J Ind Microbiol Biotechnol (2004) 31: 245–254
DOI 10.1007/s10295-004-0136-7

ORIGINAL PAPER

L. F. Silva · M. K. Taciro · M. E. Michelin Ramos
J. M. Carter · J. G. C. Pradella · J. G. C. Gomez

Poly-3-hydroxybutyrate (P3HB) production by bacteria from xylose, glucose and sugarcane bagasse hydrolysate

Received: 1 September 2003 / Accepted: 31 March 2004 / Published online: 22 June 2004

Abstract Fifty-five bacterial strains isolated from soil were screened for efficient poly-3-hydroxybutyrate (P3HB) biosynthesis from xylose. Three strains were also evaluated for the utilization of bagasse hydrolysate after different detoxification steps. The results showed that activated charcoal treatment is pivotal to the production of a hydrolysate easy to assimilate. *Burkholderia cepacia* IPT 048 and *B. sacchari* IPT 101 were selected for bioreactor studies, in which higher polymer contents and yields from the carbon source were observed with bagasse hydrolysate, compared with the use of analytical grade carbon sources. Polymer contents and yields, respectively, reached 62% and 0.39 g g^{-1} with strain IPT 101 and 53% and 0.29 g g^{-1} with strain IPT 048. A higher polymer content and yield from the carbon source was observed under P limitation, compared with N limitation, for strain IPT 101. IPT 048 showed similar performances in the presence of either growth-limiting nutrient. In high-cell-density cultures using xylose plus glucose under P limitation, both strains reached about 60 g l^{-1} dry biomass, containing 60% P3HB. Polymer productivity and yield from this carbon source reached 0.47 g l^{-1} h^{-1} and 0.22 g g^{-1}, respectively.

Keywords Poly-3-hydroxybutyrate · *Burkholderia* · Sugarcane bagasse hydrolysate · Xylose

L. F. Silva (✉) · M. K. Taciro · M. E. Michelin Ramos
J. M. Carter · J. G. C. Pradella · J. G. C. Gomez
Agrupamento de Biotecnologia,
Instituto de Pesquisas Tecnológicas do Estado de São Paulo,
Av. Prof. Almeida Prado, 532, São Paulo,
05508-901 São Paulo, Brazil,
E-mail: lukneif@ipt.br
Tel.: +55-11-37674319
Fax: +55-11-37674055

Introduction

Polyhydroxyalkanoates (PHA) are natural thermoplastics occurring in a wide variety of bacteria which synthesize these polyesters as carbon and energy storage compounds [26, 28]. The homopolymer poly-3-hydroxybutyrate (P3HB) and the copolymer poly-3-hydroxybutyrate-*co*-3-hydroxyvalerate (P3HB-*co*-3HV) are the most extensively studied PHA compounds. Since PHA can be obtained from renewable resources such as sugarcane, commercial interest in these polyesters has greatly increased during the past decade.

A process for the production of P3HB and P3HB-*co*-3HV from sugarcane carbohydrates was developed in Brazil and the technology was transferred to the company PHB Industrial S/A. The project was conceived by integrating the industrial unit with a sugarcane mill and by using local raw materials, solvents and infrastructure to generate a competitive product [13]. Nevertheless, the carbon source still represents 50% of the PHA production cost and any attempts to reduce this figure, such as the use of by-products [20], would be significant for an industrial plant. Sugarcane bagasse is the major by-product of sugar mills, representing 30% by weight of the sugarcane processed [24]. In Brazil, 300×10^6 t of sugarcane were processed in the 1999–2000 harvest (http://www.infoener.iee.usp.br/scripts/biomassa/br_cana.asp). Despite the use of bagasse in the mill to generate energy, there is still an excess of this material, estimated at 8×10^6 t in 1999, which could be used for other non-energetic uses, such as PHA production. In order to utilize bagasse, acid hydrolysis is usually performed to release sugars that can be used by microorganisms. However, acid hydrolysis of sugarcane bagasse generates a number of toxic compounds, such as acetic acid, furfural and hydroxymethylfurfural (HMF) [6]. Several procedures have been successfully applied to eliminate these toxic compounds and to permit the utilization of sugars by microorganisms [14].

In this work, a process for the production of P3HB from a sugarcane bagasse hydrolysate was developed, based on the following steps: (a) screening of soil bacteria able to grow and to accumulate P3HB from xylose and from the hydrolysate, (b) evaluation of detoxification procedures for the bagasse hydrolysate and ©) producing bioreactor cultures for the development of a high-cell-density protocol.

Materials and methods

Microorganisms

Fifty-five bacterial strains were studied, consisting of Gram-negative rods and cocci isolated from soil by Gomez et al. [8] and listed in Table 1 (see Results and discussion).

Media and growth conditions

Cells were cultured in a rotary shaker (150 rpm, 30°C) in nutrient broth (NB; 3 g l^{-1} meat extract, 5 g l^{-1} peptone, pH 6.8) or in mineral salt medium (MM; pH 7.2) [19], using as carbon source xylose or sugarcane bagasse hydrolysate. Solid media contained 15 g l^{-1} agar. PHA was detected on colonies grown on agar plates, after Sudan black B staining according to Schlegel et al. [23].

Bacterial strains IPT 048 and IPT 101 were submitted to two-step cultivations (cell growth, PHA accumulation) in a 10-l bench scale bioreactor at 30°C under conditions of controlled pH (7.0) and dissolved oxygen concentration above 20% of air saturation. The composition of the culture media used in the bioreactor experiments is described in the footnotes to the figures illustrating the respective assays.

Treatment of bagasse hydrolysate to eliminate toxic compounds

A three-step procedure based on the literature [1] was used to eliminate toxic compounds from bagasse hydrolysate. First, the hydrolysate was concentrated in porcelain capsules immersed in a water bath at 80°C. The second step consisted of CaO addition to 16 g l^{-1} and pH adjustment to 10.0 (25–30°C), followed by pH reduction to 6.0, using concentrated H_3PO_4, and solid separation by filtration. In the third step, activated charcoal was added to the filtrate (20% w/v) and the mixture was shaken at 30°C for 3 h and filtered for use in the bioreactor.

Analytical methods

Dry cell biomass (CDW) was determined after centrifugation of appropriate volumes of culture at 10,600 *g*, washing with distilled water, filtering through a 0.45-μm pore membrane and drying at 100°C. Carbohydrates were determined by liquid chromatography with a Waters 510 HPLC apparatus (Waters–Millipore, USA) equipped with a sugar separation column (Shodex SC 1011; Waters–Millipore) as described by Gomez et al. [8]. For PHA determination, samples of about 10 mg of freeze-dried cells were subjected to propanolysis and the propyl esters were detected by gas chromatography (GC) as described by Gomez et al. [9]. Nitrogen was measured using a specific electrode (Orion model 95-12) after the addition of 0.05 ml of 10 M NaOH to 5 ml of sample. Phosphorous was determined by the method of Franson [7].

The variation between replicates was less than 5% in all analytical determinations. Some experiments were run in duplicate with very good reproducibility (less than 10% variation in $Y_{3HB/Xyl}$).

Results and discussion

Screening of bacteria

A total of 54 strains grew on solid MM with xylose (5 g l^{-1})—the main sugar contained in the hydrolysate—as the sole carbon source and presented a PHA^+ phenotype (blue colonies) after staining with Sudan black B. Only one strain (IPT 052) did not grow under these conditions. Strains were then cultivated in a rotary shaker in nitrogen-limited MM [1 g l^{-1} $(NH_4)_2SO_4$] containing xylose excess (20 g l^{-1}) to promote polymer accumulation after nitrogen exhaustion (Table 1).

Strains IPT 040, IPT 041, IPT 042, IPT 043, IPT 044 and IPT 048 reached more expressive CDW values (≥4.0 g l^{-1}) and were efficient in consuming xylose (≥75%). Fifteen of the strains tested (IPT 040, IPT 041, IPT 044, IPT 056, IPT 068, IPT 071, IPT 083, IPT 084, IPT 091, IPT 095, IPT 096, IPT 097, IPT 099, IPT 109, IPT 110) reached a polymer content higher than 50% of CDW and eight (IPT 040, IPT 041, IPT 042, IPT 044, IPT 048, IPT 062, IPT 064, IPT 071) showed a P3HB yield from xylose ($Y_{P3HB/Xyl}$) ≥ 0.10 g g^{-1}, which is a reference value for non-recombinant strains from the literature [12, 20, 30].

Strain IPT 052 did not consume xylose under these conditions, confirming the preliminary results obtained with plate assays. Eleven strains promoted a decrease in pH value (below 5.0). Since a reduced pH value may interfere with cell growth and P3HB accumulation and pH can be easily controlled in a bioreactor, these low values could lead to the rejection of good strains. However, except for IPT 052 and IPT 106, all of them consumed at least 50% of the xylose offered, four of them (IPT 058, IPT 066, IPT 077, IPT 106) yielded a relatively low CDW (≤ 2.0 g l^{-1}) and only two (IPT 077, IPT 106) accumulated a very small amount of P3HB (2.0% CDW). These results indicate that the decrease in pH did not have a strong effect on substrate

Table 1 Production of P3HB by different soil bacteria in mineral medium (MM) containing xylose (20 g l^{-1}) in shaken-flask experiments. No PHA was detected in cells grown on nutrient broth (NB). The initial pH in MM was 6.9–7.0. A 5-ml culture volume in NB was inoculated into 45 ml of MM. *P3HB* Percentage of polymer (cell dry weight, CDW), $Y_{3HB/Xyl}$ P3HB yield from xylose, $\%Y_{max}$ the value of bacterial $Y_{3HB/Xyl}$ in relation to the maximum theoretical value

Strain	NBCDW (g l^{-1})	MM					
		CDW (g l^{-1})	pH final	Remaining xylose (g l^{-1})	P3HB (%)	$Y_{3HB/Xyl}$ (g g^{-1})	$\%Y_{max}$
IPT 039	1.04	3.70	5.80	0.21	24.5	0.04	33.0
IPT 040	0.76	4.92	5.46	1.73	52.3	0.13	50.8
IPT 041	0.93	4.60	5.70	2.23	54.0	0.12	45.4
IPT 042	0.87	5.03	5.77	0.91	47.4	0.11	47.3
IPT 043	0.51	4.28	4.87	1.43	30.6	0.06	39.7
IPT 044	0.90	4.84	5.83	2.37	51.1	0.12	48.0
IPT 045	0.82	3.47	5.58	1.58	43.6	0.07	32.7
IPT 048	0.81	4.54	5.42	0.37	44.4	0.11	50.5
IPT 049	0.83	3.56	5.58	1.99	48.9	0.09	37.6
IPT 052	0.46	0.14	7.00	22.00	0.0	0.00	–
IPT 053	0.86	3.45	3.90	6.50	38.9	0.08	41.8
IPT 055	0.87	3.60	5.95	6.50	44.0	0.09	41.7
IPT 056	0.75	3.62	5.43	1.20	50.5	0.09	36.4
IPT 058	0.65	1.84	3.85	0.15	15.0	0.01	13.4
IPT 059	0.77	1.20	5.47	4.99	15.2	0.01	13.2
IPT 060	0.97	1.93	6.02	8.19	47.0	0.06	26.0
IPT 061	0.72	3.05	6.34	2.82	0.00	0.00	–
IPT 062	0.60	3.43	6.24	6.26	36.8	0.10	55.2
IPT 064	0.66	3.25	4.64	7.90	36.7	0.10	55.3
IPT 066	0.69	1.74	4.64	0.54	39.0	0.04	20.8
IPT 067	0.67	3.24	4.71	0.55	22.6	0.04	35.7
IPT 068	0.72	3.42	6.43	0.57	51.4	0.09	35.8
IPT 071	0.85	3.49	4.77	1.01	66.4	0.12	37.1
IPT 072	0.70	3.33	4.27	2.06	17.5	0.03	34.5
IPT 073	0.55	2.90	5.64	1.98	16.0	0.03	37.8
IPT 074	0.70	2.55	4.85	0.25	19.2	0.02	21.0
IPT 076	0.37	2.50	6.52	0.00	17.2	0.02	23.4
IPT 077	0.56	1.57	3.92	0.00	1.7	0.00	–
IPT 078	0.62	3.04	6.37	0.25	39.5	0.06	30.9
IPT 079	0.70	2.38	6.42	0.36	7.2	0.01	27.9
IPT 080	0.51	1.68	6.11	0.69	22.8	0.02	17.7
IPT 081	1.53	2.16	6.18	7.93	24.5	0.06	49.5
IPT 083	1.19	1.27	6.44	4.92	52.3	0.05	19.5
IPT 084	1.25	1.56	6.38	5.48	55.4	0.05	18.5
IPT 086	0.81	0.21	6.43	6.53	47.4	0.01	4.3
IPT 088	0.78	1.29	5.96	7.54	30.6	0.04	26.5
IPT 091	1.34	2.21	6.32	0.36	51.1	0.05	20.0
IPT 092	1.06	1.06	6.46	0.00	0.0	0.00	–
IPT 093	1.78	1.78	6.38	0.00	22.3	0.02	18.1
IPT 095	0.51	2.21	6.13	0.00	53.5	0.06	22.9
IPT 096	0.56	1.86	6.34	0.00	58.2	0.05	17.6
IPT 097	0.50	0.86	6.45	0.57	56.7	0.02	7.2
IPT 098	0.22	1.65	6.36	0.00	47.1	0.04	17.3
IPT 099	0.42	1.68	6.32	0.00	51.9	0.04	15.7
IPT 100	0.79	0.79	6.45	0.00	41.1	0.02	9.9
IPT 101	0.54	3.55	6.61	0.00	29.6	0.05	34.2
IPT 102	0.47	1.63	6.00	3.30	3.3	0.00	0.0
IPT 103	0.60	1.63	6.45	0.00	1.6	0.00	0.0
IPT 104	0.69	3.63	6.50	0.00	37.8	0.07	37.6
IPT 105	0.60	3.04	5.50	0.00	12.3	0.02	32.7
IPT 106	0.59	0.85	4.32	17.44	1.8	0.01	66.7
IPT 108	0.48	3.05	6.50	0.00	14.6	0.02	27.6
IPT 109	0.53	3.33	6.50	0.0	54.2	0.08	30.2
IPT 110	0.53	1.71	6.52	0.00	70.8	0.05	14.5

utilization, cell growth or polymer accumulation in the majority of strains evaluated.

In order to combine different parameters for strain selection, the P3HB content (%P3HB) was plotted against P3HB yield from xylose ($Y_{3HB/Xyl}$; Fig. 1) and compared with the expected maximum theoretical yield (Y_{max}) values, which depended on the extent of P3HB accumulation. Y_{max} was estimated by considering a maximum residual biomass yield from xylose (admitted as 0.5 g g^{-1} in this case), a maximum P3HB yield from xylose (established considering metabolic pathways of −0.48 g g^{-1}) and accumulated P3HB [8]. Five strains (inside the circle in Fig. 1) accumulated a polymer content of more than 40% of CDW and reached ca. 50% of the maximum theoretical yield (Y_{max}). Thus, strains IPT 040, IPT 041, IPT 044, IPT 045 and IPT 048

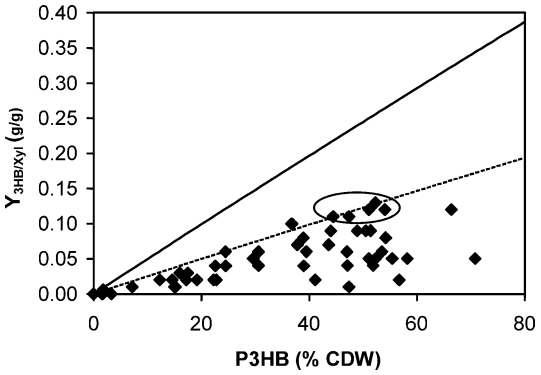

Fig. 1 Relationship between $Y_{3HB/Xyl}$ and P3HB content observed for the various strains analyzed. The *continuous line* represents the maximum theoretical value (Y_{max}) based on metabolic pathways and the *dotted line* represents 50% of this value

were selected for further study. A new experiment with shaken flasks was performed using MM containing 10 g l^{-1} xylose to compare these strains, *B. sacchari* IPT 101 and its prp mutant, IPT 189 (Table 2). Strains IPT 101 and IPT 189 were selected since they have been studied extensively and are very efficient in utilizing other sugars and propionate to accumulate PHA [3, 4, 8, 9, 25]. Two strains (IPT 045, IPT 101) did not completely consume the xylose available, with CDW values of around 3.0 g l^{-1}; and IPT 048 had a value of 4.0 g l^{-1}. All strains accumulated P3HB (35–50% CDW) with $Y_{P3HB/Xyl}$ ranging over 0.10–0.20 g g^{-1}. The $Y_{P3HB/Xyl}$ values reached by strains IPT 045 and IPT 048 represented 75% of the maximum theoretical value (Y_{max}) and IPT 101 reached 60% of Y_{max}. None of these strains reached the maximum theoretical yield of P3HB from xylose. Thus, these strains still show a potential for improved efficiency in accumulating P3HB from xylose. Strains IPT 045, IPT 048 and IPT 101 were selected for experiments using shaken flasks and the hydrolysate as carbon source since they showed higher efficiency in synthesizing P3HB in MM containing 10 g l^{-1} xylose (Table 3).

Eliminating toxic compounds from sugarcane bagasse hydrolysate

Acid hydrolysis of sugarcane bagasse generates a number of toxic compounds, such as acetic acid, furfural and HMF [6], which must be removed from the hydrolysate to allow microbial growth. Table 4 shows the hydrolysate composition after each treatment for detoxification. The procedures applied led to a ten-fold increase in xylose concentration, while furfural and HMF concentrations were greatly reduced. Although the concentrations of acetic acid increased, the total treatment led to a seven-fold reduction in the acid, considering that the solution was concentrated about ten-fold (based on the xylose concentrations). In any case, the final values were still low.

P3HB accumulation from detoxified hydrolysate

Strains IPT 045, IPT 048 and IPT 101, which best approximated the maximum theoretical efficiency of

Table 2 Production of P3HB by selected bacteria using mineral medium with xylose (10 g l^{-1}) in shaken flasks. No PHA production was detected in NB. Initial pH in MM was 6.9–7.0

Strains	NBCDW (g l^{-1})	MM					
		Final pH	Remaining xylose (g l^{-1})	CDW (g l^{-1})	P3HB (%)	$Y_{3HB/Xyl}$ (g g^{-1})	% Y_{max}
IPT 040	0.97	6.10	0.00	2.89	41.2	0.10	53.55
IPT 041	0.42	5.05	0.00	2.62	50.5	0.12	48.75
IPT 044	0.88	6.28	0.00	2.94	52.5	0.14	54.76
IPT 045	1.25	6.46	3.00	2.84	49.9	0.18	72.64
IPT 048	0.89	6.21	0.00	4.07	53.5	0.20	75.84
IPT 101	0.82	6.27	2.00	2.93	35.0	0.11	66.17
IPT 189	0.59	6.27	0.00	2.96	58.2	0.16	55.27

Table 3 Production of P3HB by selected bacteria in shaken flasks using sugarcane bagasse hydrolysate after different treatments for the elimination of toxic compounds. *C1* Hydrolysate after the concentration step, *C2* hydrolysate after $CaO/NaOH/H_3PO_4$ addition, *C3* hydrolysate after adsorption with 20% active charcoal. A 5-ml culture volume in NB was inoculated into 45 ml of MM containing C1, C2 or C3 as the carbon source

Strain	NBCDW (g l^{-1})	MM					
		C1		C2		C3	
		Final CDW	% P3HB	Final CDW	% P3HB	Final CDW	% P3HB
IPT 045	1.06	0.54	0.00	1.07	0.00	5.08	18.20
IPT 048	0.56	0.83	0.00	3.84	4.04	3.42	15.39
IPT 101	0.88	0.54	0.00	0.64	15.4	6.13	23.22

Table 4 Compounds measured in sugarcane bagasse hydrolysate after different treatments. *C1* Evaporation, *C2* $CaO/NaOH/H_3PO_4$ addition, *C3* adsorption with 20% active charcoal, *HMF* hydroxymethyl furfural, *ND* not determined

Compound	Initial	C1	C2	C3
Xylose (g l^{-1})	16.9	259.8	183.1	179.1
Glucose (g l^{-1})	9.7	184.0	140.6	129.5
Arabinose (g l^{-1})	1.4	3.8	4.2	3.6
Furfural (mg l^{-1})	244.7	86.7	ND	12.6
HMF (mg l^{-1})	103.3	116.0	ND	59.0
Acetic acid (mg l^{-1})	144.8	286.0	ND	205.0

polymer production from xylose (higher percentage of Y_{max}, Table 2), were selected for assays with the bagasse hydrolysate. The strains were cultivated in MM containing bagasse hydrolysate (after different treatments) as the sole carbon source to evaluate the detoxification procedure (Table 3). Cells cultivated in the C1 hydrolysate (after the first step of hydrolysate treatment, when the hydrolysate was concentrated in porcelain capsules immersed in a water bath at 80°C) were unable to accumulate the polymer and low biomass values were attained, demonstrating the toxic nature of the hydrolysate. The C2 hydrolysate (after precipitation by pH adjustment) allowed an important increase in the CDW of IPT 048, although the P3HB content remained low. Strain IPT 101 reached a low CDW, but accumulated P3HB corresponded to 15% of CDW. Adsorption with activated charcoal was the most important procedure for the detoxification of the bagasse hydrolysate since, after this treatment, the C3 hydrolysate supported the formation of high biomass values with a P3HB content corresponding to 15–25% of CDW. On the basis of the results obtained, bacterial strains IPT 048 and IPT 101 were selected for further study in the bioreactor.

Bioreactor experiments

Carbon source evaluation

Although the use of hemicellulose materials as a cheap carbon source has been recommended to decrease P3HB production costs, only poor results have been described in the literature [2, 10, 12, 20, 30], with maximum CDW values of 7.5 g l^{-1} and with a low polymer content (a maximum of about 49%) when wild strains were studied. The use of recombinant bacterial strains and the combination of xylose with hydrolysates permitted a greater polymer accumulation, although CDW continued to be lower than 6 g l^{-1} (Table 5). With the aim of evaluating bagasse hydrolysate as a carbon source, strains IPT 048 and IPT 101 were cultivated in a bioreactor, using glucose plus xylose (analytical grade) or C3 hydrolysate (Fig. 2). P3HB production was stimulated by nitrogen limitation. When xylose and glucose were the carbon sources, the parameters calculated for strain IPT 101 were: a maximum specific growth rate (μ_{Xrmax}) of 0.24 h^{-1}, a residual biomass (Xr, i.e. CDW minus polymer content) yield from sugar ($Y_{Xr/S}$) of 0.46 g g^{-1} and a P3HB yield from sugar ($Y_{P3HB/S}$) of 0.11 g g^{-1}. A preferential consumption of glucose was observed and, after NH_4^+ exhaustion (8 h), P3HB accumulation occurred up to 27% of CDW with partial consumption of xylose (Fig. 2a–c). Using bagasse hydrolysate as the carbon source, a similar μ_{Xrmax} value was obtained, but a significant improvement was observed for other parameters: $Y_{Xr/S} = 1.31$ g g^{-1}, P3HB = 62% CDW and $Y_{P3HB/S} = 0.39$ g g^{-1}. More than 15 ions, plus arabinose and acetic acid have been reported in the literature as components of sugarcane bagasse hydrolysates in addition to xylose and glucose; and some of these were detected in the hydrolysate used in our studies [22]. The toxic phenolic components, if present at low levels, can be utilized by bacteria in

Table 5 Comparison of the present data with data reported in the literature about P3HB production from cellulose hydrolysates or sugars obtained from the hydrolysis of these materials. *CS* Cotton seed hydrolysate, *SH* soybean hydrolysate, μ_{Xrrmax} maximum specific growth rate of residual cells (non-polymer material), μ_{Pmax} maximum specific polymer accumulation rate, P_{P3HB} polymer productivity, $Y_{P3HB/S}$ polymer yield from the carbon source

Strain	Carbon source	CDW (g l^{-1})	P3HB (%)	μ_{Xrrmax} (h^{-1})	μ_{Pmax} (h^{-1})	$Y_{P3HB/S}$ (g g^{-1})	P_{P3HB} (g l^{-1} h^{-1})	Reference
Pseudomonas pseudoflava ATCC 33668	Glucose	3.5	22.8	0.58	0.11	0.04	0.080	[2]
P. pseudoflava ATCC 33668	Xylose	4.0	27.5	0.13	0.03	0.04	0.031	[2]
B. cepacia ATCC 17759	Xylose	7.5	45	0.22	0.07	0.11	0.10	[20]
B. cepacia	Xylose		48.8	–	–	0.11	–	[30]
Escherichia coli TG1 (pSYL107)[a]	Xylose	4.75	35.8	–	–	0.097	0.028	[12]
E. coli r TG1 (pSYL107)[a]	Xylose + CSH	3.76	64.0	–	–	0.188	0.040	[12]
E. coli TG1 (pSYL107)[a]	Xylose + SH	5.95	73.9	–	–	0.226	0.070	[12]
B. sacchari IPT 101	Sugarcane bagasse hydrolysate	4.4	62	0.24	0.16	0.39	0.11	Present paper
B. cepacia IPT 048	Sugarcane bagasse hydrolysate	4.4	53	0.36	0.08	0.29	0.09	Present paper
B. sacchari IPT 101	Xylose + glucose	60	58	0.25	0.03	0.22	0.47	Present paper
B. cepacia IPT 048	Xylose + glucose	57	57	0.28	0.06	0.19	0.46	Present paper

[a]Recombinant strain

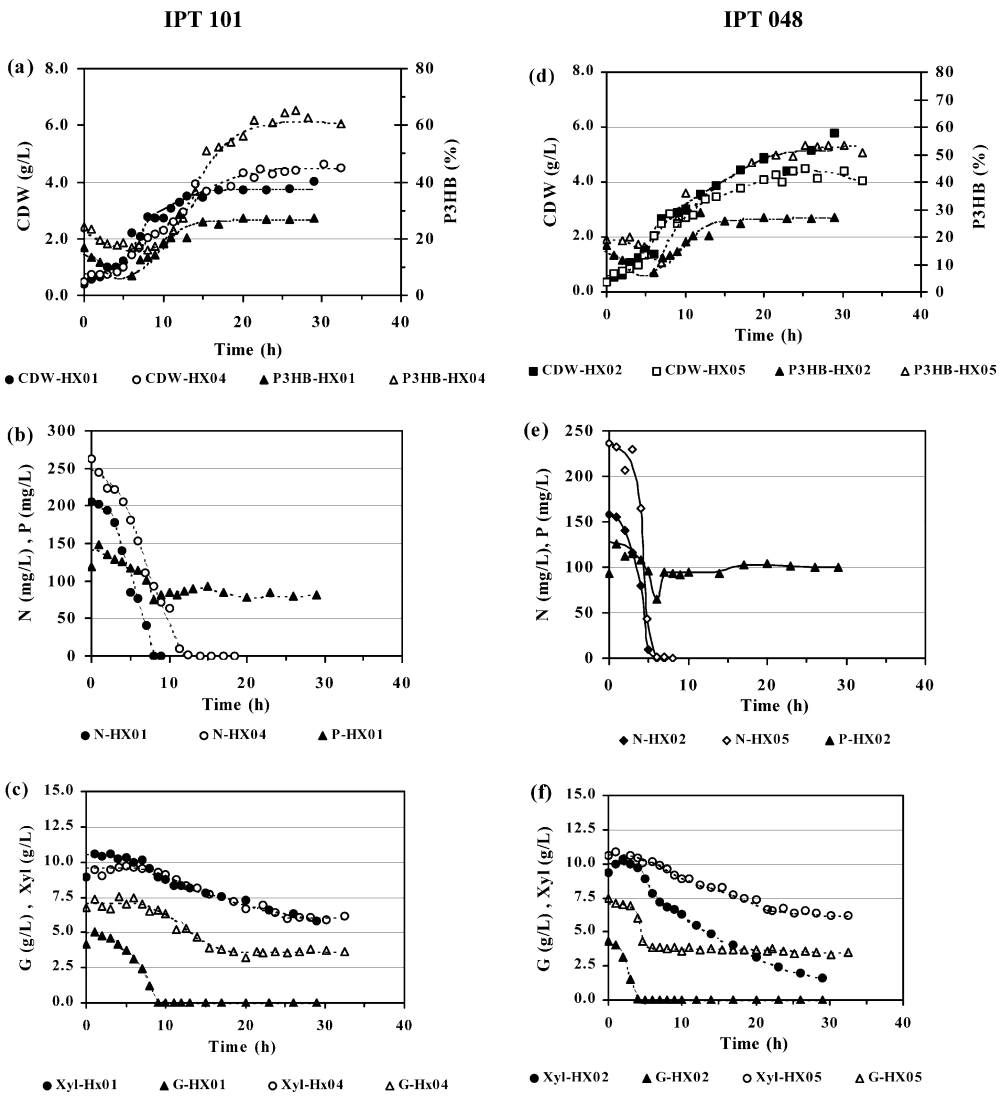

Fig. 2a–f Bioreactor experiments comparing strains IPT 101 and IPT 048 cultured in xylose and glucose (HX01, HX02) or in a detoxified sugarcane bagasse hydrolysate (HX04, HX05) under nitrogen limitation. **a, d** Polymer content (P3HB) and CDW. **b, e** N and P remaining in the medium. **c, f** Glucose (*G*) and xylose (*Xyl*) remaining in the medium. All experiments consisted of one batch with about 16 g l^{-1} carbon source. The medium used contained 0.132 g l^{-1} KH_2PO_4, 1 g l^{-1} $(NH_4)_2SO_4$, 0.109 g l^{-1} $MgSO_4{\cdot}7H_2O$, 0.06 g l^{-1} $CaCl_2{\cdot}2H_2O$, 0.02 g l^{-1} ferric ammonium citrate and 2 ml l^{-1} trace element solution. The trace element solution contained 0.3 g l^{-1} H_3BO_3, 0.2 g/l $CoCl_2{\cdot}6H_2O$, 0.1 g l^{-1} $ZnSO_4{\cdot}7H_2O$, 30.0 mg l^{-1} $MnCl_2{\cdot}4H_2O$, 30.0 mg l^{-1} $NaMoO_4{\cdot}2H_2O$, 20.0 mg l^{-1} $NiCl_2{\cdot}6H_2O$ and 10.0 mg l^{-1} $CuSO_4{\cdot}5H_2O$. The pH was automatically controlled at 7.0 by adding 4 N H_2SO_4 or 4 N NaOH

addition to acetic acid. The literature indicates that weak acids at low concentrations seem to have a stimulating effect on ethanol production by yeasts [15]. It was also observed that the addition of hemicellulose hydrolysates resulted in increased P3HB production [12].

For strain IPT 048, the parameters observed in the medium with xylose and glucose were: $\mu_{Xrmax} = 0.34\ h^{-1}$, $Y_{Xr/S} = 0.20\ g\ g^{-1}$ and $Y_{P3HB/S} = 0.15\ g\ g^{-1}$. Glucose was also preferentially consumed and polymer accumulation started after 6 h of cultivation when the N source was exhausted (Fig. 2d–f). At the end of the experiment, 37% of CDW had accumulated as P3HB. As was the case for IPT 101, an improvement in process parameters was also observed using the bagasse hydrolysate. $Y_{Xr/S}$ increased to 0.43 g g^{-1} and, after exhaustion of the N source at 7 h of cultivation, an improvement in P3HB accumulation was observed, reaching 53% CDW; and $Y_{P3HB/S}$ reached a value of 0.29 g g^{-1}.

On the basis of the present results, we may suggest that another carbon source, not detected during our experiments, was present in the hydrolysate, since higher $Y_{Xr/S}$ values were observed compared with experiments using xylose plus glucose. The increase in $Y_{P3HB/S}$ and P3HB accumulation could be attributed to nutrients, not identified but probably present in the hydrolysate, such as acetic acid or phenol compounds at low concentrations. Improvement of the process by adding nutrients to the culture medium was described by Lee [12], who observed a significant improvement in polymer content when supplementing synthetic medium with a soybean hydrolysate and a cotton seed hydrolysate (from 35% to 74% and 62%, respectively). Arabinose detected in the medium (ca. 2 g l^{-1}) was not consumed during the experiment. The present results are similar to values reported in the literature [2, 20, 30] using xylose and glucose, i.e., within a CDW range of 3.5–7.5 g l^{-1} the polymer (P_{P3HB}) productivity was about 0.10 g l^{-1} h^{-1} (Table 5).

Evaluation of limiting nutrients

Induction of P3HB biosynthesis has been claimed to be achieved using nitrogen, phosphorous, magnesium or oxygen limitation [10]. *Ralstonia eutropha* DSM 518

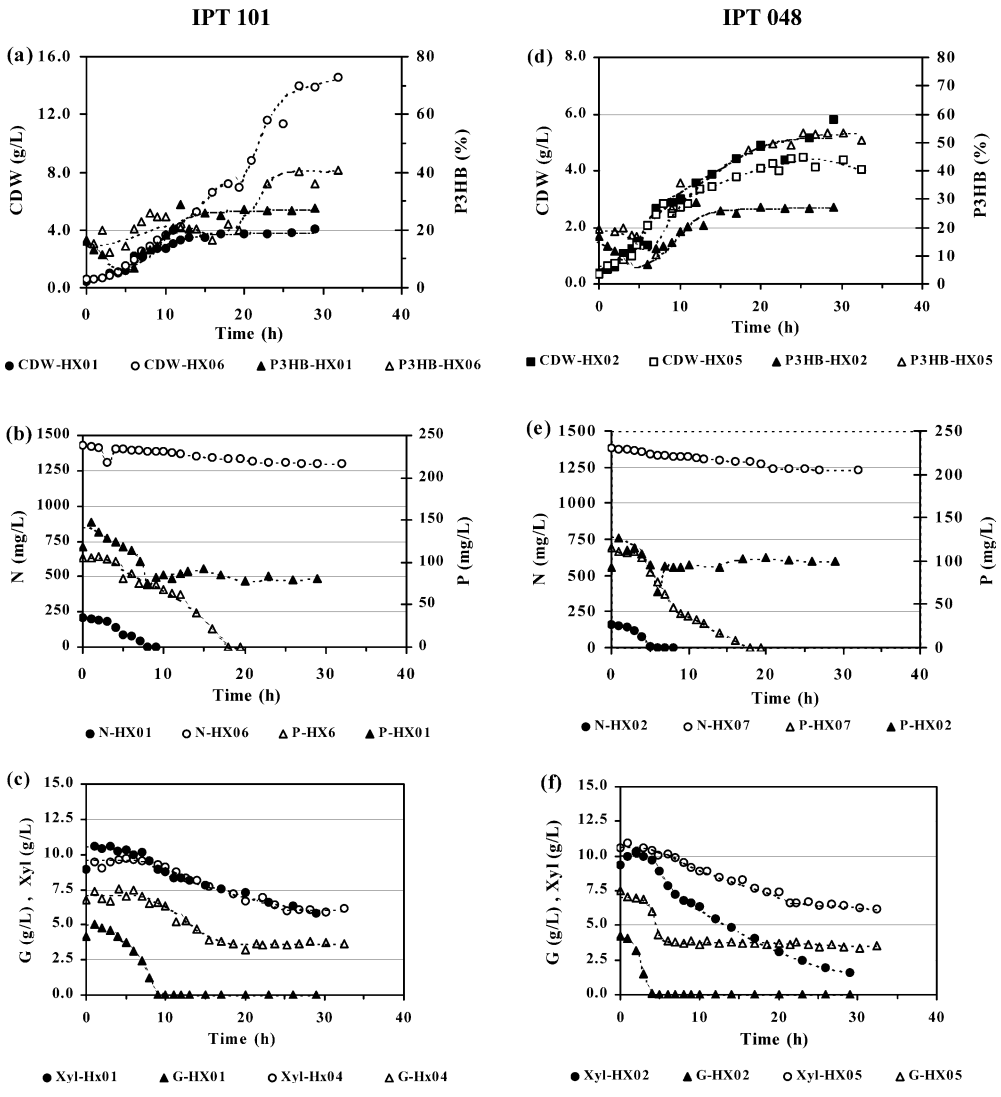

Fig. 3a–f Bioreactor experiments comparing strains IPT 101 and IPT 048 under nitrogen (experiments HX01, HX02) or phosphorus (HX06, HX07) limitation. **a, d** Polymer content (P3HB) and CDW. **b, e** N and P remaining in the medium. **c, f** Glucose (*G*) and xylose (*Xyl*) remaining in the medium. Experiments HX01 and HX02 consisted of an initial batch of xylose (10.8 g l^{-1}) plus glucose (4.8 g l^{-1}), for a total of 15.6 g l^{-1} carbon source. In experiments HX06 and HX07, the same initial batch was used, followed by a second addition of the carbon source. The medium used contained the same mineral salts concentration as in the experiment described in Fig. 2. pH was automatically controlled at 7.0 by adding 4 N H_2SO_4 or 1.5 N NH_4OH (or 4 N NaOH during N-limiting accumulation tests)

accumulated P3HB at a higher rate with SO_4^{-2} and NH_4^+ limitation, at equivalent biosynthesis rates with Mg, P and Fe limitation and at a lower rate with O_2 limitation [27]. In contrast, polyphosphate accumulation in *R. eutropha* H16 was observed to be closely related to P3HB accumulation in nitrogen-limited cultures, reducing the P3HB yield from the substrate [5].

Nitrogen-limiting experiments with xylose plus glucose (for a total of 16 g l^{-1}) with both strains were performed using a ratio between these carbohydrates similar to that found in the hydrolysate (ca. 12 g l^{-1} glucose, 4 g l^{-1} xylose). Both strains showed a preferential consumption of glucose, which was exhausted in less than 10 h, with about 7–8 g l^{-1} xylose remaining (Fig 3c,f) and nitrogen was exhausted within the same time (Fig. 3b,e). At the end of this phase, about 3 g l^{-1} CDW and 18% of polymer were achieved (Fig. 3a,d). Nitrogen exhaustion resulted in 27% polymer accumulation by strain IPT 101 (Fig. 3a) and 37% by strain IPT 048 (Fig. 3d). Both strains consumed the remaining xylose at a very low rate, particularly IPT 101. The phosphorous concentration was practically unaltered during this phase and no polyphosphate accumulation simultaneous to P3HB production was demonstrated, as previously described [5].

In the P-limiting experiments, the N source was kept non-limiting and the pH was kept constant by the addition of ammonia (Fig. 3b,e). The initial batch of carbohydrates was completely consumed (Fig 3c,f) and 8 g l^{-1} CDW with 20% polymer were achieved after 20 h (Fig. 3a,d), coinciding with P exhaustion (Fig. 3b,e). An additional carbon source was supplied and was completely consumed, resulting in 40% polymer accumulation (Fig. 3a,d, experiments HX06, HX07).

In the P-limiting experiments, the maximum specific P3HB production rate (μ_{P3HB}) reached by strain IPT 101 was approximately 0.12 h^{-1} and the rate reached by IPT 048 was 0.08 h^{-1}. During N limitation, μ_{P3HB} was lower for both strains, reaching values around 0.05 h^{-1}. The polymer yield from carbohydrates was maintained

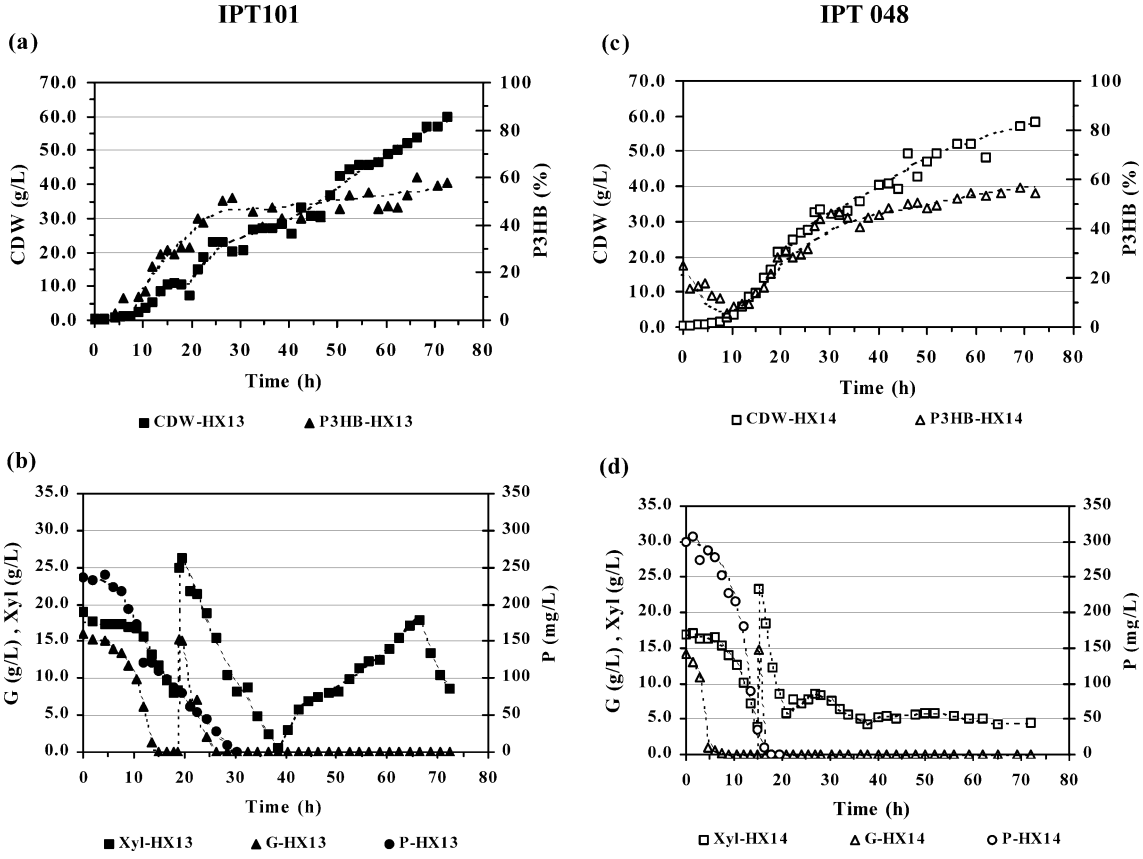

Fig. 4a–d Bioreactor experiments comparing strains IPT 101 (experiment HX13) and IPT 048 (experiment HX14) at a high cell density with phosphorus limitation. **a, c** Polymer content (P3HB) and CDW. **b, d** Glucose (*G*), xylose (*Xyl*) and P remaining in the medium. The experiments consisted of two successive batches of carbon source, each containing 15.7 g l^{-1} glucose plus 17.6 g l^{-1} xylose, followed by xylose (360 g l^{-1}) plus glucose (330 g l^{-1}) feeding at a controlled rate in order to keep the concentration of the carbon source below 10 g l^{-1}. The medium used contained 0.792 g l^{-1} KH_2PO_4, 1 g l^{-1} $(NH_4)_2SO_4$, 0.654 g l^{-1} $MgSO_4{\cdot}7H_2O$, 0.036 g l^{-1} $CaCl_2{\cdot}2H_2O$, 0.012 g l^{-1} ferric ammonium citrate and 12 ml l^{-1} trace element solution. The trace element solution contained 0.3 g l^{-1} H_3BO_3, 0.2 g l^{-1} $CoCl_2{\cdot}6H_2O$, 0.1 g l^{-1} $ZnSO_4{\cdot}7H_2O$, 30.0 mg l^{-1} $MnCl_2{\cdot}4H_2O$, 30.0 mg l^{-1} $NaMoO_4{\cdot}2H_2O$, 20.0 mg l^{-1} $NiCl_2{\cdot}6H_2O$ and 10.0 mg l^{-1} $CuSO_4{\cdot}5H_2O$. pH was automatically controlled at 7.0 by adding 4 N H_2SO_4 or 1.5 N NH_4OH

by strain IPT 048 (0.15 g g^{-1}) and was increased two-fold in the case of strain IPT 101 (0.20 g g^{-1}).

The higher values of μ_{P3HB}, polymer content and $Y_{P3HB/S}$ reached by strain IPT 101 under P limitation can be attributed to glucose availability during the accumulation phase, as opposed to the N-limiting experiments, in which only xylose was present. Bertrand et al. [2] also found a less efficient P3HB synthesis from xylose compared with glucose by *P. pseudoflava*. In strain IPT 048, only a μ_{P3HB} increase was observed, which could be attributed to the same reason.

On the basis of this set of assays results, we decided to use phosphorus limitation as an inducer of polymer accumulation.

High-cell-density experiments

The production of P3HB from xylose, glucose and cellulose hydrolysates has usually been performed under low-cell-density cultivation (below 10 g l^{-1}), reaching productivities up to 0.1 g l^{-1} h^{-1} (Table 5). High-cell-density cultures are indispensable in a PHA production process since they allow high productivity [21].

In the present study, MM with the main sugars present in bagasse hydrolysate (xylose, glucose) was used to obtain a high cell density of strains IPT 048 and IPT 101. Phosphorus limitation was applied, based on previous results. High-cell-density protocols were adapted from previous studies on the production of PHA and other bioproducts [16–18, 29]. Using six times the salt concentration used in low-density cell growth, no harmful effect was observed on strain IPT 101, with a maximum specific growth rate (μ_{Xrmax}) of 0.25 h^{-1} and a residual biomass yield from sugar similar to that obtained with a low cell density under N or P limitation ($Y_{Xr/S} = 0.4$ g g^{-1}). Cell growth proceeded until total glucose consumption (ca. 18 h), when more carbon source was added (Fig. 4a). Another growth phase took place until 30 h, when P was completely consumed. At that point, Xr reached 27 g l^{-1}, with a polymer content of 40%. After P exhaustion in the medium, polymer accumulation occurred until 70 h, reaching a total biomass (Xt) of 60 g l^{-1}, containing 58% P3HB and $Y_{P3HB/S} = 0.22$ g g^{-1}. In the high-cell-density experiment using strain IPT 048, μ_{Xrmax} decreased, compared with the experiment with P limitation and a low

cell density (0.36–0.28 h^{-1}). The residual biomass yield from carbohydrates was not constant during growth, although an average value of 0.18 g g^{-1} was calculated between 0 h and 30 h. This value is very low, compared with the $Y_{Xr/S} = 0.42$ g g^{-1} observed under low-cell-density cultivation. This result could be attributed to the elevated initial salt concentration used in experiment HX14, which was needed to obtain a high cell density. Therefore, salts should be supplied in smaller fractions during the cell growth of this strain.

Strain IPT 048 showed a preferential consumption of glucose during the experiment (Fig. 4d). At the end of the experiment (72 h), CDW reached 57 g l^{-1}, containing 57% P3HB (Fig. 4c). The procedure used led to very interesting efficiencies of both strains. After 72 h of assay, about 60 g l^{-1} cells were obtained, containing 58% P3HB. Polymer productivity was 0.5 g l^{-1} h^{-1} and polymer yield ($Y_{P3HB/S}$) was 0.46 g g^{-1}. The results presented here are the best described in the literature until now for P3HB production from xylose plus glucose or cellulose hydrolysates. The potential use of strains IPT 048 and IPT 101 using such a cheap substrate has been clearly demonstrated here; and the two strains are currently being processed in order to obtain high cell density in a bioreactor using sugarcane hydrolysate as the carbon source.

Acknowledgements FAPESP, São Paulo, Brazil, supported this work. CTC Copersucar supplied the DHR hydrolysate. M.E.M.R. was supported by CNPq.

References

1. Alves LA, Felipe MGA, Almeida e Silva JB, Silva SS, Prata AMR (1998) Pretreatment of sugarcane bagasse hemicellulose hydrolysate for xylitol production by *Candida guilliermondii*. Appl Biochem Biotechnol 70/72:89–98
2. Bertrand J, Ramsay J, Ramsay BA, Chavarrie C (1990) Biosynthesis of poly-β-hydroxyalkanoates from pentoses by *Pseudomonas pseudoflava*. Appl Environ Microbiol 56:3133–3138
3. Brämer CO, Vandamme P, Silva LF, Gomez JGC, Steinbüchel A (2001) *Burkholderia sacchari* sp. a polyhydroxyalkanoate-accumulating bacterium isolated from soil of a sugarcane plantation in Brazil. Int J Syst Evol Microbiol 51:1709–1713
4. Brämer CO, Silva LF, Gomez JGC, Steinbüchel A (2002) Identification of the 2-methylcitrate pathway involved in the catabolism of propionate in the polyhydroxyalkanoate producing strain *Burkholderia sacchari* IPT 101 and analysis of a mutant accumulating a copolyester with higher 3-hydroxyvalerate content. Appl Environ Microbiol 68:271–279
5. Doi Y, Kawaguchi Y, Nakamura Y, Kunioka M (1989) Nuclear magnetic resonance studies of poly(3-hydroxybutyrate) and polyphosphate metabolism in *Alcaligenes eutrophus*. Appl Environ Microbiol 55:2932–2938
6. Felipe MGA, Vitolo M, Mancilha IM, Silva SS (1997) Environmental parameters affecting xylitol production from sugarcane bagasse hydrolyzate by *Candida guilliermondii*. J Ind Microbiol Biotechnol 18:251–254
7. Franson MAH (1985) Standard methods for the examination of water and wastewater, 16th edn. American Public Health Association, Washington, D.C.
8. Gomez JGC, Rodrigues MFA, Alli RCP, Torres BB, Bueno Netto CL, Oliveira MS, Silva LF (1996) Evaluation of soil gram-negative bacteria yielding polyhydroxyalkanoic acids from carbohydrates and propionic acid. Appl Microbiol Biotechnol 45:785–791
9. Gomez JGC, Fontolan V, Alli RCP, Rodrigues MFA, Bueno Netto CL, Silva LF, Simões DA (1997) Production of P3HB-co-3HV by soil isolated bacteria able to use sucrose. Rev Microbiol 28:43–48
10. Gomez JGC, Bueno Netto CL (2001) Produção de poliésteres bacterianos. In: Lima A, Aquarone B, Borzani S, Schmidell L (eds) Processos fermentativos e enzimáticos. (Biotecnologia industrial vol 3) Blücher, São Paulo, pp 219–248
11. Holmes PA (1985) Applications of PHB: a microbially produced biodegradable thermoplastic. Phys Technol 16:32–36
12. Lee SY (1998) Poly(3-hydroxybutyrate) production from xylose by recombinant *Escherichia coli*. Bioprocess Eng 18:397–399
13. Nonato RV, Mantelato PE, Rossell CEV (2001) Integrated production of biodegradable plastic, sugar and ethanol. Appl Microbiol Biotechnol 57:1–5
14. Palmqvist E (1998) Fermentation of lignocellulosic hydrolysates: inhibition and detoxification. PhD thesis, Lund University, Lund
15. Palmqvist E, Hahn-Hägerdal B (2000) Fermentation of lignocellulosic hydrolysates II: inhibitors and mechanisms of inhibition. Bioresour Technol 74:25–33
16. Piccoli RAM (2000) Optimization of process production of polyhydroxyalkanoates copolymers based on a structured mathematical model. PhD thesis, University of São Paulo, São Paulo
17. Pradella JGC, Oliveira MS, Zuccolo M, Severo ACR, Bonomi A (1994). Improved culture media for growth of *Bradyrhizobium japonicum*. World J Microbiol Biotechnol 10:112–113
18. Pradella JGC, Taciro MK, Barral MF, Lemos MF, Urenha LC, Sanchez CR, Silva SR, Souza GMA (2000) Cultivation of *Bacillus thuringiensis* subsp. *kurstaki* S93 in a fed batch process: effect of the ratio glucose to yeast extract concentration in the feeding culture media. Braz J Microbiol 31:67–72
19. Ramsay BA, Lomaliza K, Chavarie C, Dube B, Batille P, Ramsay J (1990) Production of poly-β-hydroxybutyric-*co*-β-hydroxyvaleric acids. Appl Environ Microbiol 56:2093–2098
20. Ramsay JA, Hassan MCA, Ramsay BA (1995) Hemicellulose as a potential substrate for production of poly (β-hydroxyalkanoates). Can J Microbiol 41:262–266
21. Riesenberg D, Guthke R (1999) High-cell-density cultivation of microorganisms. Appl Microbiol Biotechnol 51:422–430
22. Rodrigues RCLB, Felipe MGA, Almeida e Silva JB, Vitolo M, Gómez PV (2001) The influence of pH, temperature and hydrolysate concentration on the removal of volatile and nonvolatile compounds from sugarcane bagasse hemicellulosic hydroysate treated with activated charcoal before or after vacuum evaporation. Braz J Chem Eng 18:299–311
23. Schlegel HG, Lafferty R, Krauss I (1970) The isolation of mutants not accumulating poly-β-hydroxybutyric acid. Arch Mikrobiol 71:283–294
24. Selman-Housein G, López MA, Hernández D, Civardi L, Miranda F, Rigau J, Puigdoménech P (1999) Molecular cloning of cDNAs coding for three sugarcane enzymes involved in lignification. Plant Sci 143:163–171
25. Silva LF, Gomez JGC, Oliveira MS, Torres BB (2000) Propionic acid metabolism and poly-3-hydroxybutyrate-*co*-3-hydroxyvalerate (P3HB-co-3HV) production by *Burkholderia* sp. J Biotechnol 76:165–174
26. Steinbüchel A, Valentin HE (1995) Diversity of bacterial polyhydroxyalkanoic acids. FEMS Microbiol Lett 128:219–228
27. Steinbüchel A, Schlegel HG (1989) Excretion of pyruvate by mutants of *Alcaligenes eutrophus*, which are impaired in the accumulation of poly(β-hydroxybutyric acid) (PHB), under conditions permitting synthesis of PHB. Appl Microbiol Biotechnol 31:168–175

28. Steinbüchel A (1991) Polyhydroxyalkanoic acids. In: Byrom D (ed) Biomaterials. Macmillan, New York, pp 123–213
29. Taciro MK, Pereira RPM, Pradella JGC, Netto CLB, Simões D (1997) Poly-3-hydroxibutyrate-*co*-3-hydroxyvalerate accumulation at different strategies of substrate feeding. Braz J Microbiol 28:49–53
30. Young FK, Kastner JR, May SW (1994) Microbial production of poly-β- hydroxybutyric acid from D-xylose and lactose by *Pseudomonas cepacia*. Appl Environ Microbiol 60:4195–4198