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Polyhydroxyalkanoates Production by Recombinant *Escherichia coli* Using Low Cost Substrate

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Abstract: Polyhydroxyalkanoates (PHAs) are thermoplastic, biodegradable polyesters, synthesized by 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 *Pseudomonas aeruginosa*, using hydrolyzed corn starch and soybean oil as substrate, cheese whey as supplement and acrylic acid as fatty acids β-oxidation inhibitor. Their effect 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: Dry cell weight of 1.02 g L^{-1} and 23% of PHA (9 mol% 3-hydroxybutyrate, 4.5 mol% 3-hydroxyoctanoate, 30.7 mol% 3-hydroxydecanoate and 55.8 mol% 3-hydroxydodecanoate), in mineral media containing 5% of hydrolyzed corn starch, 5% de cheese whey and 5% of soybean oil, beyond 1 mM of acrylic acid.

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

Introduction

Polyhydroxyalkanoates (PHAs) are polyesters synthesized by numerous bacteria and storaged by the form of cytoplasmatic inclusions as energy reserve material and reducing power under certain unbalanced growth conditions (Anderson and Dawes, 1990; 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 (Hoffmann *et al*., 2000). Economic evaluation of the process for the production of P(3HB) suggested that the major contributor of the overall 3(PHB) production cost was carbon substrate cost (up to 50%) (Choi and Lee, 1997).

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). Lactose is the main component of the cheese whey and many *Escherichia coli* strains can use lactose for growth. A series of papers had been reported the PHAs production by recombinant *E. coli*, in medium containing cheese whey as substrate (Lee *et al*., 1997; Choi *et al*., 1998; Kim *et al*., 2000; 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

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production (Fukui and Doi, 1998). Although have been obtained PHAs from vegetal oils and/or fats by *Pseudomonas* sp. (Kato *et al*., 1996), *Pseudomonas aeruginosa* (Eggink *et al*., 1995), *Pseudomonas resinovorans* (Cromwick *et al*., 1996; Ashby and Foglia, 1998), *Aeromonas caviae* (Shimamura *et al*., 1994); *Ralstonia eutropha* and recombinant strain (Fukui and Doi, 1998), still not was reported the production by recombinant *E. coli*.

Recently, it has been demonstrated that recombinant strains of *E. coli* harboring either PHA synthase genes *phaC1* or *phaC2* from *P. aeruginosa* are able to produce medium chain length polyhydroxyalkanoates (PHA_{MCL}) (Langenbach *et al*., 1997; Qi *et al*., 1997, 1998). At the present work, it was evaluated the PHAs production by two recombinant *E. coli* strains, containing the gene codifying for the PHA_{MCL} synthase of *P. 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 compounds in way that could be possible contribute with the reduction cost of the PHAs production.

Materials and Methods

The present study was conducted between 2003 and 2004 at the Department of Chemical Engineering and Food Engineering from Federal University of Santa Catarina, Brazil.

Strains, Plasmid, Cells Preparation and Escherichia coli Transformation

E. coli JM101 and DH10B (Stratagene, La Jolla, USA) harbouring the plasmid pBHR71 (Langenbach *et al*., 1997) were employed for these studies. Plasmid pBHR71 contains the *phaC1* gene codifying to PHA_{MCL} synthase of *P. aeruginosa*; and amp^r gene conferring resistance to ampicilin. 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 1). 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, 1% (w v^{-1}) glucose (sterilized separately), under 150 rpm agitation, in orbital agitator to 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 the experimental matrix 2^4, under 150 rpm agitation, in orbital agitator to the temperature of 37°C, during 48 h.

Cells Mass and PHAs Production

The inoculum cell concentration (2% v v^{-1}, of a suspension whose absorbance was equal to 1.2) was determined by spectrometry at 600 nm. The Dry Cell Weight (DCW) was determined through

Table 1: 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 (mM)	X_4	0	1

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

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. PHAs were qualitatively and quantitatively analyzed by Gas Chromatography (GC), as described previously by 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 1, in its respective real levels. Statistical analyses of the proposed design had been carried out using software Statistica 5.11.

Results and Discussion

In the present study, a factorial design 2^4 was applied aiming to test hydrolyzed corn starch, cheese whey and soybean oil in mineral medium and the acrylic acid addition, for the cultures of *E. coli* DH10B and JM101, harboring the plasmid pBHR71, as alternatives for reduction costs of the PHAs production. Table 2 shows the factorial design matrix and the experimental responses obtained for dry cell mass, accumulated PHA content and composition for DH10B strain, harboring the plasmid pBHR71, while in Table 3 the results are referring to JM101 strain. Table 4 exhibits the effects obtained through statistical analysis for each one of the observed responses, in both strains.

According to Table 2, the variables in study, when separately, had low influence on the DCW accumulation. Moreover, it was not obtained cellular growth in medium containing only vegetal oil as carbon source (0% HCS, 0% CW, 5% SO and 0 mM AA) (experiment 5). This observation indicates that there was no nutritional limitation, due the absence or limitation of an essential nutrient for growth, because even when none of variables in study was present (0% HCS, 0% CW, 0% SO and 0 mM AA) (experiment 1) cells could grow. It is probable that the high concentration of SO employed caused the inhibition of growth, due difficulties found in the metabolic changes between the cell membrane and medium, causing increase in membrane fluidity, leading to disruption and cell leakage and cell death (Denyer and Stewart, 1998; Haidekker *et al*., 2000). Despite the inserted plasmid in *E. coli* being responsible only for PHA_{MCL} synthesis, in the majority of the situations, the presence of 3HB was detected, even that in sufficiently low levels, what it is believed to be function of the proper endogenous metabolism of the *E. coli* strain (Amaratunga *et al*., 2000). Differently of the expected, in none of the experiments was detected the 3HHx monomer when in acrylic acid presence, since it was previously reported the synthesis of 3HHx monomer from *phaC1* synthase of *P. aeruginosa*, harbored in *E. coli* (Langenbach *et al*., 1997; Qi *et al*., 1998). Regardless of, medium containing acrylic acid provided the highest PHA accumulation percentage (22.99%) when combined to the furthermore variables (5% HCS, 5% CW, 5% SO and 1 mM AA) (experiment 16), with the presence of monomers 3HO, 3HD and 3HDD in the values of 1.04, 7.06 and 12.83%, respectively. It seems that the joined action of these complex compounds in a defined medium (MR medium) was the great responsible for the higher range of monomers synthesized. These monomers, beyond 3HHx, were previously synthesized by recombinant *E. coli*, although decanoic acid had been used as carbon source in the complex Luria-Bertani broth (LB medium) (Langenbach *et al*., 1997; Qi *et al*., 1998). Moreover, the use of the cheap MR medium instead of the expensive LB medium seems reasonable, once that the DCW obtained in the present study was sufficiently interesting (1.02 g L^{-1}), becoming this situation passive of future inquiries, aiming at an improvement of these values.

Table 2: Codified variables and obtained values for dry cell weight and accumulated PHA in accordance with factorial design 2^4 for *E. coli* DH10B (pBHR 71)

Exp.	HCS	CW	SO	AA	DCW (g L^{-1})	PHA (%)	3HB (%)	3HHx (%)	3HO (%)	3HD (%)	3HDD (%)
1	-1	-1	-1	-1	0.09	0.15	0.15	nd	nd	nd	nd
2	+1	-1	-1	-1	0.23	0.11	0.11	nd	nd	nd	nd
3	-1	+1	-1	-1	0.39	1.55	1.55	nd	nd	nd	nd
4	+1	+1	-1	-1	0.80	0.60	nd	nd	nd	0.6	nd
5	-1	-1	+1	-1	nd	nd	nd	nd	nd	nd	nd
6	+1	-1	+1	-1	1.37	0.51	0.51	nd	nd	nd	nd
7	-1	+1	+1	-1	1.05	nd	nd	nd	nd	nd	nd
8	+1	+1	+1	-1	0.83	nd	nd	nd	nd	nd	nd
9	-1	-1	-1	+1	0.32	0.09	0.09	nd	nd	nd	nd
10	+1	-1	-1	+1	0.42	0.04	0.04	nd	nd	nd	nd
11	-1	+1	-1	+1	0.73	nd	nd	nd	nd	nd	nd
12	+1	+1	-1	+1	1.05	0.50	nd	nd	nd	0.50	nd
13	-1	-1	+1	+1	0.23	1.26	0.60	nd	nd	0.26	0.40
14	+1	-1	+1	+1	0.85	nd	nd	nd	nd	nd	nd
15	-1	+1	+1	+1	0.87	1.99	1.16	nd	nd	0.65	0.18
16	+1	+1	+1	+1	1.02	22.99	2.06	nd	1.04	7.06	12.83

Exp.: Experiment; HCS: Hydrolyzed Corn Starch; CW: Cheese Whey; SO: Soybean Oil; AA: Acrylic Acid; DCW: Dry Cell Weight; PHA: Polyhydroxyalkanoate; 3HHx: 3-hydroxyhexanoate; 3HO: 3-hydroxyoctanoate; 3HD: 3-hydroxydecanoate; 3HDD: 3-hydroxydodecanoate

Table 3: Codified variables and obtained values for dry cell weight and accumulated PHA in accordance with factorial design 2^4 for *E. coli* JM101 (pBHR 71)

Exp.	HCS	CW	SO	AA	DCW (g L^{-1})	PHA (%)	3HB (%)	3HHx (%)	3HO (%)	3HD (%)	3HDD (%)
1	-1	-1	-1	-1	0.17	nd	nd	nd	nd	nd	nd
2	+1	-1	-1	-1	1.20	1.30	nd	nd	nd	1.30	nd
3	-1	+1	-1	-1	0.48	3.31	3.31	nd	nd	nd	nd
4	+1	+1	-1	-1	1.53	0.25	nd	nd	nd	0.25	nd
5	-1	-1	+1	-1	nd	nd	nd	nd	nd	nd	nd
6	+1	-1	+1	-1	3.63	0.40	0.40	nd	nd	nd	nd
7	-1	+1	+1	-1	2.19	0.34	0.34	nd	nd	nd	nd
8	+1	+1	+1	-1	0.86	nd	nd	nd	nd	nd	nd
9	-1	-1	-1	+1	0.04	0.55	0.38	nd	nd	nd	0.17
10	+1	-1	-1	+1	0.02	0.93	0.48	nd	nd	0.23	0.22
11	-1	+1	-1	+1	0.14	1.49	0.44	nd	0.18	0.65	0.22
12	+1	+1	-1	+1	0.15	0.82	0.34	nd	nd	nd	0.48
13	-1	-1	+1	+1	0.08	nd	nd	nd	nd	nd	nd
14	+1	-1	+1	+1	nd	nd	nd	nd	nd	nd	nd
15	-1	+1	+1	+1	0.08	0.28	0.28	nd	nd	nd	nd
16	+1	+1	+1	+1	0.17	0.82	0.82	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; 3HHx: 3-hydroxyhexanoate; 3HO: 3-hydroxyoctanoate; 3HD: 3-hydroxydecanoate; 3HDD: 3-hydroxydodecanoate

Table 4: Variables effects on dry cell weight and polyhydroxyalkanoate content responses for *E. coli* DH10B and JM101 (pBHR71)

Strain	DH10B		JM101	
Responses	DCM (g L^{-1})	PHA (%)	DCM (g L^{-1})	PHA (%)
Media/Interaction	0.64	1.86	0.67	0.66
(X_1) HCS	0.36	2.46	0.54	-0.18
(X_2) CW	0.40	3.18	0.06	0.52
(X_3) SO	0.27	2.96	0.41	-0.85
(X_4) AA	0.09	2.99	-1.17	-0.09
(X_1) x (X_2)	-0.20	2.67	-0.59	-0.70
(X_1) x (X_3)	0.12	2.60	0.03	0.33
(X_1) x (X_4)	-0.06	2.58	-0.55	0.24
(X_2) x (X_3)	-0.07	2.62	-0.16	-0.26
(X_2) x (X_4)	0.06	2.84	0.04	-0.03
(X_3) x (X_4)	-0.16	3.44	-0.41	0.18

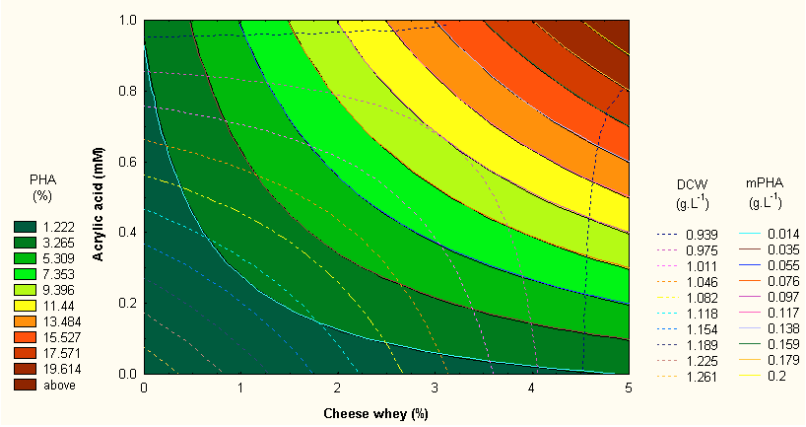
HCS: Hydrolyzed Corn Starch; CW: Cheese Whey; SO: Soybean Oil; AA: Acrylic Acid; DCW: Dry Cell Weight; PHA: Polyhydroxyalkanoate

From Table 3, we observe that the combination of variables (two by two) also brought improvements on the DCW accumulation when compared the influence of each one of them in isolated. The highest values of DCW had been obtained for the combination of 5% HCS + 5% SO (0% CW and 0 mM AA) (experiment 6) and 5% CW + 5% SO (0% HCS and 0 mM AA) (experiment 7), with 3.63 and 2.19 g L^{-1} of accumulation, respectively. However, in both cases only 3HB was detected. Moreover, it was also not obtained cellular growth in medium containing only vegetal oil as carbon source (0% HCS, 0% CW, 5% SO and 0 mM AA) (experiment 5) by the same reason reported for DH10B strain. These observations are in accordance with the results obtained by Qi *et al*. (1998), which reported that *E. coli* harboring the plasmid pBHR71 did not grown in mineral medium added only of decanoic acid. On the other hand, increasing the complexity of the medium with the addiction of glucose, yeast extract and/or tryptone favored growth and/or polymer synthesis (Langenbach *et al*., 1997; Qi *et al*., 1998). For *E. coli* the JM101, contrarily to DH10B strain, we can not affirm that increase in the complexity favored polymer synthesis. AA addition, for example, revealed toxic to this strain, having reduced drastically the DCW accumulation, that did not exceed 0.17 g L^{-1}, when present in the most complex medium (5% HCS, 5% CW, 5% SO and 1 mM AA) (experiment 16), value these equal to the medium without addition of none of the variables (0% HCS, 0% CW, 0% SO and 0 mM AA) (experiment 1). Although that, in media containing AA a higher content of monomers 3HO, 3HD and 3HDD was detected. This behavior was similar to DH10B strain, confirming the induced β-oxidation action by this acid.

The effects of each variable on the observed responses, for both strains, had been calculated, as shown Table 4. For DH10B strain, DCW was influenced mainly by the CW and HCS addition, which had contributed in 0.40 and 0.36 g L^{-1} when passed from the inferior level to the superior level. However, these variables combination presented a reduction effect of 0.20 g L^{-1} in the DCW. For the accumulated PHA percentage, the best response, among the main effects, was obtained with CW use, providing an increase in order of 3.18%. CW and AA had also been excellent, improving in 2.96 and 2.99% the PHA accumulation. Also, the combination of these variables had a still higher effect that each of them separately, being this value, 3.44%, the higher among all the furthermore effects. For JM101 strain, DCW was influenced mainly by HCS and SO addition, which had contributed in 0.54 and 0.41 g L^{-1}. For the accumulated PHA percentage, the best response was obtained with CW use, but the combination with HCS or SO had negative effect. CW was the only variable with positive effects on DCW and PHA production responses for both studies strains. Although some combinations with other variables caused negative impact on the experimental responses (DCW and %PHA), CW utilization showed interesting to be more explored in future investigations, on a concentrated form. In the present study, CW was employed as a supplement, at concentrations not enough to be utilised as an energy/carbon source. However, it seemed to provide the necessary co-factors (vitamins and precursors) for cell growth and PHA production.

The model variation in relation to the experimental data can be explained in 96.49% for the DCW and 94.11% for the accumulated PHA percentage, in *E. coli* DH10B, while in 89.52% for the DCW and 97.64% 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.

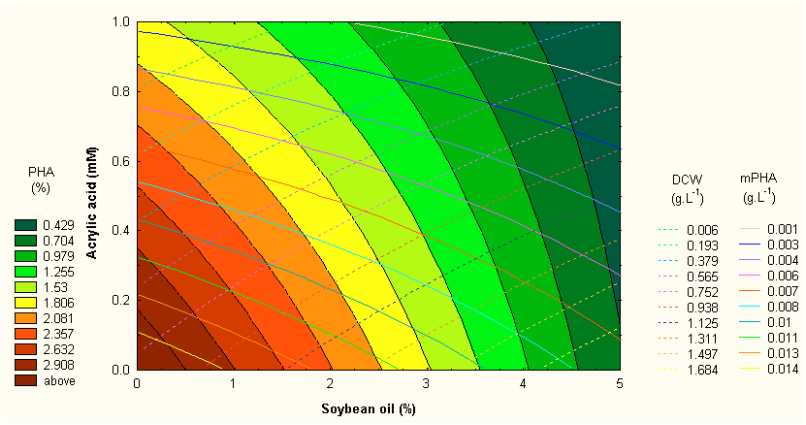
Figure 1 shows the overlapping of the three responses for *E. coli* DH10B, being the PHA percentage represented for areas, dry cell mass for hatched lines and PHA mass for full lines. It is perceives that in mineral medium containing 5% of HCS, CW and SO, beyond 1 mM of AA (0.1 mg L^{-1}) (all the parameters in its superior levels) it is possible to accumulate around 20% of PHA, 0.95 g L^{-1} of DCW and 0.2 g L^{-1} of PHA mass. In the same way, Fig. 2 shows the overlapping of the three responses for *E. coli* JM101. It is observed that in mineral medium containing 5% of CW, absentee of HCS, SO and AA, can be accumulated around 3% of PHA, 0.85 g L^{-1} of DCW and 0.015 g L^{-1} of PHA mass. The results obtained with *E. coli* DH10B are comparable with the 21% f PHA synthesized by *E. coli* LS1298 harboring the same plasmid pBHR71 in LB medium added of decanoic acid (Langenbach *et al*., 1997).



$$DCW_{DH10B} = 0.02 + 0.06.X_1 + 0.09.X_2 - 0.01.X_3 + 0.38.X_4 \quad (1)$$

$$\%PHA_{DH10B} = -1.18 + 0.52.X_1 + 0.81.X_2 + 0.50.X_3 + 2.60.X_4 \quad (2)$$

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 (hydrolyzed corn starch and soybean oil fixed in 5%), for *E. coli* DH10B harboring the plasmid pBHR71



$$DCW_{JM101} = -0.15 + 0.33.X_1 + 0.19.X_2 + 0.10.X_3 + 0.51.X_4 \quad (3)$$

$$\%PHA_{JM101} = 0.13 + 0.21.X_1 + 0.61.X_2 - 0.51.X_3 + 0.30.X_4 \quad (4)$$

Fig. 2: Response surface generated by the curves of level for DCW, percentage and accumulated PHA mass, in function of soybean oil and acrylic acid contends (cheese whey fixed in 5%, without hydrolyzed corn starch), for *E. coli* JM101 harboring the plasmid pBHR71

Acrylic acid presented a high negative effect on the DCW accumulation, mainly for JM101 strain harboring the plasmid pBHR71, having diminished in 1.17 g L^{-1}, when passed from the inferior level to the superior level. For the same plasmid in DH10B strain, the influence of acrylic acid was practically null. These results suggest that acrylic acid, in the used 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 (pBHR77), obtained results sufficiently similar, that over all confirm the toxic effect in JM101 strain. It was reported a strain-dependence effect of acrylic acid on the PHA accumulation, with larger amounts of PHA being obtained with deregulated β-oxidation mutants (Qi *et al*. 1998).

On the other hand, SO addition presented positive effect, on the DCW, for both studied strains (2.23 g L^{-1} for DH10B and 2.11 g L^{-1} for JM101), harboring the plasmid pBHR71. However, the SO and AA combination supplied a negative effect practically identical to the SO (positive) in numerical terms. This shows that in media containing SO and AA overrule the negative effect of this last one, influencing negatively the microorganism growth. Acid acrylic presented positive effect in the PHA accumulation in DH10B strain harboring pBHR71, increasing in 2.99 the accumulated PHA percentage. With respect to JM101 strain (pBHR71) it is not possible affirm the same. This result was exactly the opposite obtained by Fonseca (2003), what leads to believe that the polymer synthesis mechanism does not depend if the strain is or not affected in its cellular growth. JM101 strain, by having been more affected for the acrylic acid in the DCW accumulation, as can be observed in Table 3, had its accumulated PHA mass sufficiently reduced. Qi *et al*. (1998) reported an acrylic acid concentration of about 3.5 mM in *E. coli* RS3097 for a maximum PHA accumulation, which is a much higher value compared to the acrylic acid concentration of 1 mM from this study. It is probable that the increase in acrylic acid concentration shall improve the DCW accumulation of *E. coli* DH10B, however it is also possible to occur a reduction in the PHA content (Table 4).

It could also be observed that the fatty acid β-oxidation route was employed to provide various 3-hydroxyacyl-CoA thioesters (Qi *et al*., 1998). In both recombinant *E. coli* DH10B and JM101, the PHA synthase led to accumulation of different combination copolymers of 3-hydroxybutyrate (3HB), 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD) and 3-hydroxydodecanoate (3HDD), what suggests that the PHA synthase accepts the CoA thioesters of 3HO, 3HD and 3HDD.

Regarding PHA and DCW content, the results acquired in this study are consistent with the data obtained in cultures with other recombinant strains, harboring the same plasmid (Langenbach *et al*., 1997; Qi *et al*., 1998). 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. Considering that in a process with recombinant *E. coli*, the cost of the carbon source can be as high as 38% (Choi and Lee, 1997), use of cheaper carbon sources is an advantage, despite that, in general, the PHA content and productivity are lower than those obtainable with purified carbon substrates (Kim and Chang, 1995; Ramsay *et al*., 1995). 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. We believe that further investigations are necessary to enhance PHA synthesis and DCW accumulation in recombinant *E. coli*. However, the reactors up-scale and the low cost substrate concentration may provide much better results, making these results look very promising.

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