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Use of Vegetable Oils as Substrates for Medium-chain-length Polyhydroxyalkanoates Production by Recombinant *Escherichia coli*

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Abstract: Polyhydroxyalkanoates (PHAs) are thermoplastic, biodegradable polyesters synthesized by some bacteria from renewable carbon sources. However, their application is limited by high production costs. To reduce them, recombinant strains that use diverse carbon sources have been developed. In this work, PHA production by recombinant *Escherichia coli* (JM101), harboring the structural gene for medium-chain-length polyhydroxyalkanoate synthesis from *Pseudomonas aeruginosa*, was investigated using several vegetable oils (cotton, rice, canola, dendê (palm), sunflower, corn, olive and soybean) and cheese whey as a supplement. The highest PHA yield obtained was 11.64% PHA (20.3 mol% hydroxyhexanoate, 9.9 mol% hydroxyoctanoate, 24.0 mol% hydroxyodecanoate and 45.8 mol% hydroxydodecanoate) in a mineral medium containing 1% (v v^{-1}) dendê (palm) oil and 5% (v v^{-1}) cheese whey.

Key words: Vegetable oils, medium-chain-length polyhydroxyalkanoates, *Pseudomonas aeruginosa*, recombinant *Escherichia coli*

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are thermoplastic polymers that, due to their mechanical properties and biodegradability, have been considered promising substitutes for petrochemical-based polymers for short term applications (Weiner, 1997; Yu, 2001). Their properties can be improved by the modification of their chemical composition (Weiner, 1997; Sim *et al*., 1997). The composition of PHAs depends mainly on the PHA cell content, the carbon source employed, the cultivation conditions and the metabolic pathways involved (Madison and Huisman, 1999; Rehm and Steinbüchel, 1999).

Among the substrates utilized for PHA production are carbon dioxide, fossil resources (methane, mineral oils, lignite), renewable resources (hydrolyzed corn starch, cheese whey, glycerol) and some chemical substances (propionic acid, 4-hydroxybutiric acid) (Steinbüchel and Füchtenbusch, 1998). According to Rehm and Steinbüchel (1999) the PHA production should be centered on the use of low cost carbon sources, preferentially renewable carbon sources (carbohydrates and lipids) from agriculture.

Even though fats and oils are renewable, inexpensive agricultural products, only a few reports have described their use for PHA production (Cromwick *et al*., 1996; Ashby and Foglia, 1998; Fukui and Doi, 1998; Marangoni, 2000); however, the utilization of these substrates by recombinant *E. coli* has never been reported.

In the present study, PHA production by a recombinant *E. coli* strain (JM101), harboring the structural genes for medium-chain-length polyhydroxyalkanoate (PHA_{MCL}) synthesis from *P. aeruginosa*, was investigated using different vegetable oils as substrate and cheese whey as a supplement. The objective of this study was to evaluate the suitability of these medium components for PHA_{MCL} production.

MATERIALS AND METHODS

Strain and plasmid: *E. coli* JM101 (Stratagene, La Jolla, USA) harbouring the plasmid pBHR71 (Langenbach *et al*., 1997) was employed for these studies. Plasmid pBHR71 contains the *phaC1* gene coding for PHA_{MCL} synthase from *P. aeruginosa*.

Competent cell preparation and *E. coli* transformation: 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 *et al*., 1989).

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Culture media: Recombinant *E. coli* JM101 was cultivated in Luria Bertani broth (LB) for inoculum preparation (Sambrook *et al*., 1989) and defined mineral medium (MR) for PHA production (Lee and Choi, 2001). Commercial grade vegetable oils (cotton, rice, canola, dendê (palm), sunflower, corn, olive and soybean) as carbon sources and cheese whey as a supplement were added to MR medium at 1% and 5% (v v^{-1}), respectively. Cheese whey (94-95% water, 4.2-5% lactose, 0.8-1% proteins, 0.1% lipids and 0.7-0.8% mineral salts) was homogenized and added directly to the culture medium.

Culture conditions: Inocula were prepared in 125 mL Erlenmeyer flasks containing 25 mL of LB medium on a rotary shaker at 37°C and 150 rpm. Then, experiments were carried out in 500 mL baffled Erlenmeyer flasks containing 100 mL of MR medium with an inoculum size of 2% (v v^{-1}) (absorbance 1.2 at 600 nm) for 48 h under the same conditions.

Cell mass determination: Dry cell weight (DCW) was determined through centrifugation of a known volume of the culture broth (10,000 rpm, 4°C, 15 min), sediment drying (50°C, 24 h) and posterior weighing.

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

RESULTS AND DISCUSSION

Table 1 shows the results obtained for dry cell weight (DCW), PHA cell content and composition in cultures using different vegetable oils. The monomer units detected were 3HB, 3HHx, 3HO, 3HD and 3HDD, even though *P. aeruginosa* can synthesize monomer units up to 16 carbons long (Fukui and Doi, 1998).

Poly-3-hydroxybutyrate (PHB), a short-chain-length polymer, was detected in small amounts (1.5-2.5%), although the genes for PHA synthesis from *P. aeruginosa* are not expected to produce 3HBs. Since they might be residues resulting from metabolic pathways unrelated to polymer synthesis, they were not considered for this analysis.

Overall, cultures grew in vegetable oils gave comparable DCW values which ranged between 0.33 (canola) and 0.61 (olive) g L^{-1}. Among the cultures that achieved % PHA higher than 10% were dendê (11.64%), cotton (11.46%) and soybean (10.31%) cultures. Concerning PHA composition, the use of dendê (palm), rice and sunflower oils allowed the synthesis of all monomer units analyzed. So, these results indicate that dendê (palm) oil is a good carbon source with biotechnological potential. Studies on PHA composition from plant oils are scarce. Ashby and Foglia (1998) obtained average PHA composition of 0.5%HB, 8%HHx, 34%HO, 32.5%HD, 15.5%HDD and 9.5%C14.

In *P. resinovorans* cultures performed in a mineral medium, PHA production was enriched by the use of diverse triglyceride substrate (1% v v^{-1}) (Brandl *et al*., 1988). Similarly, Marangoni (2000) employed different vegetable oils for [P(3HB-*co*-3HV)] production in cultures of *Ralstonia eutropha*. Experiments were carried out in a mineral medium containing 1% oil (cotton, canola, dendê (palm), sunflower, corn, olive or soybean) and 30 g L^{-1} inverted sugar. The best results were obtained with sunflower oil (0.84 g L^{-1} DCW and 0.31 g polymer per gram of inverted sugar). The average values were: 0.5 g L^{-1} DCW, 0.15 g of polymer per gram of invert sugar and 30%[P(3HB-*co*-3HV)] (92% mol HB, 8% mol HV).

Cromwick *et al*. (1996) and Fukui and Doi (1998) suggested that *R. eutropha* secretes lipases in the presence of oil, which hydrolyze triglycerides to fatty acids. Then, the latter are oxidized by β-oxidation. to acetyl-CoA units. Taguchi *et al*. (1999) reported the P(3HB-*co*-3HHx) production by a recombinant *E. coli* strain (HB101) containing the 3-cetoacyl-ACP reductase gene from *E. coli* ($fabG_{Ec}$) and the poly-3-hydroxyalkanoate synthase gene from *A. caviae* ($phaC_{Ac}$), and suggested that the overexpression of the genes leads to (R)-3-hydroxyacil-CoA accumulation, a PHA precursor, via fatty acid degradation. Thus, in the present research, different vegetable oils were employed with the aim of generating acetyl-CoA units by fatty acid β-oxidation and, consequently, (R)-3-hydroxyacil-CoA for PHA synthesis in *E. coli* cultures.

Table 1: Dry cell weight, PHA cell content and composition obtained in *E. coli* JM101 (pBHR 71) cultures using several vegetable oils

Vegetable oils	DCW [1] (g L^{-1})	PHA[1] (%)	3HHx (%mol)	3HO (%mol)	3HD (%mol)	3HDD (%mol)
Cotton	0.53	11.46	nd	12.91	61.35	25.74
Rice	0.43	3.19	25.08	32.29	23.82	18.81
Canola	0.33	6.01	nd	17.14	65.22	17.64
Dendê (palm)	0.58	11.64	20.27	9.89	24.05	45.79
Sunflower	0.57	6.94	13.26	14.41	55.33	17.00
Corn	0.50	1.70	nd	42.35	57.65	nd
Olive	0.61	6.31	nd	16.00	57.69	26.31
Soybean	0.46	10.31	nd	10.48	26.28	63.24

[1] Average of duplicates; DCW: dry cell weight; PHA: polyhydroxyalkanoate; 3HHx: 3-hydroxyhexanoate; 3HO: 3-hydroxyoctanoate; 3HD: 3-hydroxydecanoate; 3HDD: 3-hydroxydodecanoate; nd: not detected

Taking into consideration that saturated fatty acids are more likely to be degraded by β-oxidation, the results obtained in this work seem quite coherent. The utilization of dendê (palm) and cotton oils, containing 51 and 27% saturated fatty acids, respectively, resulted in the highest values of % PHA (11.64 and 11.46%, respectively). Soybean oil, with the third highest percentage of saturated fatty acids among the others, presented the third best result concerning PHA cell content.

Despite having almost two times the concentration of fatty acids compared to cotton oil, dendê (palm) oil did not allow higher medium-chain-length PHA accumulation, probably due to its high saturation degree. At 37°C (cultivation temperature), low oil availability to cells might have reduced rates of transport through membranes and resulted in lower PHA accumulation.

Thus, in order to optimize β-oxidation and consequently, PHA_{MCL} synthesis, vegetable oils that present not only higher saturation degree, but also shorter fatty acids should be employed.

CONCLUSIONS

The data presented in this work demonstrate that saturated fatty acids are more likely to be oxidized by β-oxidation and generate PHAs. Besides, the level of saturated fatty acids cannot be increased indefinitely in order to obtain higher PHA accumulation. Dendê (palm) oil (which contains the highest percentage of saturated fatty acids among the other vegetable oils) allowed the highest PHA cell content (11.64%).

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