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Enhanced yield of medium-chain-length polyhydroxyalkanoates from nonanoic acid by co-feeding glucose in carbon-limited, fed-batch culture

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ABSTRACT

Medium-chain-length polyhydroxyalkanoates (MCL-PHAs) were produced in carbon-limited, single-stage, fed-batch fermentations of *Pseudomonas putida* KT2440 by co-feeding nonanoic acid (NA) and glucose (G) to enhance the yield of PHA from NA. An exponential ($\mu = 0.25\ h^{-1}$) followed by a linear feeding strategy at a NA:G ratio of 1:1 (w/w) achieved 71 g l^{-1} biomass containing 56% PHA. Although the same overall PHA productivity (1.44 g l^{-1} h^{-1}) was obtained when NA alone was fed at the same specific growth rate, the overall yield of PHA from NA increased by 25% (0.66 g PHA g NA^{-1} versus 0.53 g g^{-1}) with glucose co-feeding. Further increasing glucose in the feed (NA:G = 1:1.5) resulted in a slightly higher yield (0.69 g PHA g NA^{-1}) but lower PHA content (48%) and productivity (1.16 g l^{-1} h^{-1}). There was very little change in the PHA composition.

1. Introduction

Polyhydroxyalkanoates (PHAs) are biocompatible, biodegradable polyesters that are synthesized by many microorganisms. PHAs are categorized as short-chain-length (SCL) containing three to five carbons in their repeating units, as medium-chain-length (MCL) containing six or more monomeric carbons, and as SCL–MCL with both SCL and MCL repeating units. SCL-PHAs, such as poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), and SCL–MCL-PHAs, such as poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) can be produced on an industrial scale (Chen et al., 2001; Lee et al., 1999). However, the production of MCL-PHAs remains at the lab scale, mainly due to the high cost and toxic nature of the most suitable carbon substrates, which have until recently required complicated fermentation control if commercially acceptable product concentrations were to be attained (Sun et al., 2007b).

Increasing the yield of PHA from an aliphatic carbon source can greatly reduce the cost of production. In a two-stage, fed-batch fermentation study, we found that the PHA yield from nonanoic acid almost doubled during nitrogen-limited PHA accumulation when glucose was a co-substrate (Sun et al., 2007c). We also found that high density culture of *P. putida* KT2440 containing up to 75% MCL-PHA can be achieved using nonanoic acid as the sole carbon source during single-stage, carbon-limited, exponential growth (Sun et al., 2007a). This approach eliminates the need for sophisticated control of the carbon source concentration. This study investigates the possibility of using glucose as a co-substrate in such a single-stage, nonanoate-limited process in an attempt to reduce the amount of nonanoic acid needed for PHA production.

2. Materials and methods

2.1. Microorganism and growth medium

Pseudomonas putida KT2440 (ATCC 47054) was maintained on nutrient agar plates at 4 °C. The inoculum medium contained per liter: 4.70 g $(NH_4)_2SO_4$, 0.80 g $MgSO_4{\cdot}7H_2O$, 12.00 g $Na_2HPO_4{\cdot}7H_2O$, 2.70 g KH_2PO_4, 9 g glucose, 1 g nutrient broth. The initial fermentation medium contained per liter: 4.70 g $(NH_4)_2SO_4$, 0.80 g $MgSO_4{\cdot}7H_2O$, 9.00 g $Na_2HPO_4{\cdot}7H_2O$, 2.03 g KH_2PO_4, 1 g carbon substrates in total and 10 ml trace element solution. Each liter of trace element solution contained 10 g $FeSO_4{\cdot}7H_2O$, 3 g $CaCl_2{\cdot}2H_2O$, 2.2 g $ZnSO_4{\cdot}7H_2O$, 0.5 g $MnSO_4{\cdot}4H_2O$, 0.3 g H_3BO_3, 0.2 g $CoCl_2{\cdot}6H_2O$, 0.15 g $Na_2MoO_4{\cdot}2H_2O$, 0.02 g $NiCl_2{\cdot}6H_2O$ and 1.00 g $CuSO_4{\cdot}5H_2O$. Nonanoic acid (98%, Spectrum Chemicals) was fed in its pure form. A glucose (99.5%, Sigma–Aldrich) solution of 640 g l^{-1} was fed separately as it is immiscible with nonanoic acid. Nitrogen was supplied by using 14% (w/v) NH_4OH solution for pH control. To avoid initial precipitation of medium components, addi-

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tional $MgSO_4 \cdot 7H_2O$ (33 g l^{-1}) was mixed in the glucose feed based on a $Y_{X/Mg}$ of 240 g g^{-1} (Sun et al., 2006).

2.2. Fermentation conditions

The inoculum was pooled from two 500 ml Erlenmeyer flasks containing 150 ml of medium at 30 ± 1 °C and 200 rpm for about 12 h. Fed-batch fermentations were done at 28 ± 1 °C with 3.0 l initial working volume in a Minifors 5 l stirred tank bioreactor (Infors HT, Bottmingen, Switzerland). Data acquisition (dissolved oxygen, exit gas CO_2 content, substrate and base addition, and pH) and control was conducted with LabVIEW 6.1 (National Instruments). pH was controlled at 6.85 ± 0.05. Dissolved oxygen was measured with an Ingold polarographic probe and maintained above or at 40% air saturation (except where indicated) by gradually adjusting the agitation speed up to 1200 rpm and by automatically adjusting the flow of a mixture of air and pure oxygen via mass flow controllers while maintaining the total gas flow at 1 vvm. Exit gas CO_2 (%) was measured with an infrared CO_2 monitor (Guardian Plus, Topac Inc., Hingham, MA, USA) as a direct indication of culture activity. Control of nonanoic acid and glucose feeding was based on the mass of the substrate reservoirs. Antifoam 204 (Sigma–Aldrich) was injected manually through a sterile septum as necessary.

2.3. Substrate feeding and control methods

A total of 1 g l^{-1} of nonanoic acid (NA) and glucose (G) was added to the initial fermentation medium. The cumulative mass of carbon substrate to be fed at time t followed exponential growth according to Eq. (1).

$$\Delta S_t = \frac{\Delta X_t}{Y_{X/C}} = \frac{X_0}{Y_{X/C}}(e^{\mu t} - 1) \quad (1)$$

where

$$Y_{X/C} = Y_{X/NA} \cdot f_{NA} + Y_{X/G} \cdot f_G \quad (2)$$

ΔS_t (g) is the total carbon source required to produce biomass ΔX_t (g) at culture time t (h); $Y_{X/C}$, $Y_{X/NA}$, and $Y_{X/G}$ are the yields (g g^{-1}) of biomass from total carbon, nonanoic acid, and glucose, respectively; X_0 (g) is the initial biomass; μ (h^{-1}) is the desired specific growth rate; f_{NA} and f_G are the pre-defined fraction of nonanoic acid and glucose respectively in terms of the total mass of carbon substrates in the feed. Assuming $Y_{X/NA}$ = 0.83 g g^{-1} and $Y_{X/G}$ = 0.41 g g^{-1} (Sun et al., 2007a, 2006), $Y_{X/C}$ was calculated as 0.62 g g^{-1} for the NA:G = 1:1 (f_{NA} = 0.5 and f_G = 0.5) fermentation; and 0.58 g g^{-1} for the NA:G = 1:1.5 (f_{NA} = 0.4 and f_G = 0.6) fermentation. X_0 was set as 0.30 g (0.10 g l^{-1}) based on the amount of biomass in the inoculum and the desired μ was 0.25 h^{-1}.

The amount of each carbon substrate (nonanoic acid or glucose) required was calculated individually according to the pre-defined fraction:

$$\Delta S_{t\text{-}NA} = \Delta S_t \cdot f_{NA} \quad (3)$$

$$\Delta S_{t\text{-}G} = \Delta S_t \cdot f_G \quad (4)$$

$\Delta S_{t\text{-}NA}$ and $\Delta S_{t\text{-}G}$ (g) are the total nonanoic acid and glucose required at cultivation time t, respectively.

Fermentations with different ratios of nonanoic acid to glucose, NA:G = 1:1 and NA:G = 1:1.5, were conducted. During the NA:G = 1:1 fermentation (f_{NA} = 0.5 and f_G = 0.5), after exponentially co-feeding according to Eq. (1) for 24.9 h, the substrate feed rate (F, g l^{-1} h^{-1}) was set at 17 g l^{-1} h^{-1} till the end of the fermentation (27.3 h). The NA:G = 1:1.5 fermentation (f_{NA} = 0.4 and f_G = 0.6) was performed using only the exponential feeding strategy (Eq. (1)) until 26.45 h.

2.4. Analytical procedures

Cell dry weight was determined gravimetrically with lyophilized biomass which was obtained after centrifugation of 5 ml culture broth at 10,000 × g for 15 min and washing with distilled water. The supernatant of the centrifuged broth was frozen for later analysis of the major nutrients, nonanoic acid, and glucose. Phosphate, ammonium and glucose concentrations were determined as described by Sun et al. (2006). Nonanoic acid concentration was determined by GC analysis after methylation (Ramsay et al., 1991), using benzoic acid as the internal standard. Samples for GC analysis of cellular PHA content and composition were prepared as described by Sun et al. (2007a) and analyzed with a gas chromatograph (CP-3800, Varian Inc.) with an FID. For PHA analysis, the GC parameters were: injector temperature 250 °C, detector temperature 275 °C, 1 μl injection, and a split ratio of 10. The oven temperature profile was: 90 °C for 0.5 min, 6 °C min^{-1} to 96 °C, 7 °C min^{-1} to 131 °C, 20 °C min^{-1} to 181 °C for 5 min. The MCL-PHA standard for the GC analysis was prepared as described by Jiang et al. (2006). Its composition was confirmed by GC–MS and determined by NMR to be 65 mol% 3-OH-nonanoate (C9) and 35 mol% 3-OH-heptanoate (C7). The SCL-PHA standard (Biopol, Zeneca Bioproducts Inc.) had 81 and 19 mol% HB and HV, respectively.

PHA from harvested and dried biomass was extracted in acetone and purified (Jiang et al., 2006). The molecular weight of the extracted polymer was analyzed using a Waters 2695 Gel Permeation Chromatograph (GPC) with polystyrene standards and distilled tetrahydrofuran as solvent. Differential scanning calorimetry (DSC) to determine the glass transition temperature (T_g) and melting temperature (T_m) of the PHA produced employed a heating profile of 5 °C min^{-1} from −70 °C to 80 °C.

All analyses were in duplicate and the averages presented in the paper.

3. Results

3.1. Nonanoic acid and glucose co-feeding for MCL-PHA production

In fixed growth rate carbon-limited, fed-batch fermentations of *P. putida*, the growth rate is determined by the rate of feeding of the carbon and energy sources. The observation that both glucose and nonanoic acid concentrations increase gradually throughout the fermentations (Figs. 1 and 2) indicates that more carbon and energy is required to maintain the specific growth rate at a con-

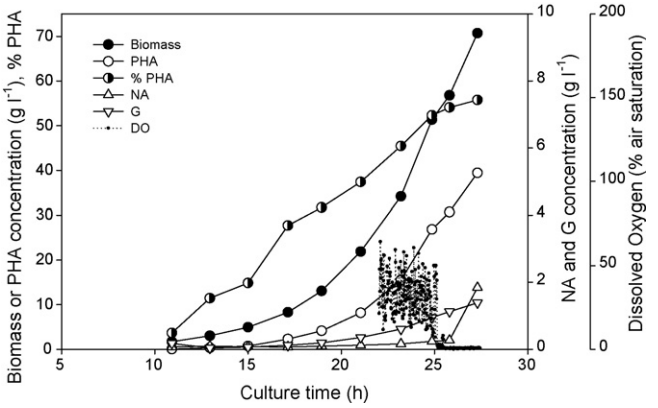

Fig. 1. Single-stage MCL-PHA production by *P. putida* KT2440 using an exponential co-feeding strategy (Eq. (1) where μ = 0.25 h^{-1}) with nonanoic acid (NA) and glucose (G) at a NA:G ratio of 1:1 (w/w) until 24.9 h followed by linear feeding at a constant rate of 17 g l^{-1} h^{-1}.