Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 5 (6): 521-529, 2010
ISSN 1816-4935 / DOI: 10.3923/jm.2006.234.242

Application of Factorial Design to Polyhydroxyalkanoate Production by Recombinant *Escherichia coli**

[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]Centro de Ciências Biológicas, Departamento de Bioquímica, Universidade
Federal de Santa Catarina, CEP 88040-900, Florianópolis, SC, Brasil

Abstract: Polyhydroxyalkanoates (PHAs) are biodegradable, thermoplastic polyesters produced from renewable carbon sources by a number of bacteria. However, their application is limited by high production costs. One of the strategies aimed to reduce their costs is the development of recombinant strains able to utilize different carbon sources. The optimal conditions for 3-hydroxybutyrate (P[3HB]) production by the recombinant *Escherichia coli* strain JM101, harboring *Ralstonia eutropha* PHA biosynthesis genes, in a defined medium containing hydrolyzed corn starch and soybean oil as carbon sources and cheese whey as a supplement had been established earlier. In order to optimize PHA production, the present study investigated the influence of other variables, such as isopropyl-1-thio-β-D-galactopyranoside (IPTG) and acrylic acid addition, temperature, inoculum size and cultivation time, on dry cell weight (DCW) and PHA accumulation through a 2^5 factorial design. Statistical models suggested that quite good PHA production performances would be obtained in *E. coli* JM101 cultures grown in a medium lacking IPTG and acrylic acid with an inoculum size of 5% (v/v) and performed at 37°C for 96 h. Theoretically, about 3.5 g L^{-1} DCW and 75% PHA could be accumulated. Acrylic acid had a negative effect on DCW at its high level (1 mmol) and was unable to promote incorporation of medium-chain-length units into PHA polymers as expected. Remarkably, IPTG, a very expensive inducer of PHA synthesis gene expression had no influence on PHA production.

Key words: Factorial design, low cost substrate, polyhydroxyalkanoate (PHA), *Ralstonia eutropha*, recombinant *Escherichia coli*

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are polymers of various hydroxyalkanoates produced by numerous microorganisms as an energy and/or carbon storage material under conditions of nutrient imbalance (Anderson and Dawes, 1990; Lee and Choi, 1999). For their physicochemical properties, some of them have been considered promising substitutes for petrochemical-based polymers provided competitive prices are set (Lee, 1996b). PHA production costs depend on polymer yields, recovery methods and, above all, the cost of the carbon source employed (Lee, 1996a), which contributes significantly to overall production costs (Choi and Lee, 1997, 1999). In order to reduce these costs, recombinant *Escherichia coli* strains, able to grow on cheap carbon sources and carry PHA synthesis genes, as well as alternative cultivation strategies have been studied (Lee, 1996b).

Studies on poly-(3hydroxybutyrate) (P[3HB]) production from cheap carbon sources by native PHA producing organisms as *Ralstonia eutropha* and *Alcaligenes latus* have been performed by some

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

Originally Published in Research Journal of Microbiology, 2006

authors, but the yields obtained on these substrates have been quite lower relative to yields on glucose (Kim and Chang, 1995; Lee, 1996b). Among the strategies to decrease PHA production costs is the utilization of food processing residues as cheap carbon sources. However, since native producing strains usually require limitation of any essential nutrient for PHA synthesis, the use of food processing residues of indefinite composition is limited. The introduction of PHA synthesis genes into *E. coli* cells enables them to produce the polymer irrespective of nutrient limitation. Thus, recombinant *E. coli* strains seem to be adequate for PHA production from food processing residues.

Research in cell growth and PHA production from food processing residues has been succeeded (Lee and Choi, 1999). If residues from the food industry were used as carbon sources, the polymer cost would be reduced and the residues, once useless, would become valuable. Although hydrolyzed corn starch is not a residue, it represents a low cost carbon source, especially in Brazil, where it is less expensive than glucose and has led to good results concerning PHA productivity (Gomez *et al*., 1996). On the other hand, whey is the major by-product of cheese or casein manufacture and represents 80-90% of the original milk volume (Yang *et al*., 1994). Lactose and lipids, able to support bacterial growth, are its main components. Moreover, many authors have reported PHA production by recombinant *E. coli* utilizing cheese whey as a substrate (Choi *et al*., 1998; Wong and Lee, 1998; Kim *et al*., 2000; Ahn *et al*., 2001). Even though fats and oils are renewable, inexpensive agricultural products, only a few reports have described their use for PHA production 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), *R. eutropha* and a recombinant *R. eutropha* strain (Fukui and Doi, 1998); however, the utilization of theses substrates by recombinant *E. coli* has not been reported.

Besides carbon sources, the addition of supplements to the medium may improve PHA production. Acrylic acid, for instance, is an inhibitor of 3-ketoacyl-CoA thiolase, an enzyme that catalyzes the final step of fatty acid β-oxidation. It was reported to increase medium-chain-length PHA production in recombinant *E. coli* cultures, through the channeling of intermediates of β-oxidation to the polymer synthesis (Qi *et al*., 1998). Isopropyl-1-thio-β-D-galactopyranoside (IPTG), in turn, stimulates gene expression (Berg *et al*., 2002). Although being an expensive inducer, many authors report its use (Taguchi *et al*., 1999, Rehm *et al*., 2002, Zheng *et al*., 2004).

In an earlier study, the effect of hydrolyzed corn starch and soybean oil as substrates and cheese whey as a supplement on P[3HB] production by a recombinant *E. coli* strain, containing PHA biosynthesis genes from *R. eutropha* carried on the plasmid pBHR68 had been investigated through a factorial design. The statistical analysis of the combined effects had indicated that high PHA production would be obtained with 5% (v/v) cheese whey, 1.5% (v/v) soybean oil and 5% (v/v) hydrolyzed corn starch (Fonseca, 2003).

In the present study, with the aim of optimizing PHA production by the recombinant *E. coli* strain JM101, containing the plasmid pBHR68, the effect of the addition of acrylic acid and IPTG, as supplements, temperature and cultivation time as well as inoculum size on DCW, PHA cell content and PHA mass was studied through a factorial design under the conditions previously established.

MATERIALS AND METHODS

Strain, Plasmid and Genetic Techniques

Recombinant *E. coli* strain JM101 (Stratagene, La Jolla, USA), harboring the plasmid pBHR68, was used in the present study. This plasmid contains the entire PHA synthesis operon from *R. eutropha* (*phaC*, *phaA* and *phaB*) (Spiekermann *et al*., 1999). The pBHR68 was kindly provided by Dr. Bernd Rehm, from Institut für Mikrobiologie, Westifaliche Wilhelms Universität (Muenster, Germany). The competent cells were prepared using a method based on membrane permeabilization by a calcium chloride solution (Hanahan, 1983) and transformed by plasmid insertion, according to a classic methodology (Sambrook and Russell, 2001).

Culture Media

Recombinant *E. coli* (pBHR68) cultures were grown in defined mineral medium (MR medium) for factorial design experiments. The MR medium contained (per L): 6.67 g KH_2PO_4, 4 g $(NH_4)_2HPO_4$, 0.8 g $MgSO_4.7H_2O$, 0.8 g citric acid, 0.01 g thiamine, 50 mg ampicilin and 0.5 mL (a 10 fold reduction in the original value of Lee and Choi, 2001) trace metal solution. It contained (per L of a 0.5 mol HCl solution): 10 g $FeSO_4$ H_2O, 2 g $CaCl_2$, 2.2 g $ZnSO_4 \cdot 7H_2O$, 0.5 g $MnSO_4.4H_2O$, 1 g $CuSO_4.5H_2O$, 0.1 g $(NH_4)_6Mo_7O_{24}.4H_2O$ and 0.02 g $Na_2B_4O_7.10H_2O$ (Lee and Choi, 2001). Thiamine and ampicillin were sterilized separately through filtration and added to the previously sterilized medium.

Inoculum Preparation

Inocula were prepared in 125 mL Erlenmeyer flasks containing 25 mL of medium added of 1% (w/v) glucose on a rotary shaker at 37°C and 150 rpm. After 24 h, samples of this culture were taken to perform absorbance measurements at 600 nm and its concentration was adjusted through dilution in order to obtain an absorbance equal to 1.2. The factorial design experiments were performed with inoculum sizes of 2% (v/v) and 5% (v/v).

Cultivation Conditions

Experiments were carried out (in duplicate) in 500 mL Erlenmeyer flasks containing 100 mL of MR medium, as well 5% (v/v) hydrolyzed corn starch (HCS), 1.5% (v/v) soybean oil (SO) and 5% (v/v) cheese whey (CW) (as established earlier by Fonseca (2003)) on a rotary shaker.

Crude cheese whey was obtained from a dairy products manufacturer in Santa Catarina State-Brazil. It was brought to the laboratory and kept at -20°C until added to the medium prior to sterilization. According to the supplier, its composition was 94-95% water, 4.2-5% lactose, 0.8-1% protein, 0.1% lipids and 0.7-0.8% mineral salts. Commercial grade soybean oil was used. A commercial preparation of hydrolyzed corn starch was diluted to 60% (v/v) of its initial concentration. Then, after sterilization, adequate volumes were added to the medium in order to obtain up to 1% (v/v) of glucose. The other experimental conditions are presented in Table 1.

Dry Cell Weight (DCW) Determination

Dry cell weight was determined through centrifugation of a known volume of the culture broth, sediment drying and posterior weighing.

PHA Determination

PHAs were qualitatively and quantitatively evaluated by gas chromatography (GC) as described previously by Timm *et al*. (1990). Cells (10 mg DCW) were suspended in 1 mL of a 15% (w/w) sulfuric acid solution in methanol and 1 mL of chloroform and afterwards the suspension was subjected to methanolysis (100°C, 4 h). The resultant methyl esters of HAs were analyzed by GC. Areas and retention times obtained from definite concentrations of standards were utilized to calculate the PHA content in samples.

Table 1: Variable limits for the full 2^5 factorial design

		Level	
Independent variable	Symbol	-1	+1
Inoculum size (% v/v)	X_1	2	5
IPTG (mmol)	X_2	0	1
Acrylic acid (mmol)	X_3	0	1
Time (h)	X_4	48	96
Temperature (°C)	X_5	30	37

Factorial Design and Statistical Analysis

A two-level factorial design was applied to evaluate the effects of acrylic acid and isopropyl-1-thio-β-D-galactopyranoside (IPTG) addition (sterilized separately through filtration), temperature of cultivation (30 and 37°C), cultivation time (48 and 96 h) and inoculum size on the responses: Dry cell weight (DCW), PHA cell content (%PHA) and PHA mass (mPHA) produced by the recombinant *E. coli* strain JM101, harboring the plasmid pBHR68. The factorial design consisted of 2^5 experiments, where 5 was the number of variables studied. Each one was tested at a low and a high value represented by the levels (+1) and (-1), respectively (Table 1). The statistical analyses of the factorial design results were performed according to Box *et al*. (1978) using the software Statistica 5.11®.

RESULTS AND DISCUSSION

Table 2 shows the design matrix of the 2^5 factorial design applied, in which the coded variables X_1, X_2, X_3, X_4 and X_5 correspond to inoculum size, IPTG, acrylic acid, cultivation time and temperature, respectively and its results on dry cell weight (DCW), PHA cell content (%PHA) and PHA mass obtained. The experiments were performed at constant concentrations of hydrolyzed corn starch (HCS), soybean oil (SO) and cheese whey (CW) since Fonseca (2003) had studied the effect of these variables, through an experimental design and concluded that the best results would be achieved in a mineral medium enriched with 5% HCS, 1.5% SO and 5% CW (37°C, cultivation time 48 h and 2% (v/v) inoculum). According to Table 2, these experimental conditions (exp. 17) led to 2.01 g L^{-1} DCW, 58.14% (%PHA) and 1.17 g L^{-1} PHA mass, which were in good agreement with the results previously predicted (1.9 g L^{-1} DCW, 50% (%PHA) and 0.95 g L^{-1} PHA mass).

Overall, %PHA ranged between 48 to 82% and the highest PHA mass achieved was 2.79 g L^{-1} in exp. 26, where most of the variables were at their high levels (+1), except for X_2 (IPTG) and X_3 (acrylic acid).

Although the highest DCW (4.53 g L^{-1}) was obtained in exp. 30, PHA mass achieved (2.23 g L^{-1}) was slightly lower than the value obtained in exp. 26. Similarly, %PHA attained its maximum value (82.31%) in experiment 10; however, only 2.39 g L^{-1} DCW and 1.97 g L^{-1} PHA mass were obtained.

The main effects of the variables X_1, X_2, X_3, X_4 and X_5 on DCW and %PHA as well as the two-factor interactions are shown in Table 3. Since the experiments were run in duplicate, the standard errors of the effects were calculated. Only the marked "a" effects were statistically significant within a 95% confidence interval. In addition, a positive effect in Table 3 means an increase in the response when a variable is changed from (-1) to (+1): for instance, when X_3 (acrylic acid) was added (from -1 to +1) to the medium DCW decreased from 2.55 g L^{-1} to 1.97 g L^{-1}. Likewise, %PHA was reduced from 61.80 to 56.44%. Among the variables which produced positive effects on DCW, X_4 was the most significative variable, followed by X_5. While X_5 influenced %PHA negatively, X_1 and X_4 had a positive effect on it, as expected.

The interactions between inoculum size vs. cultivation time (X_1 x X_3) and cultivation time-temperature (X_4 x X_5) led to the most significantly positive effects on DCW. In spite of a negative effect on DCW, the X_2 x X_5 interaction was the only one able to increase PHA production.

A multiple non linear regression analysis of experimental data presented in Table 2 was performed and generated mathematical models for the responses DCW (Eq. 1) and %PHA (Eq. 2) taking into consideration the main effects of the variables (linear terms) as well as the most significant second order interactions (interaction terms). The models obtained were:

$$DCW = 3.06-0.15X_1 + 0.09.X_2-0.12.X_3 + 0.03.X_4-0.08X_5 + 0.02X_1.X_3 + 0.0009X_2.X_3-0.001X_2.X_4 + 0.003X_3.X_5 \quad (1)$$

$$\%PHA = 123.85 - 27.24X_1 + 0.68X_2 - 0.51X_3 - 0.74X_4 - 2.21X_5 \quad (2)$$

Table 2: Coded variables and results from the 2^5 factorial design (*E. coli* JM101 (pBHR 68))

	Variables					Responses[a]		
Exp.	X_1	X_2	X_3	X_4	X_5	DCW[b] (g L^{-1})	P[3HB][b] (%)	P[3HB][c] (g L^{-1})
1	-1	-1	-1	-1	-1	2.00	60.02	1.20
2	+1	-1	-1	-1	-1	2.51	63.27	1.59
3	-1	+1	-1	-1	-1	3.07	59.37	1.82
4	+1	+1	-1	-1	-1	1.92	66.71	1.28
5	-1	-1	+1	-1	-1	0.33	61.20	0.20
6	+1	-1	+1	-1	-1	2.34	59.18	1.38
7	-1	+1	+1	-1	-1	2.11	55.86	1.18
8	+1	+1	+1	-1	-1	2.17	58.24	1.26
9	-1	-1	-1	+1	-1	3.13	69.58	2.18
10	+1	-1	-1	+1	-1	2.39	82.31	1.97
11	-1	+1	-1	+1	-1	3.17	66.29	2.10
12	+1	+1	-1	+1	-1	2.19	64.93	1.42
13	-1	-1	+1	+1	-1	2.67	68.06	1.82
14	+1	-1	+1	+1	-1	2.39	75.43	1.80
15	-1	+1	+1	+1	-1	1.68	57.97	0.97
16	+1	+1	+1	+1	-1	2.86	56.36	1.61
17	-1	-1	-1	-1	+1	2.01	58.14	1.17
18	+1	-1	-1	-1	+1	2.47	56.95	1.41
19	-1	+1	-1	-1	+1	2.62	53.14	1.39
20	+1	+1	-1	-1	+1	2.96	69.82	2.06
21	-1	-1	+1	-1	+1	2.23	58.82	1.31
22	+1	-1	+1	-1	+1	2.71	60.63	1.64
23	-1	+1	+1	-1	+1	0.25	48.85	0.12
24	+1	+1	+1	-1	+1	1.37	52.62	0.72
25	-1	-1	-1	+1	+1	3.56	64.82	2.31
26	+1	-1	-1	+1	+1	3.55	78.72	2.79
27	-1	+1	-1	+1	+1	3.58	64.16	2.30
28	+1	+1	-1	+1	+1	4.39	60.73	2.67
29	-1	-1	+1	+1	+1	2.99	65.36	1.95
30	+1	-1	+1	+1	+1	4.53	49.21	2.23
31	-1	+1	+1	+1	+1	3.20	62.51	2.00
32	+1	+1	+1	+1	+1	2.38	48.33	1.15

[a] only 3-hydroxybutyrate polymers (P[3HB]) accumulated in this study. [b] Average of duplicates; [c] Calculated from [b]

Figure 1 shows the contours of the fitted equations where the PHA cell content (%PHA) was represented by colored areas, dry cell weight (DCW) by dashed lines and PHA mass (mPHA) by continuous lines. Contours were plotted as a function of cultivation time (X_4) and temperature (X_5), at the same fixed levels. It can be seen that, at 37°C and 96 h, about 3.5 g L^{-1} DCW (dashed lines), 75% %PHA (colored areas) and 2.5 g L^{-1} mPHA (continuous lines) would be obtained.

Cultivation time presented the most significant effect on PHA accumulation. According to Table 2, 82.30% PHA was achieved after 96 h at 5% (v/v) inoculum (exp. 10), 13.61% greater than the value obtained in exp. 9. Thus, in this situation a directly proportional relationship between the variables cultivation time and temperature seems clear (data not shown). In the absence of acrylic acid, cultures grew in MR medium containing IPTG and with an inoculum size of 5% (v/v) achieved high values of DCW (4.08 g L^{-1}) and PHB mass (2.60 g L^{-1}), while any combination of inoculum size and IPTG concentrations at 1 mmol AA (high level) affected negatively all responses.

An analysis of variance (ANOVA) was performed for both responses (Table 4). According to the Fisher F-test, the models presented high correlation (R^2) coefficients and were able to explain 84.3% and 91.4% of the DCW and %P[3HB] variability, respectively. The calculated F-values were 4.7 and 9.1 times larger than the tabular value (at a significance level of 5%) which shows a very high significance of the DCW and %P[3HB] models, respectively.

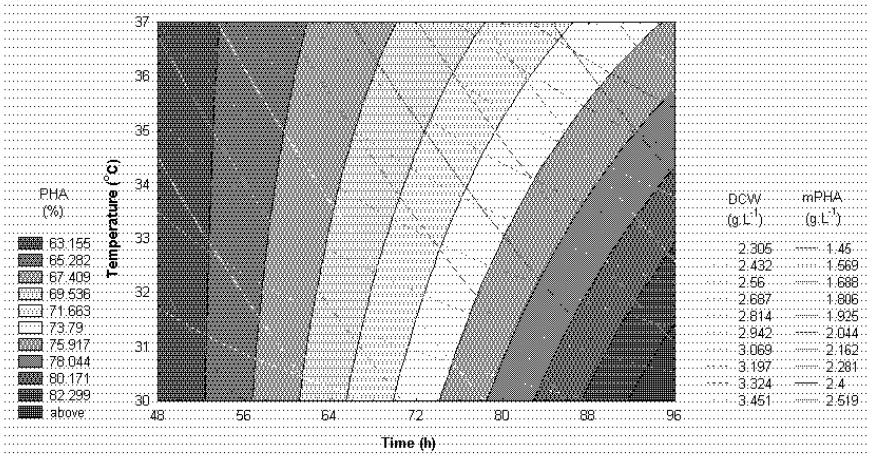

Fig. 1: Contours of the fitted response equations plotted as a function of cultivation time (AA) vs. temperature (inoculum size fixed at 5% and neither IPTG nor acrylic acid was added to the medium)

Table 3: Main effects and second order interaction effects of the variables on DCW and %PHA for *E. coli* (JM101) experiments (pBHR 68)

Responses	DCW (g L^{-1})	P[3HB] (%)
Average	2.55[a]	61.80[a]
(X_1)	0.28[a]	1.83
(X_2)	-0.11	-5.36[a]
(X_3)	-0.58[a]	-6.27[a]
(X_4)	0.97[a]	5.74[a]
(X_5)	0.49[a]	-4.49[a]
(X_1) x (X_2)	-0.21	-0.63
(X_1) x (X_3)	0.37[a]	-4.16
(X_1) x (X_4)	-0.19	-2.17
(X_1) x (X_5)	0.20	-1.68
(X_2) x (X_3)	-0.40[a]	-1.78
(X_2) x (X_4)	-0.10	-3.66
(X_2) x (X_5)	-0.29[a]	1.30
(X_3) x (X_4)	0.17	-2.26
(X_3) x (X_5)	-0.10	-1.24
(X_4) x (X_5)	0.47[a]	-1.39

[(a)] Significant effects within a 95% confidence interval (ANOVA)

Table 4: Modified ANOVA table for Eq. 1(DCW) and Eq. 2 (%P[3HB])

	Sum of squares		Mean square			F-value (calculated)	
Variation source	DCW	PHB (%)	DCW	PHB (%)	Degrees of freedom	DCW	PHB (%)
Regression	45.77	1795.5	1.83	71.82	25	8.32	16.14
Residual	8.54	169.4	0.22	4.45	38		
Total	54.31	1964. 9	-	-	63		
Correlation coefficient	0.843	0.914					

$F_{0.95,25,38} = 1.7$

According to Table 3, the increase in X_4 (cultivation time) affected positively DCW and PHA accumulation. In fact, cultivation time presented the most positive effects on both responses. On the other hand, both DCW and PHA accumulation were most negatively affected by AA addition. There was no significant change in DCW with the addition of IPTG to the MR medium.

The addition of IPTG presented a very particular effect. This inducer of the *lac* operon, utilized for the expression of PHA synthesis genes carried on the plasmid pBHR68, did not improve PHA accumulation as expected. In fact, its addition to the culture medium influenced negatively DCW and PHA accumulation (Table 3). Similarly, Kosinski *et al.* (1992) observed that IPTG might act as an *E. coli* metabolism inhibitor instead of acting as an inducer. However, the overall results show that the constructed plasmid pBHR68 may lead to significant good performances concerning PHA accumulation, even without the addition of such an expensive inducer. In weighing costs and benefits, the withdrawal of IPTG from the medium seems advantageous.

In the present study, the addition of AA aimed to inhibit β-oxidation of fatty acids from SO and as a consequence, obtain medium-chain-length polymer units, such as 3-hydroxyhexanoates, previously observed in cultures of other recombinant strains, harboring the PHA synthesis genes from *R. eutropha* carried on the same plasmid (pBHR68) (Antonio *et al*., 2000). Even though no amounts of either 3HHx or other units were detected in this study, it should be mentioned that these units had accumulated previously in experiments performed with different strains and under different conditions. Furthermore, the addition of AA had a significantly negative effect on DCW suggesting that the concentration employed might be inhibiting.

On increasing the cultivation temperature from 30 to 37°C, there was an increase in DCW but a decrease in PHA accumulation. It might be explained by the fact that the optimum growth temperature for most *E. coli* strains is 37°C, whereas the optimum temperature for PHA synthases is 30°C, which, in turn, is the optimum growth temperature for *R. eutropha* (the donor of PHA synthesis genes).

CONCLUSIONS

Regarding PHA cell content, 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, such as hydrolyzed corn starch, soybean oil and cheese whey as a supplement, making these results look very promising.

Through our study, we brought some important contributions to the choice of five variables (acrylic acid and IPTG concentrations, cultivation temperature and time, inoculum size), marginally studied so far on the polyhydroxyalkanoates production.

ACKNOWLEDGMENTS

We thank CNPq-Brazil for financial support, CAPES-Brazil for research fellowship and Prof. Dr. Bernd Rehm for kindly providing the plasmid pBHR68.

REFERENCES

Ahn, W.S., S.J. Park and S.Y. Lee, 2001. Production of poly(3-hydroxybutyrate) from whey by cell recycle fed-batch culture of recombinant *Escherichia coli*. Biotechnol. Lett., 23: 235-240.

Anderson, A.J. and E.A. Dawes, 1990. Occurrence, metabolism, metabolic role and industrial uses of bacterial polyhydroxyalkanoates. Microbiol. Rev., 54: 450-472.

Antonio, R.V., A. Steinbüchel and B.H.A. Rehm, 2000. Analysis of *in vivo* substrate specificity of the PHA synthase from *Ralstonia eutropha*: Formation of novel copolyesters in recombinant *Escherichia coli*. FEMS Microbiol. Lett., 182: 111-117.

Ashby, R.D. and T.A. Foglia, 1998. Poly(hydroxyalkanoate) biosynthesis from triglyceride substrates. Applied Microbiol. Biotechnol., 49: 431-437.

Berg, J.M., J.L. Tymoczko and L. Stryer, 2002. Biochemistry, 5th Edn, New York, WH Freeman and Company, pp: 974.

Box, G.E.P., W.G. Hunter and J.S. Hunter, 1978. Statistics for Experimenters. An Introduction to Design, Data Analysis and Model Building, New York, John Wiley and Sons Inc., pp: 678.

Choi, J. and S.Y. Lee, 1997. Process analysis and economic evaluation for poly (3-hydroxybutyrate) production by fermentation. Bioprocess Eng., 17: 335-342.

Choi, J. and S.Y. Lee, 1999. Factors affecting the economics of polyhydroxyalkanoates production by bacterial fermentation. Applied Microbiol. Biotechnol., 51: 13-21.

Choi, J., S.Y. Lee and K. Han, 1998. Cloning of the *Alcaligenes latus* polyhydroxyalkanoate biosynthesis genes and use of these genes for the enhanced production of poly (3-hydroxybutyrate) in *Escherichia coli*. Applied Environ. Microbiol., 64: 4897-4903.

Cromwick, A.M., T. Foglia and R.W. Lenz, 1996. The microbial production of poly (hydroxyalkanoates) from tallow. Applied Microbiol. Biotechnol., 46: 464-469.

Eggink, G., P. de Waard and G.N.M. Huijberts, 1995. Formation of novel poly (hydroxyalkanoates) from long-chain fatty acids. Can. J. Microbiol., 43: 14-21.

Fonseca, G.G. 2003. Polyhydroxyalkanoates production by recombinant *Escherichia coli* (in Portuguese). M.Sc. Thesis, UFSC, Florianópolis, SC, Brazil.

Fukui, T. and Y. Doi, 1998. Efficient production of polyhydroxyalkanoates from plant oils by *Alcaligenes eutrophus* and its recombinant strain. Applied Microbiol. Biotechnol., 49: 333-336.

Gomez, J.G.C., M.F.A. Rodrigues, R.C.P. Alli, B.B. Torres, C.L.B. Netto, M.S. Oliveira and L.F. Silva, 1996. Evaluation of soil gram-negative bacteria yielding polyhydroxyalkanoic acids from carbohydrates and propionic acid. Applied Microbiol. Biotechnol., 45: 785-791.

Hanahan, D., 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol., 166: 557-580.

Kato, M., T. Fukui and Y. Doi, 1996. Biosynthesis of polyester blends by *Pseudomonas* sp. 61-3 from alkanoic acids. Bull. Chem. Soc. Jap., 69: 515-520.

Kim, B.S. and H.N. Chang, 1995. Control of glucose feeding using exit gas data and its application to the production of P[3HB] from tapioca hydrolysates by *Alcaligenes latus*. Biotechnology, 9: 311-314.

Kim, B.S., B.K. O'neill and S.Y. Lee, 2000. Increasing poly(3-hydroxybutyrate) accumulation in recombinant *Escherichia coli* from whey by agitation speed control. J. Microbiol. Biotechnol., 10: 628-631.

Kosinski, M.J., U. Rinas and J.E. Bailey, 1992. Isopropyl-β-D-thiogalactopyranoside influences the metabolism of *Escherichia coli*. Applied Microbiol. Biotechnol., 36: 782-784.

Lee, S.Y., 1996a. Bacterial polyhydroxyalkanoates. Biotechnol. Bioeng., 49: 1-14.

Lee, S.Y., 1996b. Plastic bacteria?: Progress and prospects for polyhydroxyalkanoate production in bacteria. Trends Biotechnol., 14: 431-438.

Lee, S.Y. and J.L. Choi, 2001. Production of microbial polyester by fermentation of recombinant microorganisms. Adv. Biochem. Eng. Biotechnol., 71: 183-207.

Lee, S.Y. and J.L. Choi, 1999. Production and degradation of polyhydroxyalkanoate in waste environment. Waste Manage., 19: 133-139.

Qi, Q., A. Steinbüchel and B.H.A. Rehm, 1998. Metabolic routing towards polyhydroxyalkanoic acid synthesis in recombinant *Escherichia coli* (*fadR*): Inhibition of fatty acid L-oxidation by acrylic acid. FEMS Microbiol. Lett., 167: 89-94.

Rehm, B.H.A., R.V. Antonio, P. Spiekermann, A.A. Amara and A. Steinbüchel, 2002. Molecular characterization of the poly(3-hydroxybutyrate) (PHB) synthase from *Ralstonia eutropha*: *in vitro* evolution, site-specific mutagenesis and development of a PHB synthase protein model. Biochim. Biophys. Acta, 1594: 178-190.

Sambrook, J. and D.W. Russell, 2001. Molecular Cloning: A Laboratory Manual, 3rd Edn., New York., Cold Spring Harbor Laboratory Press.

Shimamura, E., K. Kasuya, G. Kobayashi, T. Shiotani, Y. Shima and Y. Doi, 1994. Physical properties and biodegradability of microbial poly (3-hydroxybutyrate-*co*-3-hydroxyhexanoate). Macromolecule, 27: 878-880.

Spiekermann, P., B.H.A. Rehm, R. Kalscheuer, D. Baumeister and A. Steinbüchel, 1999. A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalcanoic acids and other lipid storage compounds. Arch. Microbiol., 171: 73-80.

Taguchi, K., Y. Aoyagi, H. Matsusaki, T. Fukui and Y. Doi, 1999. Co-expression of 3-ketoacyl-ACP reductase and polyhydroxyalkanoate synthase genes induces PHA production in *Escherichia coli* HB101 strain. FEMS Microbiol. Lett., 176: 183-190.

Timm, A., D. Byrom and A. Steinbüchel, 1990. Formation of blends of various poly (3-hydroxyalcanoic acids) by a recombinant strain of *Pseudomonas olevorans*. Applied Microbiol. Biotechnol., 33: 296-301.

Yang, S.T., H. Zhu, Y. Li and G. Hong 1994. Continuous propionate production from whey permeate using a novel fibrous bed bioreactor. Biotechnol. Bioeng., 43: 1124-1130.

Wong, H.H. and S.Y. Lee, 1998. Poly-(3-hydroxybutyrate) production from whey by high-density cultivation of recombinant *Escherichia coli*. Applied Microbiol. Biotechnol., 50: 30-33.

Zheng, Z., M. Zhang, G. Zhang and G. Chen, 2004. Production of 3-hydroxydecanoic acid by recombinant *Escherichia coli* HB101 harboring *phaG* gene. Anton. Leeuw., 85: 93-101.