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Polyhydroxyalkanoates Production by Recombinant *Escherichia coli* Harboring the Structural Genes of the Polyhydroxyalkanoate Synthases of *Ralstonia eutropha* and *Pseudomonas aeruginosa* Using Low Cost Substrate

[1,2]Gustavo Graciano Fonseca and [2] Regina Vasconcellos Antonio
[1]Departamento de Química, Universidade de São Paulo, CEP 05424-970, São Paulo, SP, Brasil
[2]Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, CEP 88040-900, Florianópolis, SC, Brasil

Abstract: Polyhydroxyalkanoates (PHAs) are thermoplastic, biodegradable polyesters, synthesized for some bacteria from renewable carbon sources. However, its application is limited by the high cost of production. To reduce these costs, recombinant strains that use diverse carbon sources have been developed. In this study, it was studied PHAs production by recombinant *Escherichia coli* (DH10B and JM10), harboring the structural genes of the polyhydroxyalkanoate synthases of *Ralstonia eutropha* and *Pseudomonas aeruginosa*, using hydrolyzed corn starch and soybean oil as substrate, cheese whey as supplement and acrylic acid as fatty acids β-oxidation inhibitor. The effect of these on the cell mass and the PHA content had been evaluated through an experimental design 2^4. The best results had been obtained with DH10B strain, with a dry cell weight of 0.92 g L^{-1} and 5.9% of PHA (0.5 mol% 3-hydroxybutyrate, 44.9 mol% 3-hydroxyhexanoate and 54.6 mol% 3-hydroxyoctanoate), in mineral media containing 5% of soybean oil, beyond 1 mmol of acrylic acid.

Key words: Polyhydroxyalkanoates, recombinant *Escherichia coli*, *Ralstonia eutropha*, *Pseudomonas aeruginosa*, low cost substrate

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are polyesters of various hydroxyalkanoates which are synthesized for numerous microorganisms as energy reserve material, usually when an essential nutrient is limited in the presence of excess carbon source (Anderson and Dawes, 1990; Lee 1996; Lee and Choi, 1999). The composition of PHAs depends mainly on the PHA synthases, the carbon source, the cultivation conditions and the metabolic routes involved (Rehm and Steinbüchel, 1999; Hoffmann *et al*., 2000).

One of the problems that limit the PHAs commercial application is its high cost of production. Of an economic point of view, the substrate cost (mainly carbon source) contributes more significantly with the costs of total expenses of PHAs production (Choi and Lee, 1997, 1999). To reduce the substrate cost, recombinant strains that use low cost carbon sources and correspondents culture strategies have being developed (Lee, 1996).

Hydrolyzed corn starch is available in Brazil as a low-cost substrate. Good results have been obtained using this substrate as a carbon source (Gomez *et al*., 1996). Whey is the major by-product in the manufacture of cheese or casein from milk, representing 80-90% of the volume of milk transformed (Yang *et al*., 1994). It was reported a series of papers about PHAs production by recombinant *E. coli*, in media containing whey as substrate (Kim *et al*., 2000; Choi *et al*., 1998; Lee and Choi, 2001; Ahn *et al*., 2000, 2001). Fats and oils are renewable and inexpensive agricultural co-products, there have been only few reports describing the use of fats and oils for PHA production (Fukui and Doi, 1998), for example in *Pseudomonas aeruginosa* (Eggink *et al*., 1995) and *Ralstonia eutropha* (Fukui and Doi, 1998).

At the present study, it was evaluated the PHAs production by two recombinant *E. coli* strains, harboring the structural genes of the polyhydroxyalkanoate synthases of *Ralstonia eutropha* and *Pseudomonas aeruginosa*, using Soybean Oil (SO) and Hydrolyzed Corn Starch (HCS) as substrate and Cheese Whey (CW) as supplement, beyond Acrylic Acid (AA) as fatty acids β-oxidation inhibitor. The objective of this study was to evaluate the efficiency of these composites such a way that could be possible contribute with the reduction cost of the PHAs production.

Corresponding Author: Gustavo Graciano Fonseca, Departamento de Química, Universidade de São Paulo, CEP 05424-970, São Paulo, SP, Brasil Fax: +55-11-3091-2284 Tel: +55-11-3091-2282

MATERIALS AND METHODS

This study was conducted between 2003 and 2004 at Federal University of Santa Catarina, Brazil.

Strains and plasmid: The employed strains and plasmid are related in Table 1, as well as its relevant genotypical characteristics.

Competent cells preparation and *E. coli* transformation: The competent cells had been prepared using method based on the membrane permeabilization by calcium chloride solution (Hanahan, 1983) and these had been transformed by the plasmid insertion, according to classic methodology (Sambrook and Russell, 2001).

Culture media: Recombinant *E. coli* strains cultivations were performed in mineral medium (MR) (Lee and Choi, 2001). To MR medium had been also added appropriate concentrations of Hydrolyzed Corn Starch (HCS) and Soybean Oil (SO) commercials, as carbon sources and Cheese Whey (CW) as supplement (Table 2).

HCS was prepared through the dilution of a commercial preparation to a 60% (v v^{-1}) concentration. Adequate volumes of this solution had been added to the medium, in order to obtain up to 1% (v v^{-1}) of glucose, which corresponded to 5% (v v^{-1}) of HCS. CW of commercial origin was added directly in the culture medium. The CW used had 94-95% of humidity, 4.2-5% of lactose, 0.8-1% of protein, 0.1% of lipids and 0.7-0.8% of minerals salts. The preparation of this supplement consisted of the adequate homogenization and volume addition to the culture medium.

Culture conditions: The pre culture stage was carried out in 125 mL Erlenmeyer flasks, containing 25 mL of MR medium and 1% (w v^{-1}) glucose (sterilized separately) under 150 rpm agitation, in orbital agitator at the temperature of 37°C, during 24 h. The culture stage was carried out in 500 mL chicaned Erlenmeyer flasks, containing 100 mL of MR medium and furthermore substrate and supplement in definite concentrations, in accordance with an experimental matrix 2^4, under 150 rpm agitation, in orbital agitator at the temperature of 37°C, during 48 h.

Cell mass: The inoculum's cell concentration (2% v v^{-1}, of a suspension whose absorbance was equal to 1.2) was determined by spectrometry to 600 nm. The Dry Cell Weight (DCW) was determined through centrifugation of a known volume of the culture broth (10,000 rpm, 4°C, 15 min), precipitated drying (50°C, 24 h) and posterior weighing.

Table 1: Relevant genotypical characteristics of the employed plasmid and *E. coli* strains

Strain or plasmid	Relevant genotypes	References
Plasmid		
pBHR77	*amp*^{r}, *phaC1* gene from *P. aeruginosa* and PHA operon from *R. eutropha*	(Antonio *et al*., 2000)
E. coli strains		
DH10B	-	(Stratagene, La Jolla, USA)
JM101	*thi-1*	(Stratagene, La Jolla, USA)

R. eutropha operon - *phbA*, *phbB* and *phbC*: genes codifying, respectively, to acetoacetyl-CoA reductase, β-cetotiolase and PHA synthase of *R. eutropha*; *phaC1*: gene codifying to PHA_{MCL} synthase of *P. aeruginosa*; *amp*^{r}: gene confering resistance to ampicilin; *thi-1*: gene codifying to thiamine

Table 2: Variables real levels for the complete factorial design 2^4

Variable	Symbol	Inferior level	Superior level
HCS (%)	X_1	0	5
CW (%)	X_2	0	5
SO (%)	X_3	0	5
AA (mmol)	X_4	0	1

HCS: Hydrolyzed Corn Starch; CW: Cheese Whey; SO: Soybean Oil; AA: Acrylic Acid

PHAs production: PHAs were qualitatively and quantitatively analyzed by Gas Chromatography (GC), as described earlier (Timm *et al*., 1990).

Experimental design and statistical analyses: To evaluate the influence of the variables HCS, SO, CW and AA on the PHA production (PHA%) and Dry Cell Weight (DCW), was applied an experimental design 2^4, in accordance with Table 2, in its respective real levels. The statistical analyses of the proposed designs had been carried out using software Statistica 5.11.

RESULTS

Table 3 shows the factorial design matrix and the experimental responses obtained for DCW, accumulated PHA content and composition for DH10B strain, harboring the plasmid pBHR77, while in Table 4 the results are referring to JM101 strain. Table 5 exhibits the effects obtained through statistical analysis for each one of the observed responses, in both strains.

SO had a positive influence on the DCW accumulation of *E. coli* JM 101 (Table 3), reaching a maximum value of 7.87 g L^{-1} in mineral medium containing also CW and HCS (exp. 8). However, it did not have a gradual increase on the PHA accumulation. Taking in consideration the AA addition, realizes that this relation was not kept, being that the lower cellular growth was obtained in medium containing all carbon sources and supplements (exp. 8). Despite the inserted plasmid in *E. coli* being responsible also for the medium chain length PHAs synthesis (PHA_{MCL} synthase of *P. aeruginosa*), only in medium containing SO and AA (exp. 13) was detected the presence of other monomers beyond 3HB

Table 3: Codified variables and obtained values for dry cell weight and accumulated PHA in accordance with factorial design 2^4 for *E. coli* DH10B (pBHR 77)

Exp.	HCS	CW	SO	AA	DCM (g L^{-1})	PHA (%)	3HB (%)	3HHx (%)	3HO (%)	3HD (%)	3HDD (%)
1	-1	-1	-1	-1	0.20	nd	nd	nd	nd	nd	nd
2	+1	-1	-1	-1	1.59	0.3	0.30	nd	nd	nd	nd
3	-1	+1	-1	-1	0.67	nd	nd	nd	nd	nd	nd
4	+1	+1	-1	-1	2.10	0.5	0.50	nd	nd	nd	nd
5	-1	-1	+1	-1	1.10	nd	nd	nd	nd	nd	nd
6	+1	-1	+1	-1	5.79	0.62	0.62	nd	nd	nd	nd
7	-1	+1	+1	-1	7.58	1.03	1.03	nd	nd	nd	nd
8	+1	+1	+1	-1	7.87	0.43	0.43	nd	nd	nd	nd
9	-1	-1	-1	+1	0.57	0.17	0.17	nd	nd	nd	nd
10	+1	-1	-1	+1	3.48	0.37	0.37	nd	nd	nd	nd
11	-1	+1	-1	+1	0.91	0.21	0.21	nd	nd	nd	nd
12	+1	+1	-1	+1	1.08	0.35	0.35	nd	nd	nd	nd
13	-1	-1	+1	+1	0.92	5.88	0.03	2.64	3.21	nd	nd
14	+1	-1	+1	+1	3.84	0.01	0.01	nd	nd	nd	nd
15	-1	+1	+1	+1	1.33	0.31	0.31	nd	nd	nd	nd
16	+1	+1	+1	+1	0.04	0.29	0.29	nd	nd	nd	nd

Exp.: Experiment; HCS: Hydrolyzed Corn Starch; CW: Cheese Whey; SO: Soybean Oil; AA Acrylic Acid; DCW: Dry Cell Weight; PHA: Polyhydroxyalkanoate; 3HB: 3-hydroxybutyrate; 3HHx: 3-hydroxyhexanoate; 3HO: 3-hydroxyoctanoate; 3HD: 3-hydroxydecanoate; 3HDD: 3-hydroxydodecanoate; nd: not detected

Table 4: Codified variables and obtained values for dry cell weight and accumulated PHA in accordance with factorial design 2^4 for *E. coli* JM101 (pBHR 77)

Exp.	HCS	CW	SO	AA	DCM (g L^{-1})	PHA (%)	3HB (%)	3HHx (%)	3HO (%)	3HD (%)	3HDD (%)
1	-1	-1	-1	-1	0.08	nd	nd	nd	nd	nd	nd
2	+1	-1	-1	-1	1.10	0.59	0.59	nd	nd	nd	nd
3	-1	+1	-1	-1	0.46	0.24	0.24	nd	nd	nd	nd
4	+1	+1	-1	-1	1.85	0.39	0.39	nd	nd	nd	nd
5	-1	-1	+1	-1	0.95	0.50	0.50	nd	nd	nd	nd
6	+1	-1	+1	-1	5.05	1.61	1.61	nd	nd	nd	nd
7	-1	+1	+1	-1	7.39	0.35	0.35	nd	nd	nd	nd
8	+1	+1	+1	-1	7.70	0.27	0.27	nd	nd	nd	nd
9	-1	-1	-1	+1	0.005	3.07	1.78	nd	0.44	0.85	nd
10	+1	-1	-1	+1	0.02	1.66	1.27	nd	nd	0.39	nd
11	-1	+1	-1	+1	0.01	4.28	1.06	0.21	0.61	1.77	0.63
12	+1	+1	-1	+1	1.16	1.86	1.45	nd	nd	0.41	nd
13	-1	-1	+1	+1	0.10	1.31	1.31	nd	nd	nd	nd
14	+1	-1	+1	+1	0.006	1.01	1.01	nd	nd	nd	nd
15	-1	+1	+1	+1	0.26	12.66	2.19	nd	1.00	9.47	nd
16	+1	+1	+1	+1	0.08	2.64	1.47	nd	nd	0.54	0.63

Exp.: Experiment; HCS: Hydrolyzed Corn Starch; CW: Cheese Whey; SO: Soybean Oil; AA: Acrylic Acid; DCW: Dry Cell Weight; PHA: Polyhydroxyalkanoate; 3HB: 3-hydroxybutyrate; 3HHx: 3-hydroxyhexanoate; 3HO: 3-hydroxyoctanoate; 3HD: 3-hydroxydecanoate; 3HDD: 3-hydroxydodecanoate; nd: not detected

Table 5: Variables effects on dry cell mass and polyhydroxyalkanoate content responses for *E. coli* DH10B and JM101 (pBHR77)

Strain	DH10B		JM101	
Responses	DCW (g L^{-1})	PHA (%)	DCW (g L^{-1})	PHA (%)
Media/Interation	2.44	0.65	1.64	2.03
(X_1) HCS	1.56	-0.59	0.96	-1.55
(X_2) CW	0.51	-0.53	1.45	1.62
(X_3) SO	2.23	0.83	2.11	1.03
(X_4) AA	-1.84	0.59	-2.87	3.07
(X_1) × (X_2)	-1.41	0.60	-0.30	-1.54
(X_1) × (X_3)	0.09	-0.88	0.07	-0.77
(X_1) × (X_4)	-0.39	-0.80	-0.74	-1.99
(X_2) × (X_3)	0.78	-0.58	0.88	1.25
(X_2) × (X_4)	-1.87	-0.79	-1.10	1.98
(X_3) × (X_4)	-2.21	0.51	-2.29	0.65

HCS: Hydrolyzed Corn Starch; CW: Cheese Whey; SO: Soybean Oil; AA: Acrylic Acid; DCW: Dry Cell Weight; PHA: Polyhydroxyalkanoate

(small chain length) that had been 3HHx and 3HO. This occurred because practically all the carbon source have been proceeded from SO fatty acids, benefiting itself of the acrylic acid presence, that made available the use of them through the β-oxidation.

SO had also positive influence on the DCW accumulation of *E. coli* JM 101 (Table 4). The maximum value of 7.70 g L^{-1} was reached in mineral medium increased of CW and HCS (exp. 8), disclosing a sufficiently similar behavior to the one of DH10B strain. With relation to the PHA accumulation, in turn, perceives that the AA was visibly the variable of higher contribution on this response, with results almost always very superior to the ones of correspondents' media, without AA. On the other hand, the AA addition revealed toxic to this strain, having reduced drastically the DCW accumulation. Despite this, only in the media where was added AA had detention of the monomers 3HHx, 3HO, 3HD and 3HDD, in a same way that for DH10B strain (exp. 13), confirming the action of induced β-oxidation for this acid. The AA still revealed capable to be metabolized as one of the reserve carbon sources, therefore in mineral medium added of AA it was possible the polymer detention, including medium chain length (exp. 9), despite of the low DCW presented.

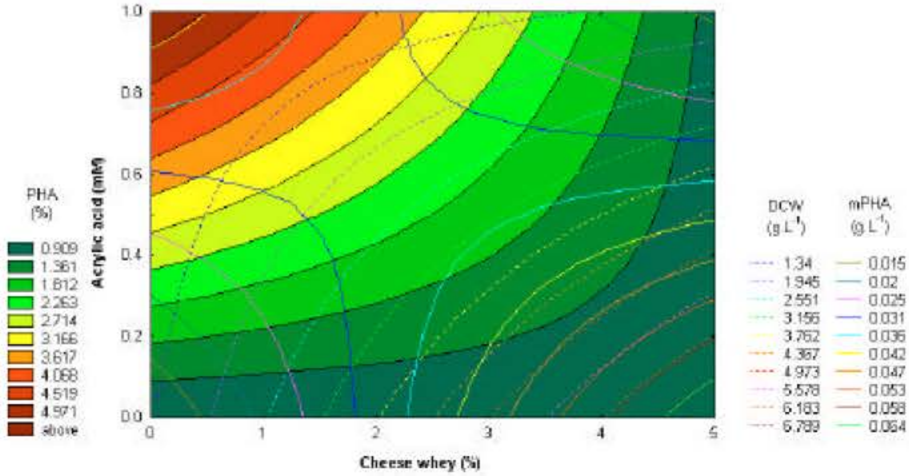

Fig. 1: Response surface generated by the curves of level for DCW, percentage and accumulated PHA mass, in function of cheese whey and acrylic acid contends (soybean oil fixed in 5%, without hydrolyzed corn starch), for *E. coli* DH10B harboring the plasmid pBHR77

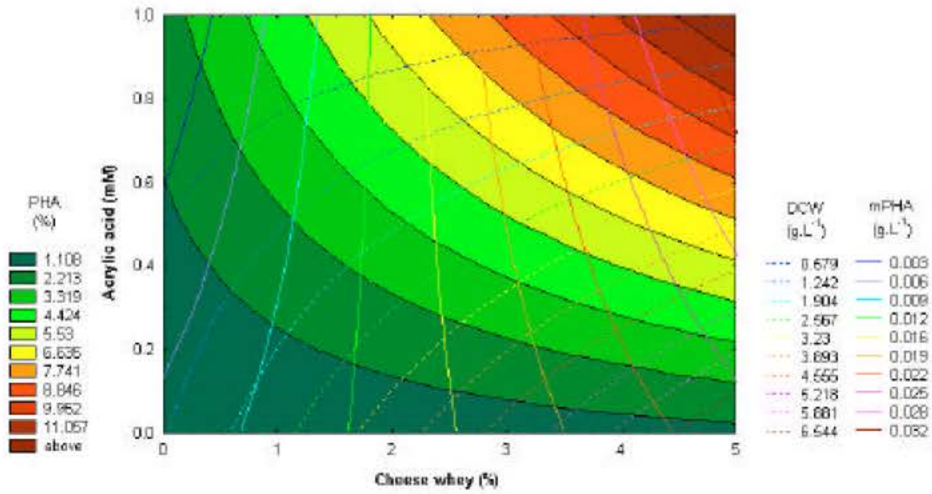

Fig. 2: Response surface generated by the curves of level for DCW, percentage and accumulated PHA mass, in function of cheese whey and acrylic acid contends (soybean oil fixed in 5%, without hydrolyzed corn starch), for *E. coli* JM101 harboring the plasmid pBHR77

The effects of each variable on the observed responses had been calculated (Table 5). For DH10B strain, DCW response was influenced mainly by the SO addition. For the accumulated PHA percentage, the best response also was obtained by SO use, but the combination with HCS or CW had negative effect. The higher negative effect was given in presence of HCS + SO. For JM101 strain, DCW response was influenced mainly by the SO addition. For the accumulated PHA percentage, the best response was obtained by the AA use, but the combination with HCS had negative effect.

The model variation in relation to the experimental data can be explained in 99.44% for the DCW and 88.88% for the accumulated PHA percentage, in *E. coli* DH10B, while in 92.77% for the DCW and 97.21% for the accumulated PHA percentage, in *E. coli* JM101. The models obtained for each one of these responses are represented, respectively, by Eq. 1 and 2, for *E. coli* DH10B and by Eq. 3 and 4, for *E. coli* JM101.

$$DCW_{DH10B} = 0.01 + 0.03X_1 + 0.17X_2 + 0.25X_3 + 0.74X_4 \quad (1)$$

$$\%PHA_{DH10B} = -0.46 + 0.24X_1 + 0.18X_2 + 0.18X_3 + 1.09X_4 \quad (2)$$

$$DCW_{JM101} = 93.6 - 17.21X_1 - 17.34X_2 - 17.24.X_3 - 87.14.X_4 \quad (3)$$

$$DCW_{JM101} = 0.50 - 0.08X_1 - 1.51X_2 - 0.10X_3 + 2.08X_4 \quad (4)$$

Figure 1 shows the overlapping of the three responses for *E. coli* DH10B. The PHA percentage is represented by areas, dry cell mass by hatched lines and PHA mass by full lines. In mineral medium containing 5% of SO, beyond 1 mmol of AA (0.1 mg L^{-1}), absentee of HCS and CW, was possible to accumulate around 5% of PHA and 1.3 g L^{-1} of DCW. In the same way, Fig. 2 shows the overlapping of the three responses for *E. coli* JM101. In mineral medium containing 5% of CW and SO, beyond 1 mmol of AA, absentee of HCS, was accumulated around 11% of PHA and 0.6 g L^{-1} of DCW.

DISCUSSION

Acrylic acid presented a high negative effect on the DCW accumulation, mainly for JM101 strain harboring the plasmid pBHR77, having diminished in 2.87 g L^{-1}, when passed from the inferior level to the superior level. For the same plasmid in DH10B strain, the influence of the acrylic acid was 1.84 g L^{-1}. These results suggests that acid acrylic, in the utilized concentration, has a toxic effect, mainly on JM101 strain, causing an inhibition of the cellular growth. Moreover, Fonseca (2003), working with the same strains in another plasmid (pBHR71), obtained results sufficiently similar, that over all confirm the toxic effect in JM101 strain.

On the other hand, the acrylic acid addition presented a positive effect, on the PHA accumulation in *E. coli* JM101 harboring pBHR77, increasing in 3.07 the accumulated PHA%. However, to *E. coli* DH10B (pBHR77) is not possible to affirm the same.

The fatty acid β-oxidation route was employed to provide various 3-hydroxyacyl-CoA thioesters, only when soybean oil was utilized as carbon source for the PHA synthase. *In vivo* PHA synthase activity was indicated by PHA accumulation and substrate specificity was revealed by analysis of the comonomer composition of the respective polyester. In recombinant *E. coli* JM101 the PHA synthase led to accumulation of different combination copolymers of 3-hydroxybutyrate (3HB), 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD) and 3-hydroxydodecanoate (3HDD), when soybean oil and/or acrylic acid were provided as carbon source and/or fatty acids β-oxidation inhibitor, respectively. These data suggested that the PHA synthase accepts, besides the main substrate 3-hydroxybutyryl-CoA, also the CoA thioesters of 3HHx, 3HO, 3HD and 3HDD.

Aiming to obtain the best media conditions, for of *E. coli* cultures, harboring the plasmid pBHR77, the best experimental responses, according to factorial design 2^4, for *E. coli* DH10, were: DCW of 1.3 g L^{-1} and 5% of PHA, in mineral medium containing 5% of soybean oil, beyond 1 mmol of acrylic acid and for *E. coli* JM101: DCW of 0.6 g L^{-1} and 11% of PHA, in mineral medium containing 5% of cheese whey and 5% of soybean oil, beyond 1 mmol of acrylic acid.

Regarding PHA and DCW contents, the results acquired in this study are consistent with the data obtained in cultures of other recombinant strains, harboring the same plasmid (Antonio *et al.*, 2000). Unlike the latter experiments, in which cells had been grown in a complex medium, our cultures were carried out in a mineral medium composed of low cost carbon sources.

The cost of the carbon source contributes significantly to the overall production cost of PHA (Yamane 1992, 1993). For the process with recombinant *E. coli*, the cost of the carbon source can be as high as 38% (Choi and Lee, 1998). Use of cheaper carbon sources can lower the production cost of PHAs. If hydrolyzed corn starch (\$ 0.22 kg^{-1}) can be used instead of glucose (\$ 0.5 kg^{-1}) for polyhydroxybutyrate (P(3HB)) production by recombinant *E. coli* without changing the fermentation performance, the production cost of PHAs will decrease to \$ 3.72 kg^{-1} P(3HB), which is \$ 1.19 kg^{-1} lower than that obtained with glucose (Choi and Lee, 1999).

Because of their low price, crude carbon substrate, such as cane and beet molasses, cheese whey, plant oils and hydrolysates of starch (corn and tapioca), cellulose and hemicellulose, can be excellent substrates for the bacteria utilizing them. Several bacteria can produce PHAs from these inexpensive carbon substrates but, in general, the PHA content and productivity are much lower than those obtainable with purified carbon substrates (Kim and Chang, 1995; Ramsay *et al.*, 1995). However, incorporation of the PHAs production process into the industry (i.e., cheese manufacturing) will be a good strategy for a cheap residue (\$ 0.07 kg^{-1}), which will reduce the cost of whey disposal and, at the same time, to produce a more valuable product (Choi and Lee, 1999).

Furthermore soybean oil seems to be also a good candidate for low cost applications since it costs \$ 0.21 kg^{-1} (USDA-United States Department of Agriculture, 1996) and our results showed that it can be utilized as substrate for the synthesis of different PHA monomers (3HB, 3HHx, 3HO, 3HD and 3HDD).

A comparison of our results with those obtained in large scale fed-batch reactors (Ahn *et al.*, 2000, 2001), using high concentrated carbon sources, even of low cost, is not practical. Nevertheless, we believe that the reactors up-scale and the low cost substrate concentration may provide much better results, making our finds look very promising.

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