μg/mL), lac promotor	This study
PAED4/PHB	8.8 kb, phb operon, Amp^r (80 μg/mL), T7 promotor	This study
pJM9131	8.55 kb, phb operon, Kan^r (50 μg/mL), its own σ^{70} promotor	Dr. Douglas D. Dennies

according to instructions provided by the supplier. A 5.2-kb *Eco*RI/*Hin*dIII restriction fragment comprising the entire *phb* operon obtained from plasmid pJM9131 was used as an insert, and plasmid pKS– carrying the lac promotor and plasmid pAED4 carrying the T7 promotor were employed as vectors. Ligation products were mixed with competent cells of *E. coli* XL-1 Blue for pKS– and *E. coli* BL21 (DE3) for pAED4, and transformants were selected on nutrient agar plates containing 80 μg/mL of ampicillin. The positive transformants were checked by restriction digestions followed by agarose gel electrophoresis and confirmed by Fourier transform infrared (FTIR) analysis.

Culture Conditions

Glycerol stocks of cells were used directly as an inoculum in all experiments. They were first inoculated into 5 mL of 2XYT growth phase media (1.6 g of tryptone, 1.0 g of yeast extract, 0.5 g of NaCl in 100 mL of distilled water) in universal bottles. Then 2 mL of the culture was transferred into 100 mL of R2T production media (20 g of glucose, 2 g of tryptone, 2 g of $[NH_4]_2SO_4$, 13.3 g of KH_2PO_4, 1.7 g of citric acid, and 1.2 g of $MgSO_4 \cdot 7H_2O$ in 1 L of distilled water with 1 mL of sterilized trace element added consisting of 1.62 g of $FeCl_3 \cdot 6H_2O$, 1.33 g of $CaCl_2 \cdot H_2O$, 0.025 g of $CoCl_2 \cdot 6H_2O$, 0.016 g of $CuSO_4 \cdot 5H_2O$, 0.012 g of $NiCl_2 \cdot 6H_2O$, and 0.01 g of $CrCl_2 \cdot H_2O$ in 100 mL 0.1 *N* HCl). Antibiotics were added according to the demand of different strains. Cultures were grown on a rotary shaker at 200 rpm and 37°C.

Batch fermentation of recombinant *E. coli* XL1-Blue using soya waste was carried out using a 3-L bioreactor (Bioengineering). Soya waste was hydrolyzed using 0.5 *M* HCl at 95°C for 8 h, filtered to remove most of the soya debris, followed by adjusting to pH 7.0 using 10 *M* NaOH and centri-

fuged at 14,333 g for 20 min to obtain clean hydrolysate. Yeast extract (1.5 g/L) and ampicillin (80 μg/mL) were added. One hundred milliliters of seed culture of *E. coli* XL1-Blue (pKS/PHB) (4% of fermentation media) was inoculated.

Analytical Procedure

Cell density was determined by measuring optical density (OD) at 600 nm. Cell dry wt (CDW) was determined as follows: Five milliliters of samples were filtered with a vacuum in a predried and preweighted 0.45-μm membrane filter. The residues were washed with 0.9% NaCl and then dried at 95°C. PHA content was obtained by recovery of the dry cells as described by Hahn et al. *(9)*. Total organic carbon (TOC) source was measured using an ASTRO 2000 TOC analyzer. Total Kjeldahl nitrogen (TKN) was obtained using a KJELTEC AUTO 1030 analyzer. Glucose concentrations were measured with a Glucose Biochemistry Analyzer 2700 Select.

FTIR Analysis of PHB Using Intact Cells

PHB was determined by FTIR analysis according to Hong et al. *(10)*. Cultures of 2–5 mL were centrifuged at 2831*g* for 15 min. The cell pellets were spread to a dot of 3–5 mm in diameter on a window of ZnSe. A mirror was used to give reflected infrared to the horizontally laid window. With a scan of 16, resolution of 4, and auto gain, spectra were recorded at wave numbers from 4000 to 400 cm^{-1} using a Mangna-IR spectrometer 750 (Nicolet).

Results and Discussion

Construction of Recombinant Strains

Using a 5.2 kb *Eco*RI/*Hin*dIII restriction fragment comprising the entire *phb* operon obtained from plasmid pJM9131 as an insert, and plasmid pKS–possessing a lac promotor and plasmid operon under control of different promotors were obtained. These constructions were confirmed by restriction digestions and electrophoresis in the agarose gel and by FTIR pAED4 possessing a T7 promotor as expression vectors whereas pKS possess a Lac promotor. Two 5.2-kb fragments carrying the *phb* operon, a 2.96-kb fragment for the vector of pKS^- and a 3.32-kb fragment for vector of pAED4 were observed on the agarose gel (Fig. 1). The characteristic band of PHB around a wave number of 1730 cm^{-1} *(10)* appeared on the FTIR spectra of these cells (Fig. 2). These results showed that the *phb* operon has been successfully inserted into the vectors and expressed in *E. coli* cells.

Comparison of the Recombinant E. coli *Strains Harboring Various Plasmids*

Comparing the yield of PHB produced by the two newly constructed strains and the formerly obtained strain from results of a flask experiment, the *E. coli* XL-1 Blue harboring plasmid pKS/PHB accumulated the highest

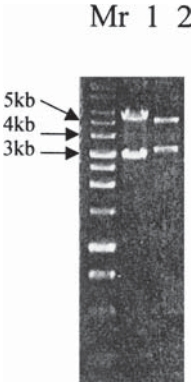

Fig. 1. Agarose gel of DNA electrophoresis after double restriction digestion showing the insert of the 5.2-kb phb operon and the two vectors. Lane 1, pKS/PHB; lane 2, pAED4/PHB.

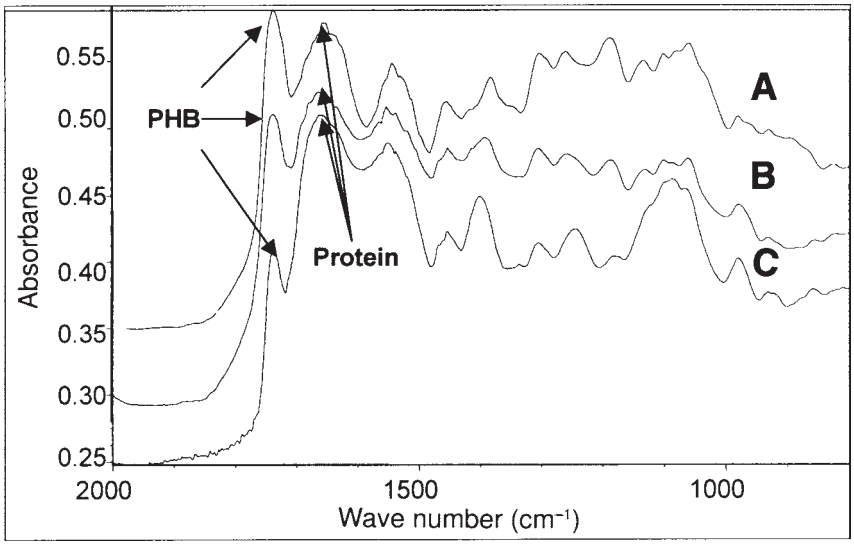

Fig. 2. FTIR spectra of the recombinant cells grown on R2T production media, showing the characteristic band of PHB on each of the spectrums. A, Spectrum of cell of *E. coli* XL-1 Blue (pKS/PHB, lac promotor); B, Spectrum of cell of *E. coli* BL21(DE3) (pAED4/PHB, T7 promotor); C, Spectrum of cell of *E. coli* HMS174 (pJM9131, its own σ^{70} promotor).

amount of PHB, according to the height ratio of PHB to protein shown on the absorbance spectra of FTIR (Fig. 2), and PHB recovery (Table 2). Two reasons might account for this. First, the expression efficiency of *phb* operon that depended on the promotors was different among the three strains. pKS/PHB possessed the lac promotor whereas the pAED4/PHB possessed the T7 promotor and pJM9131 carried its own σ^{70} promotor. It was observed that lac promotor was the most efficient on expression of *phb* operon among these three promotors. pKS/PHB was the most efficient plasmid for

Table 2
PHB Recovery (percentage of PHB/100g of cell dry wt)
in the Three Recombinant *E. coli* Strains
During Various Time Intervals of Fermentation

Time (h)	*E. coli* XL-1 Blue (pKS/PHB, lac promotor)	*E. coli* BL21(DE3) (pAED4/PHB, T7 promotor)	*E. coli* HMS174 (pJM9131, σ^{70} promotor)
6	9.46	5.56	NA
12	14.48	6.78	NA
18	23.45	11.16	NA
24	15.81	7.59	10.1
30	12.01	5.13	NA
36	NA	NA	6.88
48	11.76	NA	3.32

NA, not available.

phb operon expression among these three plasmids, and therefore the largest amount of PHB was produced. Second, the PHB synthesis in different *E. coli* strains was different because the metabolite may be different in *E. coli* XL1-Blue and *E. coli* BL21. The *E. coli* XL-1 Blue has been reported to be the best *E. coli* strain for accumulating PHB *(11)*. Plasmid pKS/PHB was also more stable than plasmid pAED4/PHB during the generation transfer. The strain containing pAED4/PHB was found to have no expression of PHB after several generations.

The lysogen BL21(DE3) contains a single copy of the gene of T7 RNA polymerase in the chromosome under control of the inducible lacUV 5 promotor. Thus, theoretically, the expression of *phb* operon, which linked to a T7 promotor in plasmid pAED4/PHB, should require the induction by isopropyl-β-D-thiogalactopyranoside (IPTG). But only a small difference was observed with and without the use of IPTG as an inducer during the course of PHB production by *E. coli* BL21(DE3) (pAED4/PHB) (Table 3). This phenomenon was also observed for the strain *E. coli* XL-1 Blue harboring pKS/PHB carrying a lac promotor during PHB production using food waste as nutrient (Fig. 3). This indicated that it might not be necessary to add IPTG during PHB production. It has been reported that the enzymes for PHA synthesis were constitutively expressed from its own promotor in *E. coli (12)*, and "leakage" of lac promotor and T7 promotor have also been reported *(13)*. Thus, leakage of the two promotors might be responsible for the constitutive expression of *phb* operon. This was useful for industrialized PHB production, since IPTG is expensive and would increase the cost of PHB production.

Production of PHB from Soy Waste Using Recombinant Strain of E. coli *XL-1 Blue (pKS/PHB) in a 3-L Fermentor*

According to the height ratio of PHB band to protein band on FTIR absorbance spectra, optimal PHB content occurred after 9 h of fermentation

Table 3
PHB Production by Recombinant *E. coli* BL21(DE3) (pAED4/PHB) With and Without Induction by IPTG

Time after adding IPTG (h)	With IPTG				Without IPTG			
	Cell dry wt (g/L)	PHB (g/L)	Non-PHB cell dry wt (g/L)	PHB content (%)	Cell dry wt (g/L)	PHB (g/L)	Non-PHB cell dry wt (g/L)	PHB content (%)
6	2.98	0.166	2.187	5.56	2.968	0.14	2.828	4.72
12	2.933	0.199	2.734	6.87	2.981	0.17	2.811	5.70
18	2.492	0.278	2.214	11.16	2.497	0.25	2.246	10.05
24	0.1858	0.141	1.717	7.59	2.501	0.20	2.298	8.12

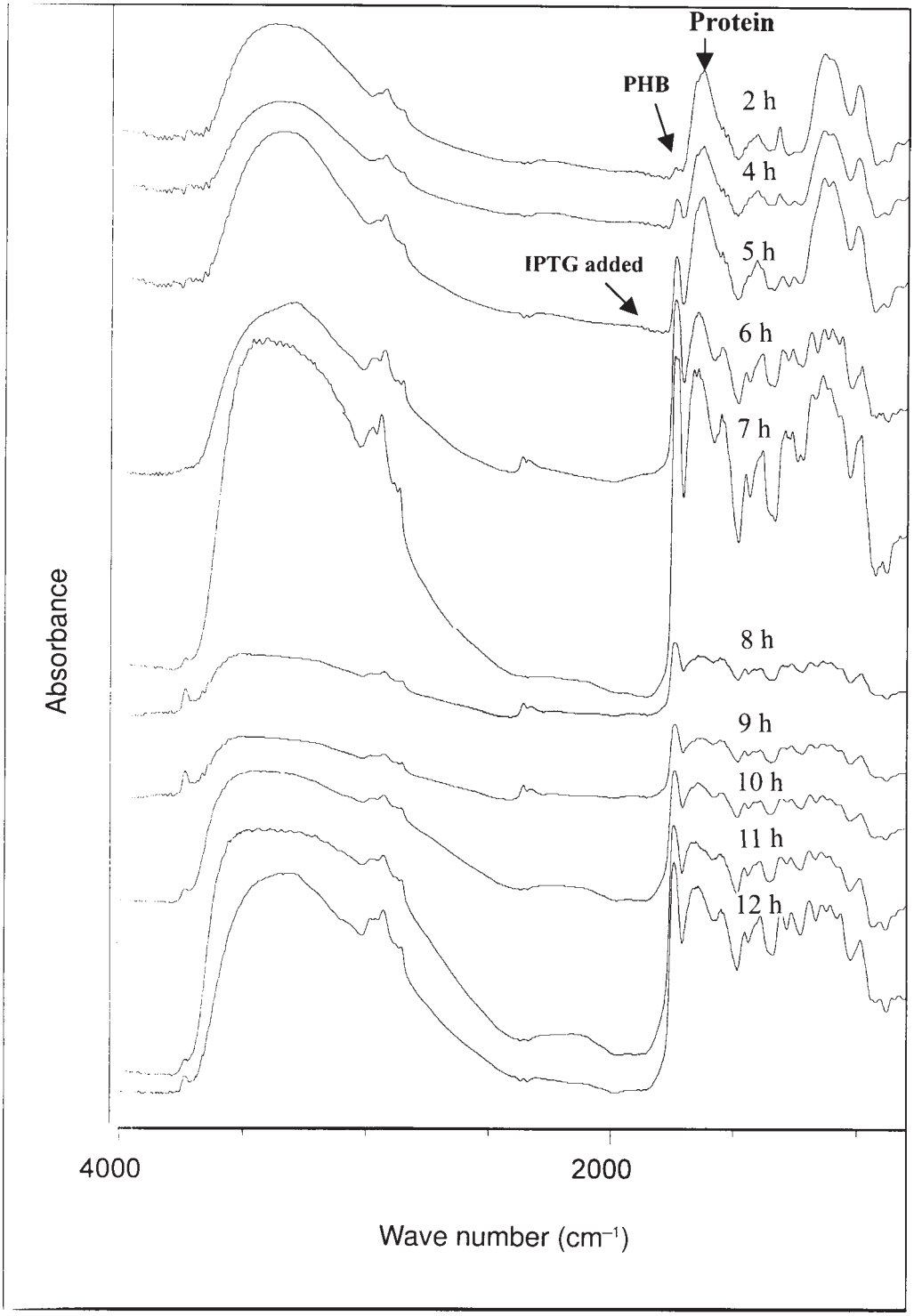

Fig. 3. FT-R spectra of cells collected at intervals of fermentation of PHB production by recombinant strain of *E. coli* XL-1 Blue (pKS/PHB) using soy waste as nutrient.

Table 4
Ratio of PHB Band to Height
of Protein Band on FTIR Absorbance Spectra

Time (h)	Height of PHB band:height of protein band
6	1.043
7	1.028
8	1.357
9	1.358
10	1.082
11	1.176
12	1.163

Table 5
Production of PHB from Soy Waste Using Recombinant Strain
of *E. coli* XL-1 Blue (pKS/PHB) in a 3-L Fermentor

Time (h)	OD600	Cell dry wt (g/L)	TOC (g/L)	TKN (g/L)	Glucose (g/L)
0	0	/	17.52	2.27	0.722
2	0.23	/	17.98	2.39	/
4	0.85	/	18.19	2.35	0.694
5	1.55	/	18.03	2.69	0.622
6	2.07	1.52	17.01	2.36	0.317
7	2.11	1.63	16.80	2.36	0.179
8	2.07	1.76	17.18	2.30	0.25
9	2.72	1.96	17.00	2.38	0.10
10	2.74	2.11	16.88	2.33	0.095
11	2.65	1.75	15.50	2.30	0.097
12	2.6	1.76	15.71	2.29	0.101
13	/	/	15.70	2.26	0.1

(Fig. 3, Table 4). IPTG was added after 5 h of fermentation. The induction of IPTG was not so significant because a small amount of PHB had been accumulated before the addition of IPTG (Fig. 3). This phenomenon was similar to that of the strain harboring pAED4/PHB.

Two PHB harvests were conducted at 9 h and 13 h. The cell dry weights were nearly the same at these two time points (3.025 and 3.032 g/L, respectively), but PHB contents were 27.83 and 24.64%, respectively, and non-PHB cell dry weights were 2.183 and 2.285 g/L each. The results might indicate that PHB consumption occurred after 9 h, at the time when glucose was nearly zero in the medium while maintaining the cell dry wt. The results of the fermentation of PHB from soy waste showed that the cell dry wt data were low but that TOC and TKN were considerably high at the course of fermentation (Table 5). This might be owing to the fact that some carbon sources were not easily consumed by the recombinant strain, according to the results of high TOC but low glucose and low cell dry wt.

According to the results of the PHB production using soy waste, the yield of PHB produced by the recombinant strain was low, but the levels of TOC and TKN were still high at harvest time. Therefore, efforts may be made to improve the consumption of other carbon sources besides glucose while using food waste as substrate.

Acknowledgment

We wish to express our sincere gratitude to the Hong Kong Polytechnic University and the University Grant Council of Hong Kong for the support of a grant for this research.

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