Production of poly-β-hydroxybutyrate on molasses by recombinant *Escherichia coli*

Fang Liu, Wenqing Li, Darin Ridgway, and Tingyue Gu*

Department of Chemical Engineering, Ohio University, Athens, OH 45701, USA. Fax: (740)593-0873;
E-mail: gu@ohiou.edu

Zhongyao Shen

Department of Chemical Engineering, Tsinghua University, Beijing 100084, P. R. China

Beet molasses successfully replaced glucose as sole carbon source to produce poly-β-hydroxybutyrate by a recombinant *Escherichia coli* strain (HMS174/pTZ18u-PHB). The fermentation with molasses was cheaper than with glucose. The final dry cell weight, PHB content and PHB productivity were 39.5 g/L, 80% (w/w) and 1 g/Lh, respectively, in a 5 L stirred tank fermenter after 31.5 h fed-batch fermentation with constant pH and dissolved O_2 content.

Introduction

Poly-β-hydroxybutyrate (PHB) is a microbial polyester produced by many bacteria and stored in cells in the form of granules (Bradel and Reichert, 1993). It is a candidate for the synthesis of environmentally benign, biodegradable plastics (Suzuki *et al.*, 1986). The cost of PHB production is rather high thus hindering its practical applications. Despite many cost-cutting efforts in the past few years, the price of "Biopol" (a commercial PHB/V copolymer) is still high, $4–8/kg compared to $0.6–0.9/kg for conventional synthetic plastics (Schubert *et al.*, 1991; Li *et al.*, 1997). The cost of the carbon source contributes significantly to the overall production costs. For the process with recombinant *E. coli*, the cost of the carbon source is as high as 30.7% of the total cost (Choi *et al.*, 1997).

Recombinant *E. coli* considered a good candidate for PHB production. High cell density *E. coli* strains have been reported (Kim *et al.*, 1992; Lee *et al.*, 1994). Unfortunately, they require expensive Luria-Bertani (LB) medium, ampicillin, and pure O_2. These are not practical in large-scale industrial production due to high costs. Recently, it was reported that some recombinant *E.coli* strains could produce PHB from whey (Lee *et al.*, 1997). Molasses, which is a common industrial by-product, is much cheaper than glucose, and has been extensively used as a carbon source in yeast fermentation. Reports on using molasses as the sole carbon source to produce PHB by *Alcaligenes eutrophus* have also appeared in the literature in recent years (Beaulieu *et al.*, 1995; Li *et al.*, 1997).

We have previously constructed a recombinant *E. coli* HMS174/pTZ18u-PHB which can produce PHB on glucose mineral medium without the use of antibiotics during fermentation. The research reported here investigated the possibility of adding beet molasses as the sole carbon and energy source to a mineral medium to produce PHB using this *E. coli* strain in a 5 L stirred tank fermenter.

Materials and methods
Plasmid and microorganism
The recombinant *E. coli* strain HMS174/pTZ18u-PHB was used. The strain contains the plasmid pTZ18u-PHB, which carries *A. eutrophus* PHB biosynthesis genes (phbA, phbB, and phbC), and ampicillin resistance.

Culture media and conditions
Inocula of the recombinant *E. coli* were prepared by cultivating the cells at 37°C and 150 rpm for 12 h in a 300 mL shake flask containing 50 mL of the nutrient-rich medium with 100 μg ampicillin/mL. Each liter of the medium contained: 1 g glucose, 1 g yeast extract, 5 g polypeptone, 3 g beef extract, and 5 g NaCl. The fermentation medium was a mineral medium supplemented with suitable amount of molasses. Each liter of the medium contained: 4.8 g $Na_2HPO_4 \cdot 12H_2O$, 2.65 g KH_2PO_4, 4 g $(NH_4)_2SO_4$, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.01 g $CaCl_2 \cdot 2H_2O$, and 1 mL trace element solution. Each liter of the trace element solution contained: 0.3 g H_3BO_3, 0.2 g $CoCl_2 \cdot 6H_2O$, 30 mg $ZnSO_4 \cdot 7H_2O$, 30 mg $MnCl_2 \cdot 4H_2O$, 30 mg $NaMoO_4 \cdot 2H_2O$, 20 mg

NiCl$_2$·6H$_2$O, and 10 mg CuSO$_4$·5H$_2$O. The medium was sterilized for 20 min at 121°C. Molasses was hydrolyzed at 100°C and pH 1.5 (adjusted with 98% sulphuric acid) for 30 min. Then, pH was adjusted to 7.5 with Ca(OH)$_2$ after cooling to about 60°C. 12 h later it was centrifuged at 6000 rpm for 10 min to remove the precipitated CaSO$_4$. The supernatant was sterilized for 30 min at 112°C and added to the medium at room temperature. Before sterilization, the pH of the medium was adjusted to 7.0 with 2 M NaOH.

The shake flask culture was carried out in 300 mL shake flasks filled with 75 mL of the medium. The fed-batch culture was carried out in a 5 L microprocessor-controlled fermenter. The initial volume was 1.5 L with 26.5 g pretreated beet molasses/L. The inoculum size was 10% of the fermenter's working volume. To keep the DO level above 20%, the agitation speed was increased from 300 rpm to 850 rpm. The temperature, aeration rate were controlled at 37°C and 1.5 vvm, respectively. The pH was maintained at 6.8 by adding 4 M NaOH/KOH.

Analytical methods

Cell growth was monitored using optical density measured by a spectrophotometer at 600 nm. Dry cell weight was determined by centrifuging 1 mL of sample, washing with acetone once to remove moisture, then drying at 60°C for 24 h. The dried cell mass was analyzed for its PHB concentration by degrading PHB to crotonic acid at 90 °C for 1 h with 98% sulphuric acid, and then measuring the absorbance of the crotonic acid at 208 nm. PHB content was defined as the percentage of PHB weight to dry cell weight. Residual biomass was equal to dry cell weight minus PHB weight. Molasses concentration (expressed in reducing sugar concentration) was assayed with 3, 5-dinitrosalicylic acid method (Zhang, 1986).

Results and discussion
Utilization of different carbon sources

Beet molasses contains 30–50% (w/v) sucrose. The sucrose decomposes to glucose and fructose after hydrolysis. To confirm the feasibility of using molasses to replace glucose in the production of PHB by recombinant *E. coli*, dry cell weight and PHB accumulation were determined on different carbon sources in shake flask cultures. It was found that the cells could efficiently utilize glucose (99%), fructose (97%) and sucrose hydrolyzate (96%), but the utilization efficiency on sucrose was very low (20%). Therefore, beet molasses must be hydrolyzed before use.

Influence of molasses concentration on cell growth and PHB biosynthesis

Experimental data on dry cell weight, PHB content and reducing sugar utilization efficiency under different initial molasses concentrations were compared (Table 1). It was found that cell growth (residual biomass) at initial molasses concentrations of 40 g/L and 60 g/L was lower than that at 20 g/L roughly before 45 h. This suggests that the system has substrate inhibition. The higher the molasses concentration, the stronger the inhibition. With the use of the reducing sugar, the inhibition gradually disappeared around 45 h, and the residual biomass increased considerably and reached their maximum values after about 80 h. However, the PHB accumulation at initial molasses concentrations of 40 g/L and 60 g/L exceeded that at 20 g/L after about 15–20 h. The maximum PHB content under 20 g/L, 40 g/L, and 60 g/L reducing sugar concentrations were 68%, 77%, and 85%, respectively. It was shown that a lower molasses concentration promoted the growth of the recombinant *E. coli*, while a higher molasses concentration enhanced PHB synthesis.

Fed-batch culture in shake flasks

As shown in Table 2, three fed-batch methods were compared with batch cultures with initial molasses concentrations of 20 g/L and 40 g/L. The molasses was fed at three different times during fermentation.

It was found that after 68 h of cultivation, the dry cell weight, PHB content, and reducing sugar utilization efficiency of the first and second fed-batch strategies were similar to those of the batch culture using 40 g/L initial molasses concentration, but the dry cell weight, PHB content, and reducing sugar utilization efficiency of the third strategy were similar to those of the batch culture using 20 g/L initial molasses concentration. This indicated that the feed time had a significant effect on the cell growth. The molasses should be added before 20 h. If it was fed after 20 h, the cells could not utilize it efficiently. This is probably because the ability of the cells to divide

Table 1 Comparison of different initial molasses concentrations.

Molasses concentration (g/L)	Maximum dry cell weight (g/L)	Maximum residual biomass (g/L)	Maximum PHB content (% w/w)	Reducing sugar utilization efficiency (% w/w)
20	6.5	2.4	68	93
40	12.3	3.5	77	89
60	16.7	4.1	85	87

Table 2 Different fed-batch strategies in shake flask.

Culture method	1	2	3	4	5
Initial molasses concentration (g/L)	20	20	20	20	40
Time at first feeding (h)	4.5	6.5	20	–	–
Time at second feeding (h)	8	10	32	–	–
Time at third feeding (h)	10	20	44	–	–
Fed molasses at first feeding (g/L)	10	10	10	–	–
Fed molasses at second feeding (g/L)	5	5	5	–	–
Fed molasses at third feeding (g/L)	5	5	5	–	–
Dry cell weight at 68 h (g/L)	11.2	10.9	4.7	5.5	10.5
PHB content (% w/w)	80	81	75	67	79
Reducing sugar utilization efficiency (% w/w)	91	92	43	41	91

Numbers in brackets are relative yields calculated by GC/MS analysis.

was affected when a large number of PHB granules accumulated inside the cells. The higher the PHB content, the lower the cell growth rate. After 20 h cultivation with 20 g/L initial molasses concentration, the PHB content was above 60% which is close to its maximum (Table 1). The cell division ability and the cell growth rate reduced such that the cells could no longer utilize the fed molasses to increase the cell mass and to synthesize PHB. Therefore, the selection of suitable feeding time was important for obtaining optimal cell growth and PHB synthesis.

pH-DO-stat fed-batch culture in a stirred tank fermenter

A pH-DO-stat (i.e., constant pH and DO) fed-batch culture was carried out in a 5 L stirred tank fermenter to achieve a high cell density and PHB production with molasses. Molasses and ammonium sulfate were fed into the reactor intermittently by monitoring DO and pH as feedback parameters. When either the carbon source or nitrogen source became exhausted, pH and DO values rose quickly. Molasses and ammonium sulfate were then added manually. The molasses and ammonium sulfate concentrations in the feeding solution were 332 g/L and 200 g/L, respectively. The results are shown in Figure 1.

At the beginning of culture, the molasses concentration in the medium was 26.5 g/L. Before 12 h, pH and DO were kept constant, the culture was carried out in a batch mode. When pH and DO rose rapidly at around 12 h, it was switched to a fed-batch culture mode. To confirm the effect of high molasses concentrations on the PHB accumulation, the molasses concentration in the culture broth was gradually increased to about 144 g/L at 19 h. It was observed that the residual biomass was inhibited by high molasses concentrations. However, PHB content increased until the end of the culture. The final dry cell weight, PHB content, PHB productivity, and reducing sugar utilization efficiency were 39.5 g/L, 80%, 1 g/Lh , and 76%, respectively.
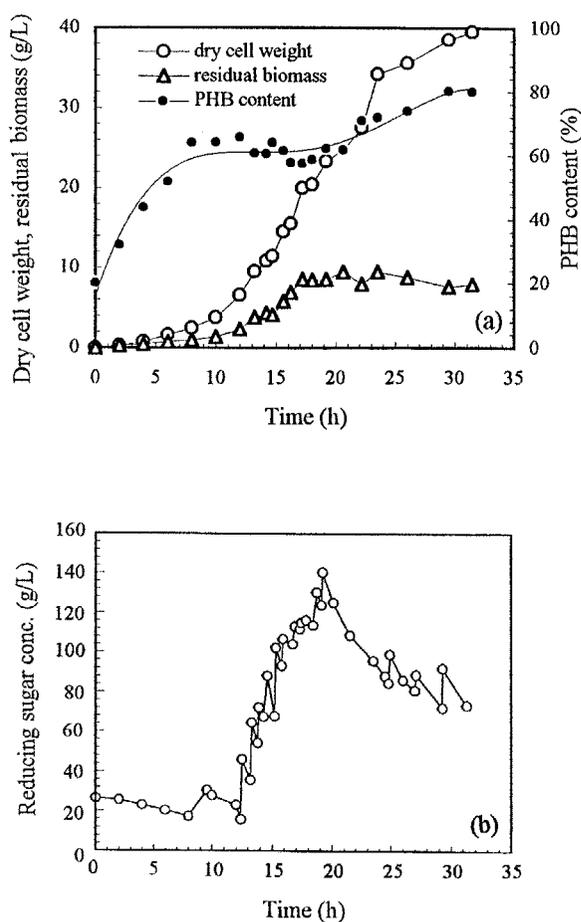

Figure 1 pH-DO-stat fed-batch culture of recombinant *E. coli* in a 5 L fermenter: (a) dry cell weight, PHB content, and residual biomass vs. time; (b) reducing sugar concentration vs. time.

Conclusions

The experimental results demonstrated that recombinant *E. coli* HMS174/pTZ18u-PHB could utilize molasses effi-

ciently as the sole carbon source instead of glucose to produce PHB, but molasses must be hydrolyzed to glucose and fructose before use. The molasses concentration had a significant effect on the synthesis of PHB and the growth of cells. High molasses concentrations yielded high PHB contents. Enhanced cell density and PHB productivity were achieved by pH-DO-stat fed-batch culture in a 5 L stirred tank fermenter. A fed-batch feeding strategy was developed to improve the cell growth and the production of PHB by overcoming substrate inhibition.

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