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Production and Characterization of Poly(3-Hydroxybutyrate) from Oleic Acid by *Ralstonia eutropha*

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Summary

The aim of this research is to investigate the influence of oleic acid concentration on the cell growth and the physical properties of the polymer formed by cultures of *Ralstonia eutropha* in mineral medium. Cells were cultivated in Erlenmeyer flasks with 300 mL of mineral medium, containing glucose and fructose as a carbon source (30 g/L) and ammonium sulphate (5.0 g/L) as a nitrogen source. Oleic acid was added as nutritional supplement in different concentrations (0, 0.3, 0.9, 1.5 and 3.0 g/L) and the cells were incubated at 30 °C and 150 rpm. The films prepared by casting were evaluated by X-ray diffraction, thermogravimetry and differential scanning calorimetry. These results indicate that the increase of oleic acid concentrations leads to a higher specific growth rate and cell productivity. The characterization of the films revealed that the increase of the concentration of oleic acid from 0 to 1.5 g/L has no influence on thermal behaviour and crystallinity degree. However, the thermal stability, melting temperature, glass transition temperatures and crystallinity degree decreased when 3.0 g/L of oleic acid were used.

Key words: biopolymers, *Ralstonia eutropha*, oleic acid, characterization

Introduction

Plastic materials are widely used in everyday life primarily because they are strong, light, inexpensive, and chemically inert. However, the persistence of plastics in the environment is increasingly considered as a source of ecological problem (*1*). After use, it is thrown away into the garbage and sanitary embankments already saturated with plastic residues, causing an accumulation of garbage, hindering the circulation of liquids and gases and retarding the stabilization of the organic matter (*2*). The environmental impact caused by the disposal of plastics and the progress of medicine have motivated the development of biodegradable and biocompatible materials (*3*).

An overwhelming number of different polyhydroxyalkanoates (PHAs), comprising approx. 150 different hydroxyalkanoic acids as constituents, has been isolated from numerous microorganisms during the last 25 years (*4*). Accumulation of PHAs in the bacterial cell usually occurs when at least one of the nutritional elements is limited during the growth phase and the carbon source is provided in excess (*5*). The polyesters accumulated in the cytoplasm are deposited as insoluble inclusions referred to as PHA granules (*4*). They can be completely degraded to water and carbon dioxide under aerobic conditions and to methane under anaerobic conditions by microorganisms in the soil, sea, lake water and sewage (*6*).

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All PHAs share some properties which recommend them for some applications and make them interesting to industry (*7,8*). They are thermoplastic and/or elastomeric, enantiomeric pure chemicals consisting, in general, only of R-stereoisomer, non-toxic, biocompatible, insoluble in water and exhibit a rather high degree of polymerization (*9*). Several PHAs, in particular the homopolyester poly(3-hydroxybutyrate), P(3HB), and the copolyester poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), P(3HB-*co*-3HV), have been used in the production of various materials, and they are considered for several applications in the packaging industry, medicine, pharmacy, agriculture and food industry, or as raw materials for the synthesis of enantiomerically pure chemicals and production of paints (*10*). P(3HB) films may also be of use in food packagings with low oxygen diffusion (*11*).

The use of PHAs as substitutes for conventional plastics in a wide range of applications has been hampered due to the high production cost of PHAs compared to petrochemically based polymers (*12*). From an economical point of view, to commercialize the production of P(3HB), it is very important to develop a new process with low cost and low pollution to recover P(3HB) from microorganism cells (*13*). It has been estimated that about 40 % of the total production cost of P(3HB) can be attributed to the raw material used. Thus, the use of a cheaper carbon source is required in order to reduce the high production cost of P(3HB) (*14*). There have been several reports on the production of P(3HB) from cheap carbon sources by wild type P(3HB) producers (*5*). However, especially when fatty acids or vegetable oils are used, specific growth rate, P(3HB) concentration and P(3HB) content obtained were considerably lower than those obtained using purified carbon substrates. Much effort has been devoted to reduce the cost of PHA by the isolation of genetically engineered strains and development of more efficient fermentation and recovery processes (*15*).

Previous study (*16*) showed that supplementation of cultures producing P(3HB-*co*-3HV) with oleic acid increased the polymer productivity, however, the authors observed that the oleic acid acts as an inducer for 3HB fraction in the polymer. The use of fatty acids and soy oil as nutritional supplements for the production of P(3HB) using hydrolyzed starch as carbon source resulted in 50.5 and 56.7 % of P(3HB) content and 0.37 and 0.49 g/(L·h) of polymer, respectively (*17*).

The aim of this research is to investigate the influence of different concentrations of oleic acid as a nutritional supplement in cultures of *Ralstonia eutropha* in mineral medium and to evaluate the characteristics of the films obtained by casting. Films were evaluated by X-ray diffraction, differential scanning calorimetry and thermogravimetric analysis.

Materials and Methods

Microorganism, culture medium and conditions

An intracellular producer of P(3HB) and glucose--utilizing mutant, *Ralstonia eutropha* DSM 545, was used throughout the experiments. The strain was grown in nutrient broth (NB) medium, containing 5.0 g/L of meat peptone and 3.0 g/L of meat extract at 30 °C for 24 h. The subsequent culture medium had been described previously (*18*), with the following composition (in g/L): nitroacetic acid 0.19, ammonium iron(III) citrate 0.06, $MgSO_4 \cdot H_2O$ 0.5, $CaCl_2 \cdot 2H_2O$ 0.01, $(NH_4)_2SO_4$ 5.0 and 1 mL of trace elements solution. After autoclaving the culture medium, a sterile solution of phosphate was added aseptically to the culture medium to obtain a final concentration of 8.95 g/L of $Na_2HPO_4 \cdot 12H_2O$ and 1.5 g/L of KH_2PO_4. A sugar solution of glucose and fructose (30 g/L) was utilized as carbon source. The inoculum ratio was 10 %, which was equivalent to 2.0 g/L of cells. The cells were cultivated in shake flasks of 1 L, at 30 °C and 150 rpm for 24 h.

Biomass

Cell density was determined by turbidimetry (600 nm), using an LKB Biochrom spectrophotometer (Biochrom Ltd, UK). During the exponential phase (until 6 h of culture), it was possible to determine the cell concentration (g/L) from the cell growth by using a linear equation which correlates absorbance and concentration.

Inverted sugar

Residual sugar concentration was measured using the dinitrosalicylic acid (DNS) method, which determines the concentration of reduced sugar.

Recovery of the polymer

For the P(3HB) recovery, a new method described previously (*19*) was used. At the end of the cultivation the culture broth with the cells was frozen at –20 °C for subsequent extraction stages. The culture broth was defrosted, centrifuged at 8000 rpm and the cells were washed twice with distilled water. A mass of 0.7 g of beads, 0.75 mm in diameter, was added to 4.0 mL of cell suspension and 1 mL of chloroform. Then the cells were ruptured in a bead mill (Retsch, USA) for 20 min at 100 rpm. The cells were centrifuged again at the same rotation speed and then the intermediated phase compound, containing P(3HB) dissolved in chloroform, was separated.

Preparation and characterization of the films

The films synthesized with different concentrations of oleic acid (0, 0.3, 0.9, 1.5 and 3.0 g/L) were obtained by casting. The polymer was solubilized in chloroform by magnetic mixing for 1 h to be completely homogenized. This solution was mixed with *n*-hexane and then filtered. The filtrate was prepared at the concentration of 1 % (*m/V*), dissolved with chloroform, poured on Petri dishes and placed in an evaporator system for 2 days, with saturated atmosphere of chloroform. The films were vacuum-dried for 24 h at 40 °C and stored in a desiccator. All the samples were analyzed by X-ray diffraction, differential scanning calorimetry and thermogravimetric analysis.

X-ray diffraction

To investigate the crystallinity of polymeric films, X-ray diffraction measurements were carried out on a Shimatzu diffractometer X-ray XRD 6000 model using CuK_α radiation. The scattering angle (2 θ) covered was from 10 to 70°.

Differential scanning calorimetry (DSC)

Calorimetric measurements were performed with a DSC Thermal Analyzer 2920 (TA Instruments, USA) under argon atmosphere. Samples of 7.2–7.8 mg were sealed in aluminum pans, heated from 25 to 190 °C at a rate of 10 °C/min (first scan) and kept at this temperature for 2 min. Subsequently, the samples were cooled to –100 °C at a rate of 20 °C/min and kept at this temperature for other 2 min. The crystallized samples were heated to 190 °C at a rate of 10 °C/min (second scan). From the data of melting enthalpy (ΔH_m), crystallization entalphy (ΔH_c) and calculated melting enthalpy considering the 100 % polymer crystalline, ΔH_m^0 = 142 J/g for the P(3HB) (*20*), it was possible to obtain the crystallinity degree (χ_c) of P(3HB) following the Eq. 1:

$$\chi_c = \frac{\Delta H_m - \Delta H_c}{\Delta H_m^0} \times 100/\% \qquad /1/$$

Thermogravimetric analysis (TGA)

The TGA analyses were carried out with the Proteus® Software for Thermal Analysis, NETZSCH–Gerätebau GmbH (Selb, Germany). Samples of 25 mg were heated from room temperature to 600 °C at a heating rate of 10 °C/min under argon atmosphere.

Results and Discussion

The effect of oleic acid supplementation in the growth phase

Considering the low solubility of oleic acid at 30 °C, which has no influence on the initial absorbance, it can be observed in Fig. 1 that an increase in oleic acid concentration leads to an increase of the specific growth rate, which is confirmed by the crescent slopes obtained at the beginning of the process for the different oleic acid concentrations tested. Akiyama *et al.* (*21*) evaluated the synthesis of poly(3-hydroxyalkanoates) by *Alcaligenes* species grown on various carbon sources such as *n*-alkanoic acids (3.0 g/L) of carbon numbers ranging from C2 to C22, plant oils and animal fats as unique carbon source. *n*-Alkanoates with chain lengths ranging from C11 to C19 yielded higher dry cell matter, over 2 g/L.

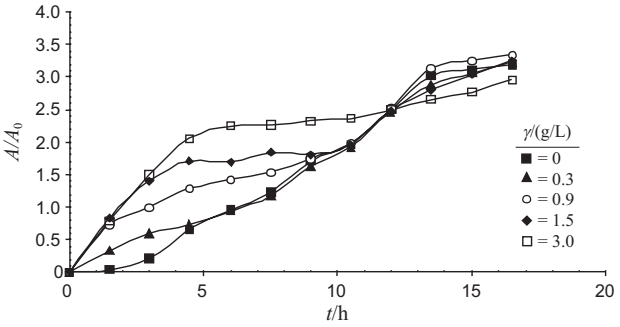

Fig 1. Cell growth of *Ralstonia eutropha* (A/A_0) with time for different concentrations of oleic acid used as supplement at 30 °C

The results obtained in this work allowed reaching a biomass concentration of 6.96 g/L for 6 h of cultivation, with glucose and fructose being used as substrate (30 g/L) and oleic acid (3.0 g/L) as nutritional supplement. From Fig. 1, we can also notice two exponential phases. The value of the first exponential phase increased as the oleic acid concentration increased (Table 1). Considering the first exponential phase and the biomass produced in the medium containing 3.0 g/L of oleic acid in 6 hours of cultivation, the cell yield ($\eta_{X/S}$) was equivalent to 1.40 g/g, if just glucose and fructose were considered as substrate. This result shows clearly that the microorganism used another substrate. According to Roels (*22*), it is possible to express the cell yield in c_{cell}/c_{subs} units (c_{cell}/c_{subs}=mol of substance when only one carbon is considered), so that the value obtained considering glucose and fructose used was 1.6 c_{cell}/c_{subs}. Supposing that the oleic acid content was also consumed, $\eta_{X/S}$ obtained was 0.77 c_{cell}/c_{subs}, which is near the theoretical value (0.6 c_{cell}/c_{subs}) suggested by Roels.

Table 1. Biomass concentration (γ) and cell productivity (P) (for 6 hours of cultivation), specific growth rate (μ), and cell yield ($\eta_{X/Sglu}$) (g/g), $\eta_{X/Sglu}$ (*c*/*c*) and $\eta_{X/(Sglu+Soleic)}$ (*c*/*c*) for crescent concentrations of oleic acid at 30 °C

γ(oleic acid)	γ(biomass)	P	μ	$\eta_{X/Sglu}$	$\eta_{X/Sglu}$	$\eta_{X/(Sglu+Soleic\ acid)}$
g/L	g/L	g/(L·h)	1/h	g/g	*c*/*c*	*c*/*c*
0	1.02	0.17	0.25	0.25	0.30	0.30
0.3	1.02	0.17	0.15* 0.28**	0.51	0.60	0.47
0.9	2.10	0.35	0.16* 0.31**	0.70	0.83	0.53
1.5	2.64	0.44	0.35* 0.22**	0.75	1.03	0.50
3.0	6.96	1.16	0.45* 0.09**	1.40	1.60	0.77

*first growth phase; **second growth phase

The results suggest that the oleic acid content is utilized in the first exponential phase. Considering that the fatty acid oxidation is extremely exergonic, *i.e.* the oxidation of fatty acid generates metabolic energy, an increase in oleic acid concentration stimulates the cell growth.

The effect of oleic acid supplementation on the physical properties of the polymer

In order to characterize the polymer synthesized with different oleic acid concentrations, X-ray diffraction, differential scanning calorimetry and thermogravimetric analysis were conducted. The X-ray diffractogram for the polymer synthesized by *Ralstonia eutropha* without oleic acid (Fig. 2) showed diffraction peaks in 2 θ equivalent to 13.5, 16, 20, 21.5, 22.5, 27 and 31, with crystallinity degree equal to 66.5 %. The profile of the diffractogram is similar to those presented by Galego *et al.* (*23*) and Yoshie and Inoue (*24*). As the acid concentration increased, the crystallinity degree decreased up to 52.5 % with 3.0 g/L of oleic acid (results not shown).

The glass transition temperature (t_g) of polyesters was identified from differential scanning calorimetry analysis. Fig. 3 shows differential scanning calorimetry curves according to the different conditions evaluated. It can be seen that the values of t_g varied between –5 and 3 °C (characteristic of P(3HB) (*25*)), except for the film obtained with 3.0 g/L of oleic acid, where t_g value was –10 °C, suggesting a more flexible polymer, although this value is lower for P(3HB) according to the literature (–5 to 5 °C) (*26*). It can also be observed that the melting temperature (t_m) did not vary with the concentration of oleic acid from 0 to 1.5 g/L. Nevertheless, the film obtained with the concentration of oleic acid of 3.0 g/L showed an 11 °C lower t_m if compared with the control sample and a shoulder at t_m of 149 °C, which is quite lower, indicating that smaller crystals are melted.

These results are in accordance with values of t_m obtained by Akiyama *et al.* (*21*). They observed a decrease in t_m from 179 to 116 °C when oleic acid was used as the only carbon source at the same concentration in the *Alcaligenes* sp. AK201 culture. This low t_m value is also

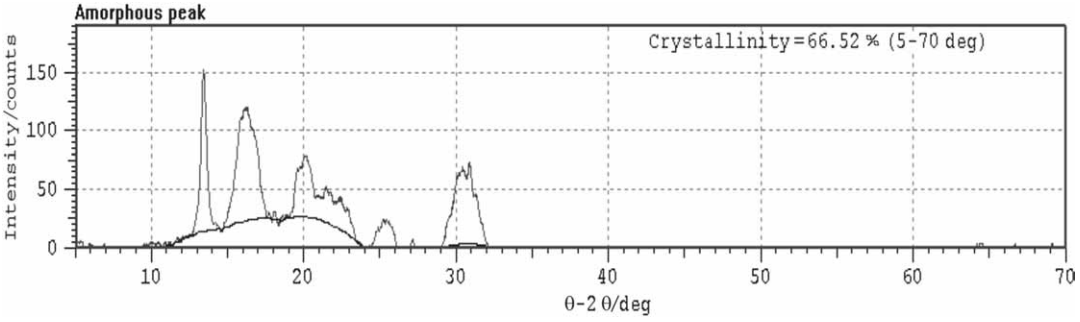

Fig. 2. X-ray diffraction of polymer synthesized by *Ralstonia eutropha* without oleic acid supplementation

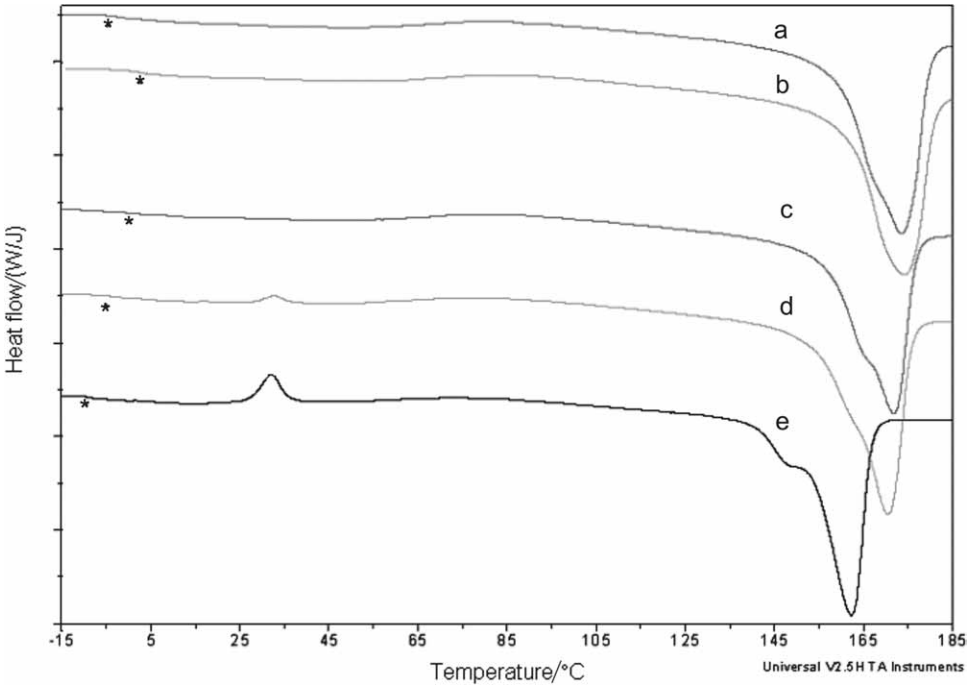

Fig. 3. DSC curves of P(3HB) films (second heating), in relation to oleic acid concentrations (in g/L): a) 0, b) 0.3, c) 0.9, d) 1.5, e) 3.0, showing the glass transition temperature t_g (*)

Table 2. Data of glass transition temperature (t_g), crystallyzation temperature (t_c), crystallization enthalpy (ΔH_c), melting temperature (t_m), melting enthalpy (ΔH_m) and crystallinity degree (χ_c) determined by DSC curves (second heating) of the P(3HB) films synthesized by *Ralstonia eutropha* with crescent concentrations of oleic acid

γ(oleic acid)/(g/L)	t_g/°C	t_c/°C	ΔH_c/(J/g)	t_m/°C	ΔH_m/(J/g)	χ_c/%
0	–4	—	—	173	100	70
0.3	2	—	—	174	94	66
0.9	0	—	—	172	89	62
1.5	–5	33	1	171	91	63
3.0	–10	32	5	149 162	80	53

found in the literature for the P(3HB) polymer (*9,24*). The synthesis of a polymer with low t_m is interesting because the material processability is improved.

From Table 2 it can be seen that the melting enthalpy (ΔH_m) and consequently the crystallinity degree decrease with the increase of oleic acid concentration. Compared to the control sample, a decrease of approx. 25 % was observed when 3.0 g/L of oleic acid were used. In the samples from 0 to 1.5 g/L of oleic acid, about 10 % variation of the crystallinity degree was observed, showing a slight decrease of crystallinity in this range.

Fig. 4 shows the thermogravimetric curves presenting only one stage of mass loss. The same onset temperature (t_{onset}) was observed for samples containing from 0 to 1.5 g/L of oleic acid. However, a decrease of 10 °C in the thermal stability was verified for the film with 3.0 g/L of oleic acid, as can be seen in Table 3.

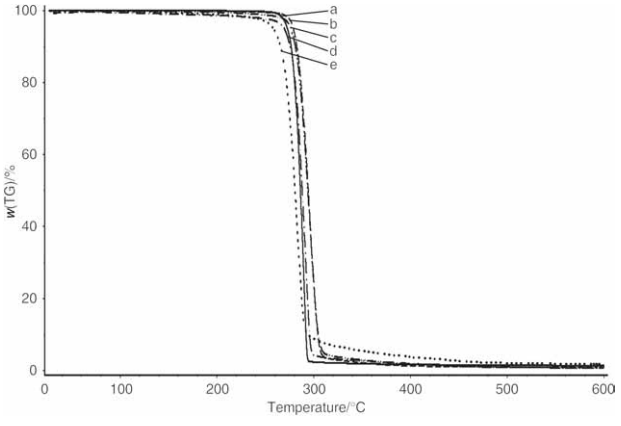

Fig 4. TGA curves of P(3HB) synthesized by *Ralstonia eutropha* at different concentrations of oleic acid (in g/L): a) 0, b) 0.3, c) 0.9, d) 1.5, e) 3.0

Table 3. Data of onset temperature (t_{onset}) and peak temperature (t_{peak}) obtained by TGA analysis for the polymer synthesized by *Ralstonia eutropha* at different concentrations of oleic acid

γ(oleic acid)/(g/L)	t_{onset} /°C	t_{peak}/°C
0	280	290
0.3	284	298
0.9	283	296
1.5	280	292
3.0	270	284

Conclusions

Increasing oleic acid concentration used as nutritional supplement in a mineral medium containing glucose and fructose as a carbon source in the culture of *Ralstonia eutropha* improved the cell productivity and specific growth rate. By the analysis of cell yield, it was demonstrated that oleic acid was also consumed at the first cellular exponential phase. Characterization by X-ray, differential scanning calorimetry and thermogravimetric analysis of the films obtained from the different concentrations of oleic acid, revealed that no significant variation was observed from 0 to 1.5 g/L of oleic acid; however, with 3.0 g/L of oleic acid a film with lower crystallinity degree, glass transition and melting temperatures and thermal stability was obtained.

References

1. L. Liu, S. Li, H. Garreau, M. Vert, Selective enzymatic degradations of poly(L-lactide) and poly(ε-caprolactone) blend films, *Biomacromolecules*, *1* (2000) 350–359.
2. D.S. Rosa, G. Carraro, D.F. Penteado, B.L.M. Franco, Evaluation of the degradation of LDPE, PCL and PHB in soil compounds, *Revista Projeções*, *18* (2000) 111–118 (in Portuguese).
3. C. Marangoni, A. Furigo Jr., G.M.F. de Aragão, Oleic acid improves poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) production by *Ralstonia eutropha* in inverted sugar and propionic acid, *Biotechnol. Lett. 22* (2000) 1635–1638.
4. A. Steinbüchel, Perspectives for biotechnological production and utilization of biopolymers: Metabolic engineering of polyhydroxyalkanoate biosynthesis pathways as a successful example, *Macromol. Biosci. 1* (2001) 1–24.
5. C.S.K. Reddy, R. Ghai, Rashmi, V.C. Kalia, Polyhydroxyalkanoate: An overview, *Bioresour. Technol. 87* (2003) 137–146.
6. S. Khanna, A.K. Srivastava, Statistical media optimization studies for growth and PHB production by *Ralstonia eutropha*, *Process Biochem. 40* (2005) 2173–2182.
7. Y. Doi: *Microbial Polyesters*, Wiley VHC, New York, USA (1990).
8. G.A.M. van der Walle, G.J.H. Buisman, R.A. Westhuis, G. Eggink, Development of environmentally friendly coatings and paints using medium-chain-length poly(3-hydroxyalkanoates) as the polymer binder, *Int. J. Biol. Macromol. 25* (1999) 123–128.
9. D. Jendrossek, A. Shirmer, H.G. Schlegel, Biodegradation of polyhydroxyalkanoic acids, *Appl. Microbiol. Biotechnol. 46* (1996) 451–463.

10. B.H.A. Rehm, A. Steinbüchel: PHA Synthases: The Key Enzymes of PHA Synthesis. In: *Biopolymers, Vol. 3a*, A. Steinbüchel, Y. Doi (Eds.), Wiley-VCH, Weinheim, Germany (2002) pp. 173–215.

11. H.J. Rehm: G. Reed: *Biotechnology***,** *Vol. 6*, Wiley-VCH, Weinheim, Germany (1988).

12. Y.L. Sang, J.I. Choi, Effect of fermentation performance on the economics of poly(3-hydroxybutyrate) production by *Alcaligenes latus*, *Polym. Degrad. Stab. 59* (1998) 387–393.

13. Y. Chen, H. Yang, Q. Zhou, J. Chen, G. Gu, Cleaner recovery of poly(3-hydroxybutyric acid) synthesized in *Alcaligenes eutrophus, Process Biochem. 36* (2001) 501–506.

14. B.S. Kim, Production of poly(3-hydroxybutyrate) from inexpensive substrates, *Enzyme Microb. Technol. 27* (2000) 774–777.

15. S.Y. Lee, J.I. Choi, H.H. Wong, Recent advances in polyhydroxyalkanoate production by bacterial fermentation: Mini--review, *Int. J. Biol. Macromol. 25* (1999) 31–36.

16. C.R. Squio, Strategies for feeding and use of nutritional supplements for the production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) by *Ralstonia eutropha*, *MSc Thesis*, Federal University of Santa Catarina, Florianópolis, SC, Brazil (2003) (in Portuguese).

17. R.C. Rodrigues, Culture conditions for the production of poly(3-hydroxybutyrate) by *Ralstonia eutropha* from food--industry residues, *MSc Thesis*, Federal University of Santa Catarina, Florianópolis, SC, Brazil (2005) (in Portuguese).

18. G.M.F. de Aragão, N.D. Lindley, J.L Uribellarrea, A. Pareillelux, Maintaining a controlled residual growth capacity increases the production of polyhydroxyalkanoate copolymers by *Alcaligenes eutrophus*, *Biotechnol. Lett. 18* (1996) 937–942.

19. M.C. Formolo, D. Capanema, V.H. Grigull, G. Apati, S.A. Furlan, A.L.S. Schneider, G.M.F. Aragão, A.P.T. Pezzin, New strategy for extraction of poly(3-hydroxybutyrate) synthesized by *Ralstonia eutropha*, *Proceedings of the VIII Congresso Brasileiro de Polímeros (CBPOL)*, Águas de Lindóia, Brazil (2005).

20. T. Tsuge, Metabolic improvements and use of inexpensive carbon sources in microbial production of polyhydroxyalkanoates, *J. Biosci. Bioeng. 94* (2002) 579–584.

21. M. Akiyama, Y. Taima, Y. Doi, Production of poly(3-hydroxyalkanoates) by a bacterium of the genus *Alcaligenes* utilizing long-chain fatty acids, *Appl. Microbiol. Biotechnol. 37* (1992) 698–701.

22. J.A. Roels: *Energetics and Kinetics in Biotechnology*, Elsevier Biomedical Press, Amsterdam, The Netherlands (1983).

23. N. Galego, C. Rozsa, R. Sanchez, J. Fung, A. Vazquez, J. Santo Tomas, Characterization and application of poly(β--hydroxyalkanoate) family as composite materials, *Polym. Test. 19* (2000) 485–492.

24. N. Yoshie, Y. Inoue: Structure, Composition and Solution Properties of PHAs. In: *Biopolymers, Vol. 3b*, A. Steinbüchel, Y. Doi (Eds.), Wiley-VCH, Weinheim, Germany (2002).

25. A.J. Anderson, E.A. Dawes, Occurrence, metabolism, metabolic role and industrial uses of bacterial polyhydroxyalkanoates, *Microbiol. Rev. 54* (1990) 450–472.

26. K. Sudesh, T. Fukui, K. Taguchi, T. Iwata, Y. Doi, Improved production of poly(4-hydroxybutyrate) by *Comamonas acidovorans* and its freeze-fracture morphology, *Int. J. Biol. Macromol. 25* (1999) 79–85.