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High-Cell-Density Cultivation of *Pseudomonas putida* IPT 046 and Medium-Chain-Length Polyhydroxyalkanoate Production From Sugarcane Carbohydrates

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Abstract

We studied high-density cultures of *Pseudomonas putida* IPT 046 for the production of medium-chain-length polyhydroxyalkanoates (PHA_{MCL}) using an equimolar mixture of glucose and fructose as carbon sources. Kinetics studies of *P. putida* growth resulted in a maximum specific growth rate of 0.65 h^{-1}. Limitation and inhibition owing to NH_4^+ ions were observed, respectively, at 400 and 3500 mg of NH_4^+/L. The minimum concentration of dissolved oxygen in the broth must be 15% of saturation. Fed-batch strategies for high-cell-density cultivation were proposed. Pulse feed followed by constant feed produced a cell concentration of 32 g/L in 18 h of fermentation and low PHA_{MCL} content. Constant feed produced a cell concentration of 35 g/L, obtained in 27 h of fermentation, with up to 15% PHA_{MCL}. Exponential feed produced a cell concentration of 30 g/L in 20 h of fermentation and low PHA_{MCL} content. Using the last strategy, 21% PHA_{MCL} was produced during a period of 34 h of fed-batch operation, with a final cell concentration of 40 g/L and NH_4^+ limitation. Using phosphate limitation, 50 g/L cell concentration, 63% PHA_{MCL} and a productivity of 0.8 g/(L·h) were obtained in 42 h

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of fed-batch operation. The PHA_{MCL} yield factors from consumed carbohydrate for N-limited and P-limited experiments were, respectively, 0.15 and 0.19 g/g.

Index Entries: Polyhydroxyalkanoate; *Pseudomonas putida*; high cell-density; sugarcane; kinetics studies.

Introduction

Microorganisms produce polyhydroxyalkanoates (PHAs), usually as carbon and energy reserve granules, under conditions of nutritional limitation of N, P, S, or Mg and in excess carbon source *(1)*. PHA is a biodegradable polymer that can be used in several applications such as in the manufacture of bottles, films, and fibers for biodegradable packaging. The latex of PHA can be applied to the surface of paper or cardboard to form a water-resistant layer. PHA also finds a vast field of applications in the medical area such as in the manufacture of bony plates, surgical sutures, and controlled drug-release preparations *(2)*.

Historically, poly(3-hydroxybutyrate) (PHB) has been the most studied in this class of biodegradable polymers, and copolymers of PHB can be formed starting from the addition of another substrate resulting in polymers containing 3-hydroxyvalerate or 4-hydroxybutyrate. These polymers form a class known as short-chain-length PHA (PHA_{SCL})-containing hydroxy acid monomers with three- to five-carbon length. Medium-chain-length PHAs (PHA_{MCL}) are produced by *Pseudomonas sensu stricto* using several carbon sources from agricultural origin and also petrochemical compounds (alkanes, carboxylic acids, alcohols, and carbohydrates, among others) and have attracted great interest because several different hydroxy acid monomers with 6- to 14-carbon length can be incorporated into such polymers *(3)*. Preusting et al. *(4)* used a two-phase continuous system to obtain PHA_{MCL} from octane with a productivity of about 0.18 g of PHA/(L·h). Preusting et al. *(5)* obtained in a similar system productivity ranging from 0.45 to 0.58 g of PHA/(L·h). Recently, Jung et al. *(6)*, using a two-stage continuous process, obtained a productivity of 1.06 g of PHA/(L·h), the highest value of PHA_{MCL} productivity reported for a continuous system.

In two-stage fed-batch cultivation using glucose in the growth phase and octanoate as main carbon source in the accumulation phase, Kim et al. *(7)* showed a production of 55 g/L of dry biomass and productivity of 0.92 g of PHA/(L·h). De Koning et al. *(8)* cultivated *Pseudomonas putida* in a 300-L fed-batch bioreactor using a mixture of fatty acids as carbon source and obtained a cell concentration of 40 g/L with a productivity of 0.40 g of PHA/(L·h). Weusthuis et al. *(9)* cultivated a *P. putida* strain using oleic acid as carbon source in a fed-batch process and obtained a 92 g/L cell concentration with a PHA_{MCL} productivity of 1.6 g/(L·h). The highest productivity value in a fed-batch cultivation for PHA_{MCL} production was reported by Lee et al. *(10)*, who obtained 1.9 g/(L·h) using a *P. putida* strain, phosphate limitation, and oleic acid as carbon source. Few studies have been carried out concerning PHA_{MCL} production from carbohydrates.

Table 1
Culture Medium for *P. putida* IPT 046 Bioreactor Experiments, With a Working Volume of 8.0 L

Component	Batch	Initial batch	Fed batch, Strategy I	Fed batch, Strategies II and III
Glucose	20–160 g	80 g	570 g	680 g
Fructose	20–160 g	80 g	570 g	680 g
KH_2PO_4	1.4–11.2 g	12 g	33.4 g	38 g
Na_2HPO_4	—	28 g	—	—
$(NH_4)_2SO_4$	10.5–83.7 g	24 g	41.9 g	45.0 g
$MgSO_4 \cdot 7H_2O$	0.7–11.2 g	1.6 g	17.7 g	21.0
$CaCl_2 \cdot 2H_2O$	0.007–0.17 g	0.08 g	0.158 g	0.180
Ammoniac ferric citrate	0.02–0.17 g	0.48 g	0.51 g	0.58
Trace element solution	16 mL	8 mL	84 mL	84 mL

Sugarcane hydrolysate carbohydrates presented as an equimolar mixture of glucose and fructose can be used to produce PHA industrially *(11)*. Recently, our group isolated a *P. putida* strain, named IPT 046, able to consume glucose and fructose and to accumulate up to 60% PHA_{MCL} in shaker flask experiments *(12)*. The characterization of produced PHA_{MCL} has been described *(13)*.

The objective of the present investigation was to study the bioreactor cultivation performance of *P. putida* IPT 046 in order to obtain high cellular density. This was done by testing the possibility of PHA_{MCL} production from an equimolar mixture of glucose and fructose as the only carbon sources.

Materials and Methods

Microorganism

P. putida IPT 046 *(12)* was maintained as frozen dried cells in glycerol at –80°C.

Culture Conditions

Inocula were prepared by suspension and incubation of plated *P. putida* IPT 046 cells in nutrient agar for 48 h at 30°C, followed by transfer to nutrient broth and incubation for 9 h in a shaker (250 rpm, 30°C). Transfer (10% [v/v]) and incubation for 14 h (250 rpm, 30°C) in minimal culture medium were carried out in the culture medium as shown in Table 1.

An inoculum volume was transferred to the bioreactors to obtain an initial cell concentration of about 0.2 g dry wt/L. Experiments in the bioreactors used an 8.0-L working volume. The mechanically stirred bioreactors (10-L volume) were Biostat ED (B. Braun Biotech.) and Microferm Fermentor (New Brunswick Scientific). Each bioreactor was coupled to a Hartmann-Braun outlet gas analyzer for CO_2 and O_2 analysis.

The temperature was 30°C and the pH was maintained at 7.0 by the addition of 4 *N* H_2SO_4 and 4 *N* NaOH to the batch experiments, and NH_4OH solution (7% [v/v]) to the fed-batch experiments.

Batch experiments were performed varying the initial carbohydrate concentration and proportionally the other culture medium composition as shown in Table 1. Fed-batch experiments used three distinct feed strategies, as explained in Theoretical Calculation. These procedures started with an initial batch and the utilized culture medium in this phase is presented in Table 1. The amounts of mineral culture medium components added for these strategies are presented in Table 1. The trace element solution consisted of 0.3 g/L of H_2BO_4, 0.2 g/L of $CoCl_2 \cdot 6H_2O$, 0.1 g/L of $ZnSO_4 \cdot 7H_2O$, 0.03 g/L of $NaMoO_4 \cdot 2H_2O$, 0.2 g/L of $NiCl_2 \cdot 6H_2O$, 0.01 g/L of $CuSO_4 \cdot 5H_2O$, and 0.03 g/L of $MnCl_2 \cdot 6H_2O$. Carbohydrate feed concentration was 350 g/L (glucose = 175 g/L and fructose = 175 g/L) for pulse-feed procedure (strategy I) and 500 g/L (glucose = 250 g/L and fructose = 250 g/L) for strategies II and III.

The accumulation phase of the experiments started by changing the pH control solution from NH_4OH to NaOH to induce a nitrogen-limited culture, or by using a limited amount of phosphate in the culture. Culture media composition for the N-limited and P-limited experiments is described in Table 1. In the phosphorous-limited experiment, the amount of KH_2PO_4 was reduced to 21.6 g. The same concentrated carbohydrate solution, 700 g/L (glucose = 350 g/L and fructose = 350 g/L), was fed to the bioreactor at a specific feed rate of *Vesp* = 0.22 g of carbohydrate/(g of residual biomass·h), after exhaustion of nitrogen or phosphorus, calculated as in Eq. 1 (see Theoretical Calculation).

Analytical Methods

The cell concentration was determined by centrifugation (10,000*g*, 10 min^{-1}), and the pellet was recovered by filtration through a previously weighed membrane (0.45 μm pore; Millipore) and dried to a constant weight at 105°C. The supernatant of the centrifuged material was used to determine glucose; fructose; acetic, pyruvic, and butyric acid; and ammonium concentrations. Glucose and fructose were evaluated using high-performance liquid chromatography, organic acids using gas chromatography (GC) (HP 6890 series II equipped with column HP-5), and ammonium nitrogen using the Kjeldahl method *(14)*. PHA_{MCL} concentration was attained by propanolysis of previously weighed lyophilized cells followed by GC *(15)*. The residual cell concentration was calculated as the difference between cell concentration and PHA_{MCL} concentration. Phosphorous concentration was evaluated according to Franson *(16)*.

Theoretical Calculation

Specific Feed Rate

The specific feed rate was calculated as Eq. 1:

$$Vesp = \phi S_O / XrV \quad (1)$$

Dissolved Oxygen

A Monod relationship between specific growth rate and dissolved oxygen (DO) concentration as a percentage of air saturation O_2 can be written as follows:

$$\mu_{xr1} = \mu_{max} \frac{O_2}{O_2 + K_{O2}} \tag{2}$$

High Cell-Density

The following strategies were used to attain high cell-density: strategy I, pulse feed followed by a constant feed; strategy II, constant flow feed; strategy III, exponential flow feed. For strategy II, the carbohydrate mass balance in the bioreactor gives

$$ds/dt = \phi S_O - Vr_{SX} \tag{3}$$

If there is no accumulation of substrate, $ds/dt = 0$, then Eq. 3 becomes

$$\phi S_O = Vr_{SX} \tag{4}$$

Equation 4 can be written as

$$\phi = VdX_r/dt/S_O Y_{X/S} \tag{5}$$

For constant feed rate, Eq. 5 might be integrated to give

$$x_r = x_{rO} + Y_{Xr/S}\phi S_O t \tag{6}$$

At a constant feed rate and no accumulation of substrate, a linear growth characterized by a constant growth rate dx_r/dt occurred. To set up the experimental flow rate ϕ, two constraints were used:
(1) The constant growth rate is limited by the maximum specific fructose uptake rate, because it is assumed that $ds/dt = 0$. Therefore,

$$dX_r/dt = \mu_{Xr2} X_r \tag{7}$$

(2) The constant growth rate is limited by the amount of available oxygen in the bulk phase. Therefore,

$$dX_r/dt = k_{La}(O_2^* - O_{2CRIT})Y_{Xr/O}/V \tag{8}$$

For strategy III, the carbohydrate mass balance in the bioreactor gives

$$\phi = \mu_{Xr2} X_O V_O \exp(\mu_{Xr2} t)/(S_O - S)Y_{Xr/G+F} \tag{9}$$

Table 2 summarizes the parameters used for the described strategies I–III.

Results

Effects of Carbohydrates

Experiments with increasing initial carbohydrate concentrations were run to identify the effects of carbohydrate concentration on bacterial

Table 2
Summary of Parameters Used for Feed Strategy Procedures

Parameter	Strategy I: pulse + constant feed	Strategy II: constant feed	Strategy III: exponential feed
μ_{Xr1}	0.6 h^{-1}		
μ_{Xr2}	0.2 h^{-1}	0.2 h^{-1}	0.2 h^{-1}
$Y_{Xr/G}$	0.45 g/g		
$Y_{Xr/F}$	0.33 g/g		
$Y_{Xr/G+F}$		0.4 g/g	0.4 g/g
Lag phase	2 h		
V_O	7.0 L	6.0 L	6.0 L
X_O	0.2 g/L	7.0 g/L	7.0 g/L
S_O	350 g/L	500 g/L	500 g/L
S_I			4.0 g/L
$K_{La\max}$	1200 h^{-1}	1200 h^{-1}	1200 h^{-1}
O_{2CRIT}	14.6%	14.6%	14.6%
$Y_{Xr/O}$	0.0264 g/(mmol·L)	0.0264 g/(mmol·L)	0.0264 g/(mmol·L)

growth. Preferential consumption of the sugars was observed in all experiments. An example of this behavior is the experiment with a 10 g/L initial carbohydrate concentration. Preferential consumption of glucose occurred up to 6 h of fermentation, and fructose as the second carbon source was totally consumed only when the glucose had been exhausted from the culture media (Fig. 1A). During this period, the culture media supported exponential growth up to 2 g of biomass/L for a fermentation time of 6 to 7 h with almost no fructose consumption with μ_{Xr1} = 0.63 h^{-1}. The growth rate sharply decreased to μ_{Xr2} = 0.23 h^{-1} until fructose exhaustion with an increase in cell concentration up to 3.9 g of biomass/L (Fig. 1B).

The same behavior was observed for the other carbohydrate concentrations. Independent experiments with glucose or fructose as the sole carbon source confirmed the differences in μ_{Xr} on these carbohydrates (Table 3). Table 3 summarizes the results concerning the effects of initial carbohydrate concentration on *P. putida* IPT 046 in batch cultivation.

A lower carbohydrate concentration yielded a lower cell concentration, but the sugar consumption pattern was almost the same, with preferential and more effective growth on glucose than on fructose. The maximum specific growth and consumption rate on carbohydrates were obtained for glucose + fructose culture media with an initial sugar concentration of 10–20 g/L. In this situation, μ_{Xr1} was 0.61–0.65 h^{-1}, and the specific glucose consumption rate was μ_G of 1.47 h^{-1}, these being the maximum values observed for these parameters.

Growth solely on 15 g/L of glucose or on 15 g/L of fructose confirmed preferential consumption of the former. A lower value of μ_{Xr2} for fructose (0.14 h^{-1}) was attained, compared with glucose. The specific carbohydrate consumption rate for fructose (μ_F of 0.36 h^{-1}) was also much lower than for

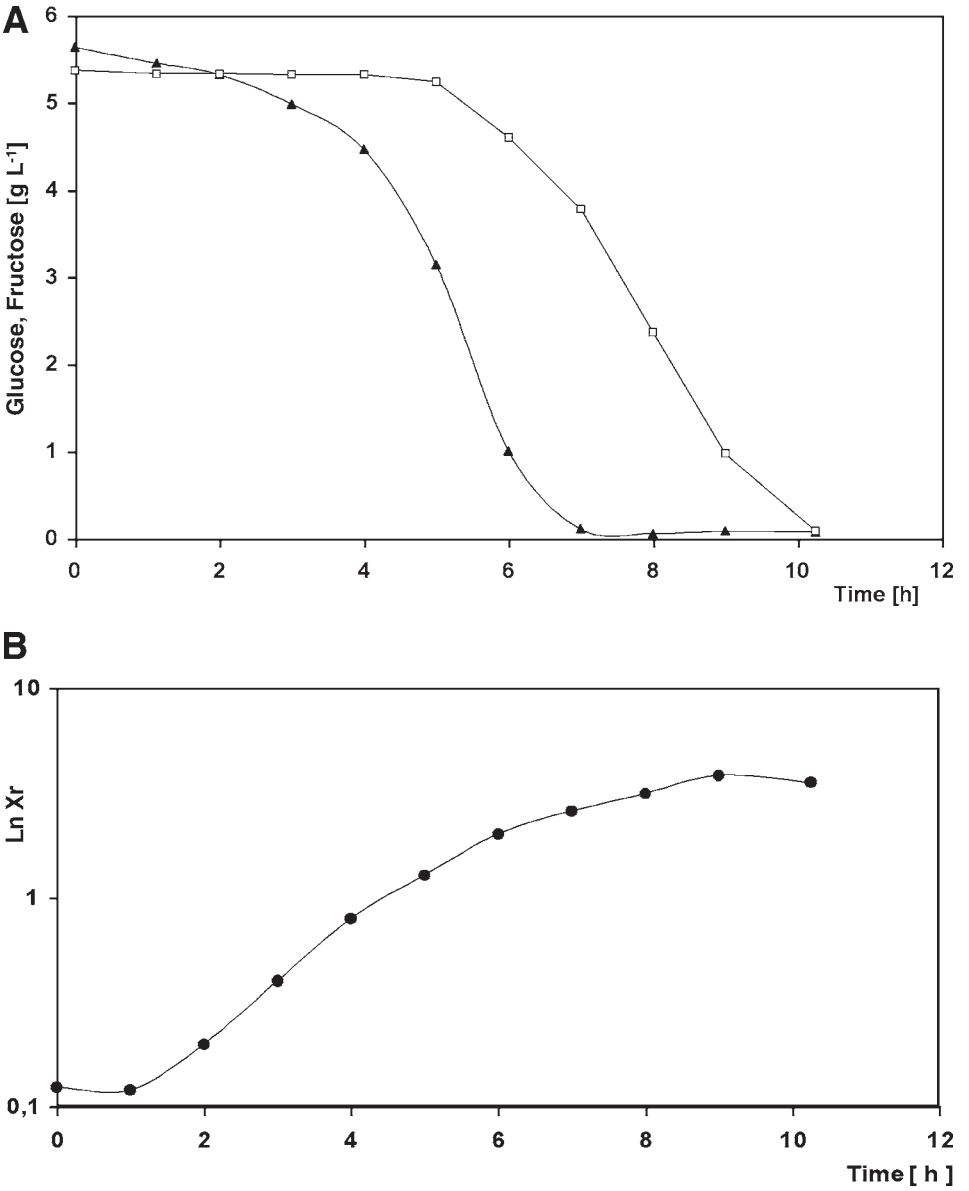

Fig. 1. **(A)** Glucose (▲) and fructose (□) consumption and **(B)** growth evolution of *P. putida* IPT 046 in culture medium with initial glucose plus fructose concentration (1:1) of 10 g/L.

glucose (μ_G of 1.30 h^{-1}). Regardless of the initial carbohydrate concentration used, a lag phase above 2 h was always observed.

Biomass productivity (P_{Xr}) increased with initial carbohydrate concentration up to an initial glucose + fructose concentration of 30 g/L, reaching 10.2 g of cells/L at 11.4 h of fermentation time, with P_{Xr} = 1.0 g of cells/(L·h).

Table 3
Effect of Initial Carbohydrate Concentration on Growth Parameters of *P. putida* IPT 046 Batch Cultivation

Carbohydrate concentration (g/L)	μ_{Xr1} (h^{-1})	μ_{Xr2} (h^{-1})	μ_G (h^{-1})	μ_F (h^{-1})	$Y_{Xr/G}$ (g/g)	$Y_{Xr/F}$ (g/g)	Lag phase (h)	*Xr* final (g/L)	Time (h)	P_{Xr} (g/L·h)
G + F = 5	0.52	0.20	1.32	0.45	0.40	0.44	2	2.3	8.00	0.28
G + F = 10	0.63	0.26	1.34	0.50	0.51	0.61	2	3.9	9.00	0.43
G = 15	0.54	—	1.30	—	0.38	—	2	6.0	8.50	0.71
G + F = 15	0.61	—	0.94	0.40	0.52	0.32	2	5.6	11.25	0.50
F = 15	—	0.14	—	0.36	—	0.42	4	6.1	22.5	0.27
G + F = 20	0.65	0.20	1.47	0.38	0.47	0.38	2	7.5	11.00	0.68
G + F = 30	0.50	N.I.	0.64	0.36	0.33	0.18	2	10.2	11.42	0.89
G + F = 40	0.43	N.I.	0.92	0.29	0.56	0.15	5	14.4	17.00	0.84

[a]G, glucose; F, fructose.
[b]N.I., exponential growth not identified.

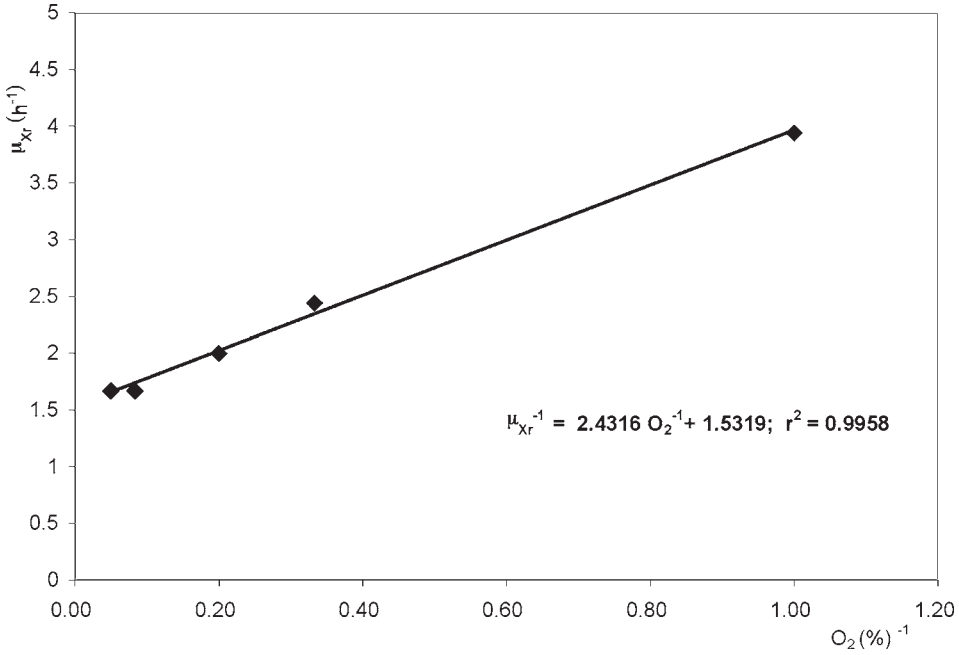

Fig. 2. Lineweaver-Burke plot for specific growth rate, μ_X, and DO concentration, O_2, according to Eq. 1.

Interestingly, the biomass yield from carbohydrate was 0.3–0.6 g/g, with no evident correlation with initial sugar concentration. For the highest initial sugar concentration (40 g/L) used, fructose biomass yields ($Y_{Xr/F}$) were very low, <0.2 g of cells/g of consumed fructose, and glucose biomass yields ($Y_{Xr/G}$) were very substantially high, over 0.5 g of cells/g of consumed glucose. Nevertheless, the calculated mean value of carbohydrate-to-biomass yield ($Y_{Xr/G+F}$) for the experiments was about 0.4 g of biomass/g of consumed carbon source (Table 3).

Intracellular PHA_{MCL} content in all batch experiments was always below detectable levels. In addition, no detectable amounts of organic acids were found during the experiments, indicating no catabolic effects for *P. putida* IPT 046.

Effects of DO and NH_4^+

Experiments with different values of DO concentration and initial NH_4^+ concentration were run to identify the effects on bacterial growth.

A Lineweaver-Burke plot of μ_{Xr1} against O_2 from experiments run at 1, 3, 5, 12, and 20% DO concentration showed that a Monod relationship fitted the experimental data (Fig. 2). The values of the estimated parameters were $\mu_{MAX} = 0.65\ h^{-1}$ and $K_{O_2} = 1.46\%$. Critical DO concentration (O_{2CRIT}) estimated as 10 K_{O_2} was 14.6% of air saturation.

For 1 and 3% DO concentrations, larger lag phases were observed. P_{Xr} and μ_{Xr1} were harmed by the lack of oxygen dissolved in the culture

Table 4
Effect of NH_4^+ and DO Concentration on Growth Parameters for Batch Cultivation of *P. putida* IPT 046

NH_4^+ (mg/L) or O_2 (%)	μ_{Xr1} (h^{-1})	μ_N (h^{-1})	$Y_{Xr/N}$ (g/g)	q_{O2} (mmol/[g·h])	q_{CO2} (mmol/[g·h])	$Y_{X/O}$ (g/mmol)
NH_4^+ = 400	0.60	0.21	5.52	22.7	25.0	0.0264
NH_4^+ = 1500	0.65	0.10	5.50	14.30	15.70	0.0414
NH_4^+ = 3500	0.51	0.11	4.63	10.00	7.60	0.0510
O_2 = 1.0	0.25		8.65			
O_2 = 3.0	0.41		7.82			
O_2 = 5.0	0.50		8.28			
O_2 = 12.0	0.60		5.85			
O_2 = 20.0	0.65		5.50			

media. The experiment with a 5% DO concentration presented a lag phase of 3 h (1 h more than the standard experiment) and $\mu_{Xr1} = 0.50\ h^{-1}$, less than for the standard condition, $\mu_{Xr1} = 0.65\ h^{-1}$. Nevertheless, a limitation of DO concentration down to 1% saturation did not induce PHA or organic acid accumulation. The qO_2 and qCO_2 determinations were not reliable because of extreme variation in airflow rate during the experiments.

Initial NH_4^+ concentrations of 400, 1500, and 3500 mg/L showed that excess ammonium ions may influence negatively *P. putida* growth. A reduction in growth parameters (μ_{Xr1}, μ_N, qO_2, qCO_2) and nitrogen yield factor was observed with increasing ammonium concentrations, suggesting an optimum value of about 400 mg/L (Table 4).

Cultivation at High Cell-Density

High-cell-density assays were carried out in light of the aforementioned experiments. DO concentration was maintained above the estimated O_{2CRIT} concentration, 15% of air saturation, by adjusting airflow rate, agitation frequency, and carbohydrate flow rate. Ammonium concentration was maintained above 400 mg/L using an NH_4OH aqueous solution (7% [v/v]) supplemented to the bioreactor using the automatic pH control. Carbohydrate limitation and inhibition levels were assumed to be 5 and 20 g/L, respectively. Further experimental conditions are described in Materials and Methods.

Pulse Feed Followed by Constant Feed

Strategy I was set up in order to obtain a carbohydrate nonlimited, noninhibitory situation until the maximum oxygen transfer of the bioreactor was attained. At first, a batch with an initial glucose + fructose concentration of 20 g/L was processed until glucose exhaustion at 9 h. At this point, successive carbohydrate pulses were fed to the bioreactor until maximum system aeration/agitation was achieved. This was reached soon after the third carbohydrate pulse at 14 h. Afterward, a constant car-

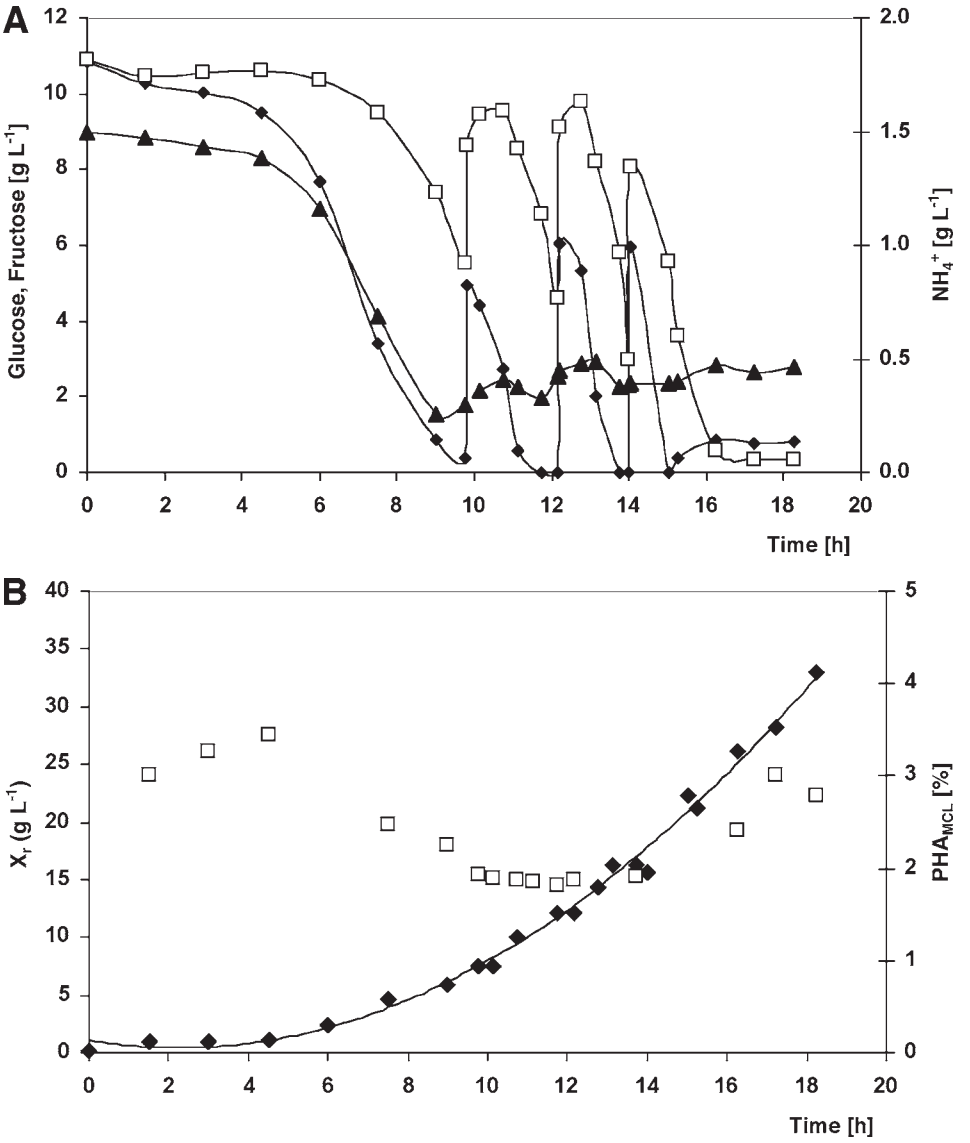

Fig. 3. Cell growth in pulse + constant feed of *P. putida* IPT 046: **(A)** concentration of glucose (◆), fructose (□), and NH_4^+ (▲); **(B)** PHA_{MCL} accumulation (□) and concentration of residual cell (◆).

bohydrate feed rate was established to provide an unlimited DO concentration until the end of the experiment. For this purpose, carbohydrate concentration must be limited (Fig. 3A).

Up to a 32 g/L cell concentration was obtained at 18 h of fermentation, with a low PHA_{MCL} content (<3%) and virtually no accumulation of organic acids (Fig. 3B). Cell mass productivity at the end of the experiment was 1.83 g/(L·h). On-line measurement of CO_2 evolution rate and oxygen uptake rate was a very useful tool to calculate the time for the carbohydrate

pulse. The maximum volumetric oxygen transfer coefficient, k_La, reached its maximum value between 14 and 16 h, with a value of about 1200 h^{-1}. Pulse followed by constant feed is a highly effective method to attain high cell concentrations, but for the precise time of pulse feed, it is necessary to use an expensive CO_2/O_2 analyzer coupled to the bioreactor. Moreover, it is a time-consuming procedure that requires a full-time equipment operator or on-line carbohydrate analysis.

Constant Feed

Strategy II, set up in order to overcome the complicated pulse procedure, also attained high productivity. At first, a batch with an initial glucose + fructose concentration of 20 g/L was processed until carbohydrate exhaustion at 9 h. At this point, a constant feed was set up at a previously calculated culture medium flow rate, ϕ, as shown in Materials and Methods.

Using the values presented in Tables 2 (NH_4^+ = 1500 mg/L) and 4, the linear growth rate calculated from Eqs. 6 and 7 were dX_r/dt = 1.40 g/(L·h), and dX_r/dt = 1.32 g/(L·h), respectively. Equation 4 was then used to estimate the culture medium flow rate, ϕ.

During the constant-feed phase, 9–28 h of fermentation (Fig. 4A), carbohydrate was not accumulated in the culture medium. Linear growth was also observed after 9 h of fermentation with a constant growth rate very close to that calculated from Eqs. 6 and 7, 1.5 g/(L·h).

Up to a 35 g/L cell concentration was obtained in 27 h of fermentation, with medium PHA_{MCL} content (up to 15%) and virtually no accumulation of organic acids (Fig. 4). Cell productivity at the end of the experiment reached 1.3 g/(L·h).

A lower carbohydrate cell yield was obtained mainly because of PHA_{MCL} accumulation during constant growth rate and occurred even at an unlimited NH_4^+ concentration above 0.5 g/L (Fig. 4B).

Exponential Feed

Strategy III also reached high productivity. At first, a batch with an initial glucose + fructose concentration of 20 g/L was used until carbohydrate exhaustion at 9 h. At this point, a culture medium exponential feed was set up as previously estimated by Eq. 9 in Materials and Methods.

During the exponential feed phase, 9–20 h of fermentation, carbohydrate did not accumulate in the culture medium and remained below 4.0 g/L. An unlimited NH_4^+ concentration above 0.4 g/L was observed (Fig. 5A).

Exponential growth was also observed after 9 h of fermentation with a specific growth rate very close to that calculated from Eq. 9, approx 0.2 h^{-1}. Up to a 30 g/L cell concentration was obtained in 20 h of fermentation, with a low PHA_{MCL} content (<3%) and virtually no accumulation of organic acids (Fig. 5B). Cell mass productivity at the end of the experiment reached 1.5 g/(L·h). The carbohydrate-to-biomass yield $Y_{Xr/G+F}$ = 0.4 g/g confirmed the value for previous low-cell-concentration experiments.

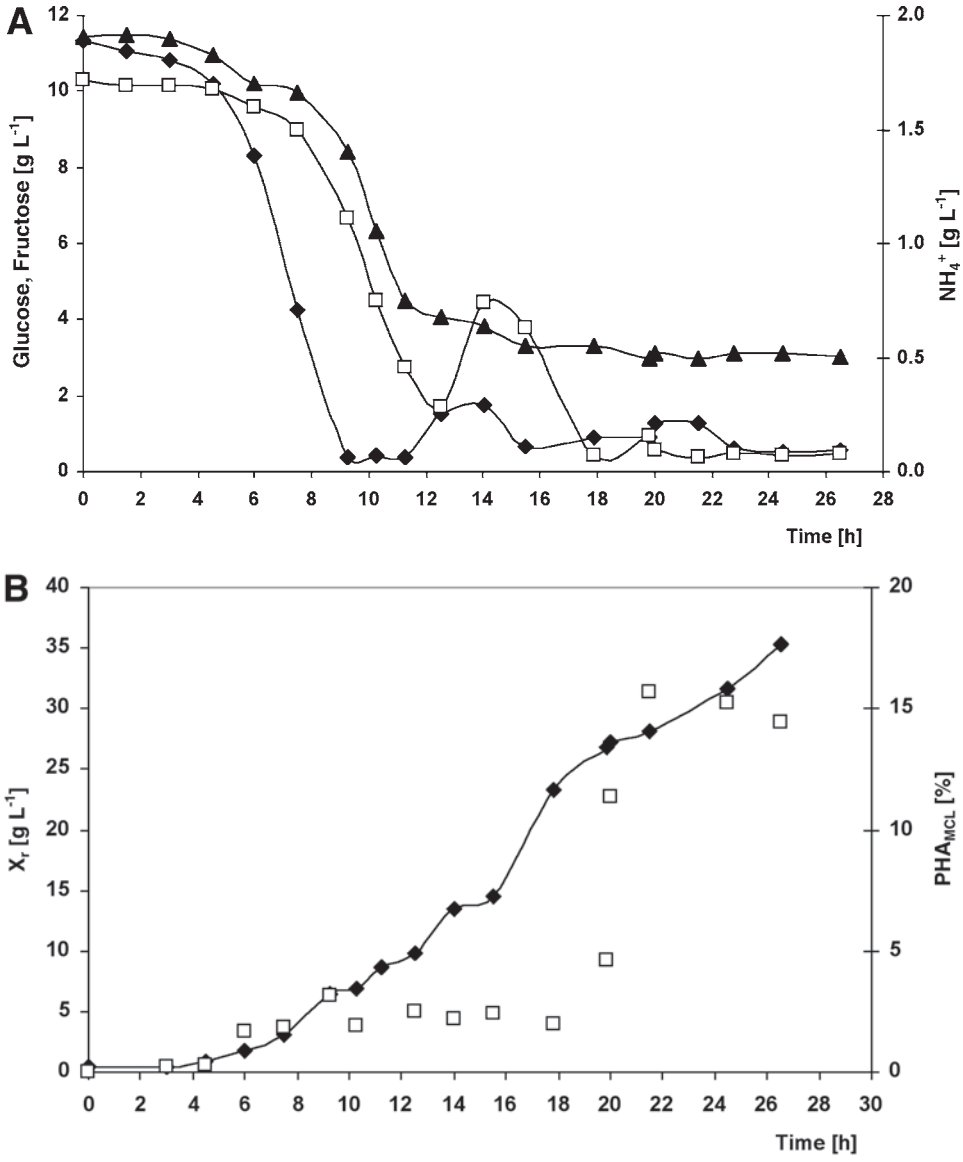

Fig. 4. Cell growth in constant feed of *P. putida* IPT 046: **(A)** concentration of glucose (◆), fructose (□), and NH_4^+ (▲); **(B)** PHA_{MCL} accumulation (□) and concentration of residual cell (◆).

Production of PHA_{MCL}

Production of PHA_{MCL} was performed using the aforementioned growth protocols and submitting the growing culture to nitrogen or phosphorus limitation in separate experiments.

In the nitrogen-limited experiment, limitation took place after exponential feed cultivation at 22 h. At the end of this experiment, a 50 g/L total

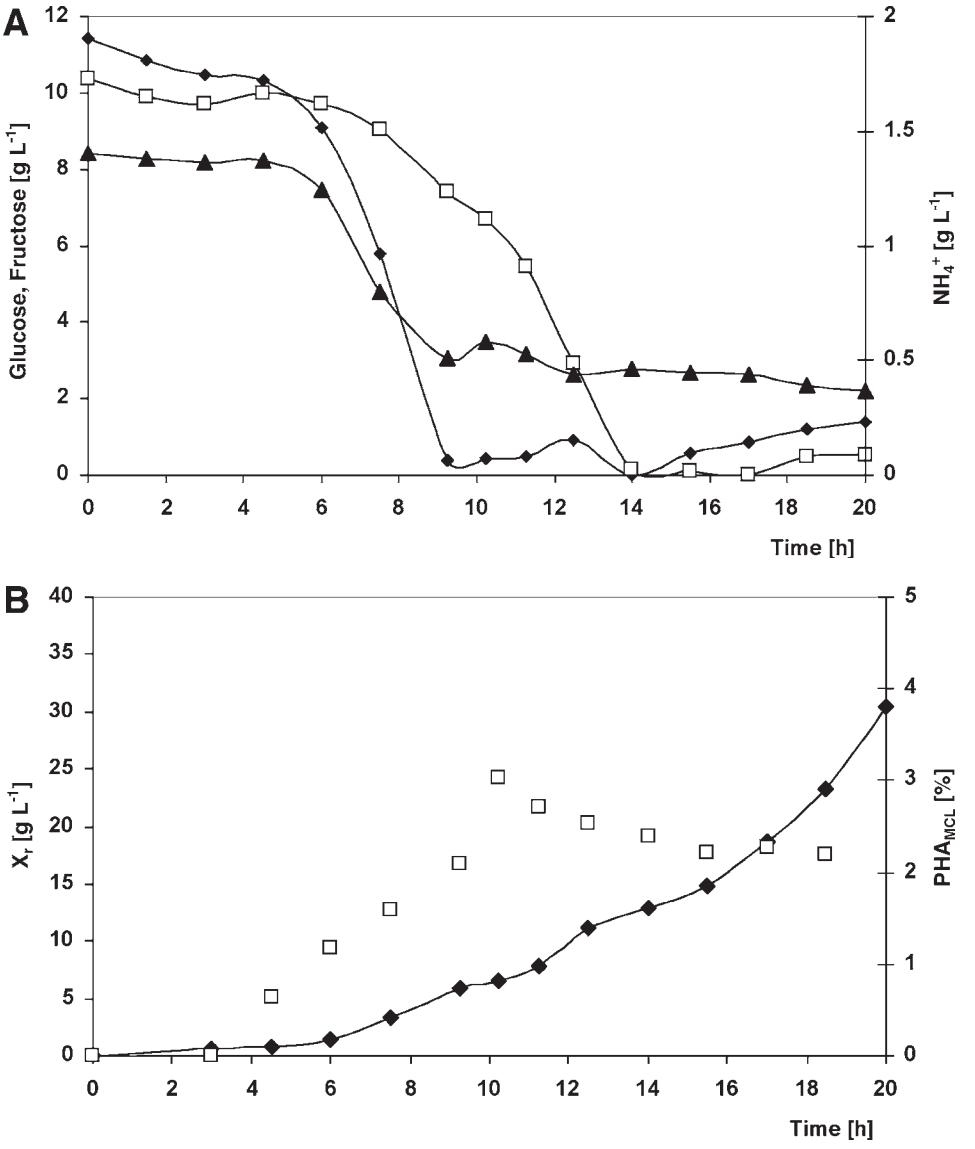

Fig. 5. Cell growth in exponential feed of *P. putida* IPT 046: **(A)** concentration of glucose (◆), fructose (□), and NH_4^+ (▲); **(B)** PHA_{MCL} accumulation (□) and concentration of residual cell (◆).

cell concentration and 21% PHA_{MCL} cell content were obtained in 35 h, leading to PHA_{MCL} productivity of 0.3 g/(L·h) (Fig. 6). The PHA_{MCL} yield from consumed carbohydrate in the accumulation phase was 0.15 g/g, in agreement with previous shake-flask experiments *(12)*.

In the phosphorous-limited experiment, limitation took place after constant-feed cultivation at 16 h. At the end of this experiment, a 51 g/L total cell concentration and 63% PHA_{MCL} cell content were obtained in 40 h,

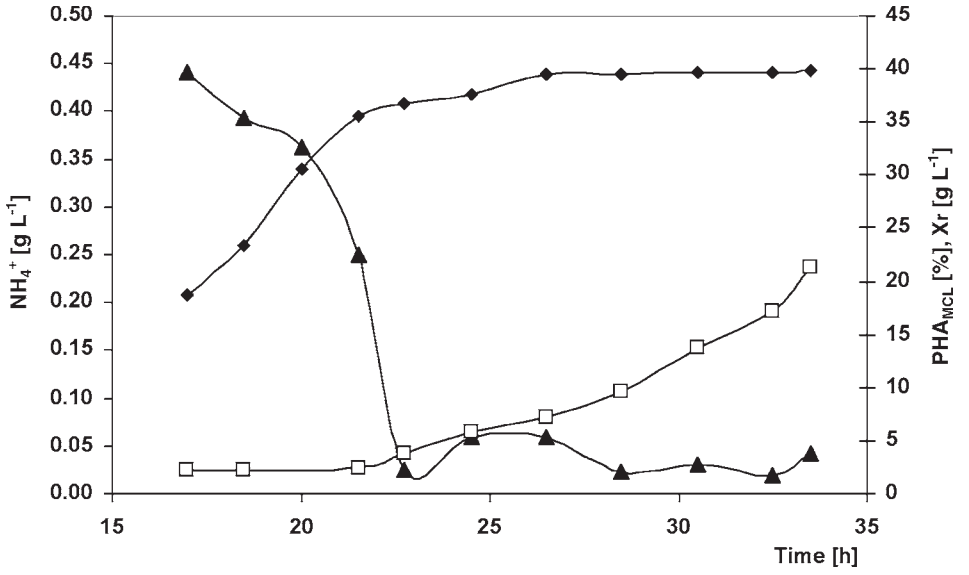

Fig. 6. PHA_{MCL} accumulation using *P. putida* IPT 046 previously grown using fed-batch strategy III (exponential feed) up to 22 h, followed by NH_4^+ limitation: PHA_{MCL} accumulation (□); concentration of residual cell (◆); concentration of NH_4^+ (▲).

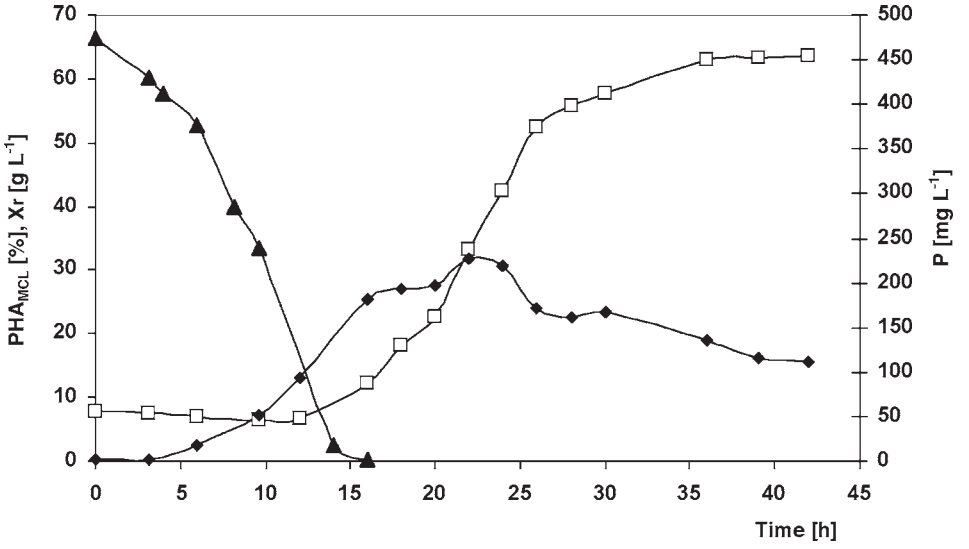

Fig. 7. PHA_{MCL} accumulation using *P. putida* IPT 046 previously grown using fed-batch strategy II (constant feed) from 10 to 42 h, with accumulation owing to phosphate limitation: PHA_{MCL} accumulation (□); concentration of residual cell (◆); concentration of phosphorus (▲).

leading to PHA_{MCL} productivity of 0.8 g/(L·h) (Fig. 7). The PHA_{MCL} yield from consumed carbohydrate in the accumulation phase was 0.19 g/g. The decrease in residual cell mass from 25 h (Fig. 7) was owing to cell lysis.

Discussion

Sucrose is a carbohydrate source derived from sugarcane at a very low price (<US$0.30/kg) in tropical countries. Glucose + fructose equimolar syrup is now routinely obtained from sugar in alcohol mills by acid hydrolysis and is therefore a cheap raw material for economical PHA production *(17)*. Of course, high biomass and product yields from raw material are also crucial to setting up an economical process.

The carbohydrate-to-biomass yield was about 0.4 g/g, regardless of the adopted strategy for cell cultivation; these values were higher than those found for *Pseudomonas* cultivated on fatty acids. As mentioned before, no detectable amounts of organic acids were found during the experiments, indicating no catabolic effects for *P. putida* IPT 046, and this could be the reason for the very high biomass yield obtained on the used culture media. It should be mentioned that acetic acid may be found as an intermediary metabolite in *P. aeruginosa* (http://pseudroyc.pseudomonas.com).

A high cell density and high PHA content must also be obtained to bring the PHA price to reasonable levels (<US$3.0/kg). The design of high-cell-density cultivation must take into account mainly oxygen mass transport and limiting substrate flow to the bulk culture medium phase, and because of economical reasons, pure oxygen for aeration purposes must be avoided during PHA production. DO concentration is a key parameter for the development of high-cell-density cultivation of obligate aerobic microorganisms. High O_2 demand and increasing difficulty for O_2 transport and dispersion owing to modification of the physical properties of the fermentation broth (mainly viscosity and surface tension) limit the final cell concentration.

Most of the literature data concerning O_{2CRIT} have been from studies on the production of the short-chain-length PHA, PHB.

Sonnleitner et al. *(18)* determined a maximum specific rate of oxygen consumption ($qO_{2\,MAX}$) for *Ralstonia eutropha* at 25% of air saturation, and Bitar and Underhill *(19)* found a μ_{MAX} at 60% of air saturation for the same microorganism. Gaudin et al. *(20)* and Piccoli *(21)* found that for *R. eutropha* a DO concentration above 7–10% of air saturation was sufficient to maintain an unlimited situation. Our data support the latter researchers' findings, because for *P. putida* we obtained μ_{MAX} at 15% of air saturation (Fig. 2).

Regarding the carbon source, it is well known that high and low carbohydrate concentrations may influence negatively specific cell growth rate and yield factors. A minimum glucose concentration of 10 g/L has been reported in the literature for *R. eutropha* high-cell-density cultivation *(22)* and of 5–10 g/L for an equimolar glucose plus fructose concentration in PHB production *(21,23)*. In the present study, the maximum specific growth rate was attained with a glucose + fructose concentration between 10 and 20 g/L ($\mu_{Xr1} = 0.65\ h^{-1}$, Table 3). However, a minimum concentration

of glucose just above 3 g/L appeared to be sufficient to maintain the exponential growth of *P. putida* as shown, for example, in Fig. 1.

In the present study, pulse feed strategy I was based on these assumptions, and an exponential growth took place before oxygen limitation was attained at 14 h of fermentation (Fig. 3). When a carbohydrate-limiting flow was used in strategies II and III (Figs. 4 and 5), lower specific growth rates were observed. In exponential-feed strategy III, an exponential growth also occurred but at a much lower μ_{Xr} than for the nonlimiting carbohydrate procedure of pulse-feed strategy I.

All adopted strategies yielded a cell concentration of >30 g/L at the end of the experiment and a high cell productivity above 1.3 g/(L·h). Furthermore, in all used strategies carbohydrate concentration was <1 g/L at the end of growth phase and the biomass had a low PHA_{MCL} content. Therefore, we believed that different growth strategies led to the same cell condition at the end of the growth phase, and it was assumed that the used growth strategy may not influence the accumulation phase.

PHA_{MCL} productivity of about 2 g/(L·h) has been reported in the literature using oleic acid as carbon source *(9,10)*, but a much lower value was observed using carbohydrates *(7)*. The present results are the highest productivity reported for PHA_{MCL} production using simple sugars so far. As indicated in Fig. 7, a nonoptimized procedure for PHA_{MCL} production could attain a high PHA_{MCL} accumulation (>60% of total cell) and 0.8 g/(L·h) PHA_{MCL} productivity. Although we believe the concentration of NH_4^+ was limiting for growth (<50 mg/L), as shown in Fig. 6, this could be one of the reasons that nitrogen-limited culture had a lower PHA content than phosphate limitation.

In conclusion, we observed that it is possible to obtain high-cell-density culture of *P. putida* IPT 046 able to accumulate a high PHA_{MCL} amount in the biomass using carbohydrate as the carbon source. In the described procedure, some constraints, such as differences in sugar uptake rate and minimum oxygen requirement, must be obeyed, and simply open-loop fed-batch techniques *(24)* may be used in order to achieve high cell concentration. Different carbohydrate feed strategies led to almost the same high-cell-density growth. Phosphorous-limited culture appeared to be highly effective in attaining high PHA_{MCL} content.

The results of the present study make *P. putida* IPT 046 an interesting candidate for economical PHA_{MCL} production using sugarcane glucose-fructose syrup as the carbon source. We are currently working on optimizing the process in order to obtain higher PHA_{MCL} productivity.

Nomenclature

CER = CO_2 evolution rate (mol/[L·h])
k_{La} = volumetric coefficient for oxygen transfer (h^{-1})
K_{O_2} = constant for Monod equation
OUR = oxygen uptake rate (mol/[L·h])

P_{Xr} = active biomass productivity (g/[L·h])
qCO_2 = specific CO_2 evolution rate (mol/[g·h])
qO_2 = specific oxygen uptake rate (mol/[g·h])
r_{SX} = substrate consumption rate for growth (g/[L·h])
s = substrate (g)
S_I = initial amount of substrate (g)
S_O = initial concentration of substrate (g/L)
T = time (h)
V = volume (L)
V_O = initial volume (L)
$Vesp$ = specific feed rate (g/[g·h])
xr = active biomass (g)
xr_o = initial amount of active biomass (g)
Xr = concentration of active biomass (g/L)
X_O = initial concentration of biomass (g/L)
$Y_{Xr/F}$ = conversion yield of fructose to biomass (g/g)
$Y_{Xr/G}$ = conversion yield of glucose to biomass (g/g)
$Y_{Xr/G+F}$ = conversion yield of carbohydrate to biomass (g/g)
$Y_{Xr/O}$ = conversion yield of oxygen to biomass (g/g)
$Y_{Xr/S}$ = conversion yield of substrate to biomass (g/g)
μ = specific growth rate (h^{-1})
μ_F = specific consumption rate of fructose (h^{-1})
μ_G = specific consumption rate of glucose (h^{-1})
μ_N = specific consumption rate of ammonium ions (h^{-1})
μ_{Xr1} = specific growth rate on glucose (h^{-1})
μ_{Xr2} = specific growth rate on fructose (h^{-1})
ϕ = feed rate (L/h)

Subscripts

CRIT = critical
MAX = maximum

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References

1 Anderson, A. J. and Dawes, E. A. (1990), *Microbiol. Rev.* **54(4),** 450–472.
2. Steinbüchel, A. (1991), in *Biomaterials: Novel Materials from Biological Sources*, Byrom, D., ed., Macmillan, Basingstoke, UK, pp. 123–213.
3 Madison, L. L. and Huisman, G. W. (1999), *Microbiol. Mol. Biol. Rev.* **63,** 21–53.
4 Preusting, H. J., Kingma, J., and Witholt, B. (1991), *Enzyme Microb. Technol.* **13,** 770–780.
5 Preusting, H. J., Hazenberg, W., and Witholt, B. (1993), *Enzyme Microb. Technol.* **15,** 311–316.

6 Jung, K., Hazenberg, W., Prieto, M., and Witholt, B. (2001), *Biotechnol. Bioeng.* **72,** 19–24.
7 Kim, G. J., Lee, I. Y., Yoon, S. C., Shin, Y. C., and Park, Y. H. (1997), *Enzyme Microb. Technol.* **20,** 500–505.
8. De Koning, G. J. M., Kellerhals, M., Van Meurs, C., and Witholt, B. (1997), in *International Symposium on Bacterial Polyhydroyxalkanoates*, Eggink, G., Steinbüchel, A., Poirier, Y., and Witholt, B., eds., NRC Research Press, Ottawa, pp. 137–141.
9. Weusthuis, R. A., Huijberts, G. N. M., and Eggink, G. (1997), in *International Symposium on Bacterial Polyhydroxyalkanoates*, Eggink, G., Steinbüchel, A., Poirier, Y., and Witholt, B., eds., NRC Research Press, Ottawa, pp. 102–109.
10 Lee, S. Y., Wong, H. H., and Choi, J. (2000), *Biotechnol. Bioeng.* **68,** 466–470.
11. Bueno Netto, C. L., Craveiro, A. M., Pradella, J. G. C., Oliveira, M. S., Maiorano, A. E., Pinto, A. G., and Matsubara, R. M. S. (2000), Patent PI 9103116-8.
12. Gomez, J. G. C., Costa, M., Pradella, J. G. C., Silva, L. F., Torres, B. B., and Schenberg, A. C. G. (2000), in Abstracts of 8th International Symposium on Biological Polyesters.
13. Sánchez, R. J., Schripsema, J., Silva, L. F., Taciro, M. K., Pradella, J. G. C., and Gomez, J. G. C. (2003), *Eur. Polym. J.* **39,** 1385–1394.
14. Treadwell, F. P. (1956), *Tratado de Química Analítica in Gravimetria o análisis por pesadas*, Ed. Manuel Marín y CIA, Barcelona, pp. 54–56.
15 Riss, V. and Mai, W. (1988), *J. Chromatogr.* **445,** 285–289.
16. Franson, M. A. H. (1985) *Standard Methods for the Examination of Water and Wastewater*, 16th ed., American Public Health Association, Washington, DC.
17 Nonato, R. V., Mantelato, P. E., and Rossell, C. E. V. (2001), *Appl. Microbiol. Biotechnol.* **57,** 1–5.
18 Sonnleitner, B., Heinzle, E., Braunneg, G., and Laferty, R. M. (1979), *Eur. J. Appl. Microbiol. Biotechnol.* **7,** 1–10.
19. Bitar, A. and Underhill, S. (1990), *Biotechnol. Lett.* **12,** 563–568.
20. Gaudin, P., Bueno Netto, C. L., Pradella, J. G. C., Taciro, M. K., Piccoli, R. A. M., Pereira, R. P. M., Silva, E. S., and Santos, A. L. (1993), XVII Congresso Brasileiro de Mocrobiologia, X Simpósio Nacional de Fermentações-Anais.
21. Piccoli, R. A. M. (2000), PhD thesis, Escola Politécnica da Universidade de São Paulo.
22 Lee, J. H., Hong. J., and Lim, H. C. (1997), *Biotechnol. Bioeng.* **56,** 697–705.
23. Piccoli, R. A. M., Bueno Netto, C. L., Pradella, J. G. C., Taciro, M. K., Pereira, R. P. M., Urenha, L. C., and Simões, D. A. (1998), IV Seminário de Hidrólise Enzimática de Biomassa **1,** 141–143.
24. Riesenberg, D. and Guthke, R. (1999), *Appl. Microbiol. Biotechnol.* **51,** 422–430.