Articles from ISBP 2004

Production of Polyhydroxyalkanoates from Agricultural Waste and Surplus Materials†

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Received August 31, 2004; Revised Manuscript Received November 16, 2004

To be competitive with common plastics, the production costs of polyhydroxyalkanoates (PHAs) have to be minimized. Biotechnological polymer production occurs in aerobic processes; therefore, only about 50% of the main carbon sources and even a lower percentage of the precursors used for production of co-polyesters end up in the products wanted. A second cost factor in normally phosphate-limited production processes for PHAs is the costs for complex nitrogen sources. Both cheap carbon sources and cheap nitrogen sources are available from agricultural waste and surplus materials and make a substantial contribution for minimizing PHA production costs. In this study, fermentations for PHA production were carried out in laboratory-scale bioreactors on hydrolyzed whey permeate and glycerol liquid phase from the biodiesel production using a highly osmophilic organism. Without any precursor, the organism produced a poly[3(hydroxybutyrate-*co*-hydroxyvalerate)] copolyester on both carbon sources. During the accumulation phases, a constant 3-hydroxyvalerate content of 8−10% was obtained at a total PHA concentration of 5.5 g/L (on hydrolyzed whey permeate) and 16.2 g/L (glycerol liquid phase). In an additional fermentation, an expensive nitrogen source was substituted by meat and bone meal beside the glycerol liquid phase as a carbon source, resulting in a final PHA concentration of 5.9 g/L.

1. Introduction

To achieve biodegradable polymeric materials that can act as alternatives to common plastics derived from petrol, many studies have looked at the possibilities of microbial production of polyhydroxyalkanoates (PHAs).[1] Such polyesters are produced by numerous prokaryotic strains from renewable resources such as carbohydrates under unfavorable conditions: surplus of carbon source and limitation of an essential compound, for example, nitrogen, phosphorus, or oxygen.[2] The bacterium that produces these compounds completely degrades them to water and CO_2 if external carbon is limited, thus, embedding them into nature's closed cycle of carbon. Ongoing studies do not only aim at sustainable aspects of PHAs but also consider economic factors that until today are the major disadvantages in replacing common materials.[3,4]

A total of 40 420 800 tons of whey is produced per year as a byproduct of the cheese industry in the EU. From it, an annual amount of 13 462 000 tons, containing about 619 250 tons of sugar, is nowadays not utilized for further production of lactose and, therefore, constitutes a surplus product.[5] On the other hand, studies show that the price of lactose from whey can be estimated to only $116.7/ton compared to $493/ton pure glucose, which is a common substrate for biotechnological purposes.[6] The direct utilization of whey permeate as the sole carbon source for high-scale PHA production is only investigated by recombinant *Escherichia coli*.[7,8] In this study an osmophilic wild-type strain that is still under detailed characterization was used.

The crude whey was separated from proteins (retentate fraction) and concentrated (permeate fraction) directly at a dairy company and was obtained by the authors with a lactose concentration of 20% (w/v) that constitutes the upper limit of lactose solubility in water. This permeate was used as the carbon source for the investigation of PHA accumulation.

The same organism was used for PHA production on the glycerol liquid phase (GLP), a byproduct of the biodiesel production from plant oils and tallow, containing about 70% (w/w) glycerol. In all of Europe, a total production of biodiesel can be estimated for 2005 at 1 925 000 metric tons and for 2008 at 2 649 000 metric tons, corresponding to 192 500 and 264 900 metric tons of glycerol, respectively.[9] Currently the GLP constitutes a surplus material. The utilization of this product leads to an enormous cost advantage compared with commercially available pure glycerol, possessing a market value of 900 EURO per metric ton (year 2002).[10] Pre-experiments done by the authors

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† This paper was presented at the ISBP 2004 (International Symposium on Biological Polyesters), held in Beijing, China, August 22−28, 2004.

10.1021/bm049478b CCC: $30.25
Published on Web 01/08/2005

showed that the application of GLP has no negative impact on cultivation of the osmophilic organism compared with pure glycerol.

Severe problems have arisen during the last decennium in the EU from the topic *bovine spongiphorme encephalophathie*. At its peak, the disease had infected 3500 head of cattle weekly in the U.K.[11] In 2001, this encouraged several scientists at Graz University of Technology to find methods for safe utilization of meat and bone meal (MBM).[12] To produce alternative cheap nitrogen sources, MBM proven to be free of prions was chemically hydrolyzed and successfully used as a complex substrate for the osmophilic organism.

Aim of the Study. This work was performed to examine the use of surplus agricultural materials that can replace expensive carbon or nitrogen sources for biotechnological production of PHA. This way, agricultural waste and problematic surplus materials that until today have to be disposed are converted into high-value and sustainable bioplastics.

2. Materials and Methods

Media Composition. For cultivation of osmophilic strains, a common medium supplemented with the carbon and nitrogen sources of interest was applied. The exact types and amounts of carbon, nitrogen, and phosphorus sources are described together with the experiments.

Determination of Glucose, Galactose, and Glycerol. HPLC equipment consisting of a thermostated Aminex HPX 87H column (thermostated at 80 °C; Biorad, Hercules, U.S.A.), a HP 7673 controller, a JASCO 880-PU intelligent HPLC pump (JASCO Labor-und Datentechnik GmbH, Gross-Umstadt, Germany), and a BISCHOFF RI-Detector 8110 (BISCHOFF Analysentechnik und -geräte GmbH, Leonberg, Germany) were used. The analytes were eluted with 0.005 M H_2SO_4 (flow rate 0.60 mL min^{-1}) and integrated by a SIC chromatocorder 12 (Sic System Instruments, Dover, MA, U.S.A.).

Determination of PHA. Intracellular PHA in lyophilized biomass samples were transesterificated by acidic methanolysis. Analysis was performed with a HP 5890 Series II gas chromatograph (30-m HP5 column, protected by a 5-m HP1 capillary precolumn; Hewlett-Packard, U.S.A.). The analytes were detected by a flame ionization detector; the carrier gas helium was used with a split ratio of 1:10.[13]

Isolation of PHA. The cells cultivated in bioreactors were pasteurized in situ, centrifuged, frozen, and lyophilized for 24 h. After degreasing the biomass by overnight Soxleth extraction with ethanol, the PHAs were Soxleth extracted overnight with $CHCl_3$. The purity of the extracted material as well as the completeness of the extraction was determined by gas chromatography.

Determination of Molecular Mass Distribution and Thermal Analysis Characterization. Molecular weight data were obtained from measurements on a Jasco PU-1580 HPLC connected to Jasco 830-RI detector (JASCO Labor-und Datentechnik GmbH, Gross-Umstadt, Germany) and equipped with two PLgel 5-μm mixed-C columns (Agilent Technologies, U.S.A.). $CHCl_3$ was used as a solvent (1.0 mL min^{-1}). Monodisperse polystyrene standards were used for calibration (obtained from Polymer Standards Service GmbH, Germany).

Thermal analysis characterization was performed on a Mettler TA 4000 System instrument (Mettler Toledo) consisting of a DSC-30 differential scanning calorimeter, TGA-50 furnace with an M3 microbalance, and TA72 GraphWare software. differential scanning calorimetry samples of approximately 5 mg were weighed in 40-μL aluminum pans; an empty pan was used as the reference. Measurements were carried out under an 80 mL min^{-1} nitrogen flow rate according to the following protocol: first, second, and third heating from −30 to 200 °C at 10 °C min^{-1}; first cooling (quenching after the first heating) from 200 to −30 °C at 100 °C min^{-1}; and the second cooling from 200 to −30 °C at 10 °C min^{-1}.

Hydrolysis of Lactose in Whey Permeate. Lactose hydrolysis in whey permeate was performed by adding 2.5 mL of a solution of β-galactosidase (EC 3.2.1.23; Maxilact 2000, 2000 NLU/g, prepared from *Klyveromyces lactis*) in glycerol per liter whey permeate (DSM Food Specialities, U.K.).[14] After continuous stirring in glass vessels (25 h, pH = 6.5, T = 38−40 °C), the efficiency of lactose hydrolysis was monitored via HPLC. The hydrolysis was necessary because of the strain's inability of direct lactose conversion.

Determination of Free Amino Groups in Hydrolyzed MBM. Samples were centrifuged at 4000 rpm for sufficient time to separate the particles in solution. Residual solids were removed by filtration. A total of 10 μL of filtrate was added to 1 mL of OPA reagent (80 mg OPA, *o*-phthaldialdehyde; Merck, Darmstadt, Germany) mixed in 2 mL of 95% ethanol, 10 mL of 10% sodium dodecyl sulfate, 0.2 mL of mercaptoethanol, and 50 mL of 0.1 M sodium borate adjusted to 100 mL with water). After 2 min, absorbance was measured (340 nm) against water as the zero reference.

Determination of Optical Density (OD). OD was measured at λ = 420 nm against deionized water as the zero reference on a Hitachi U-1100 spectrophotometer.

Determination of Protein. After ultrasonic disruption of the cells, the protein concentration was measured according to Lowry et al.'s method.[15]

Fermentation Strategy. In a 42-L bioreactor (MBR Bioreactor AG Switzerland), a highly osmophilic organism was cultivated for production of poly[3(hydroxybutyrate-*co*-hydroxyvalerate)] {poly[3(HB-*co*-HV)]} on hydrolyzed whey permeate (see section 3.1) or GLP (see sections 3.2 and 3.3) as the main carbon source. At the start of the fermentations, the medium was supplemented with 10 g/L of sugars from hydrolyzed whey lactose (see section 3.1) or glycerol from GLP (see sections 3.2 and 3.4), respectively. Nitrogen was supplied by adding yeast extract (2.5 g/L) and casein peptone (2.5 g/L) at the beginning of each fermentation. To expedite phosphorus-limited conditions soon, the only phosphate source was yeast extract. On the basis of analytical data (HPLC) after each sampling, further additions of substrates were done if necessary. The cells were cultivated under controlled conditions of pH (7.0), temperature (37 °C), and oxygen tension (control of dissolved oxygen by agitation speed of stirrer; constant aeration rate of 10 mL min^{-1}).

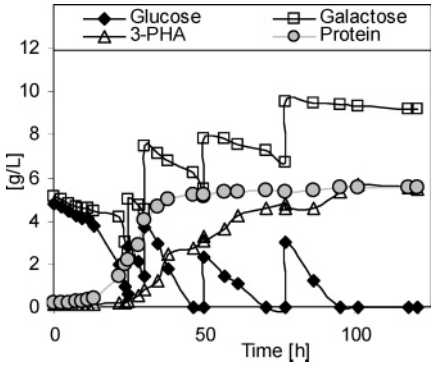

Figure 1. PHA production on whey: time curves of substrates and products during a fed-batch fermentation. Increases in substrate concentrations are due to refeeding of hydrolyzed whey permeate.

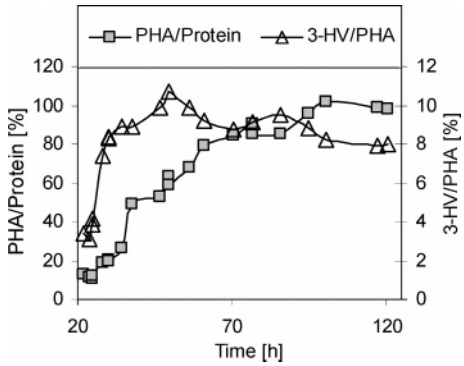

Figure 2. Percentages of 3-HV in polymer and PHA/protein ratio.

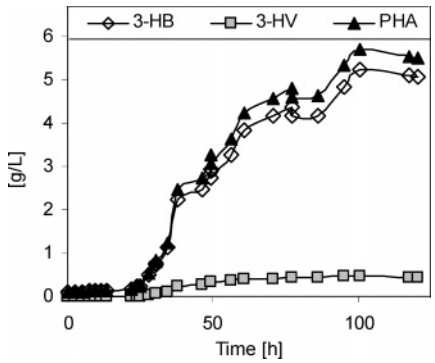

Figure 3. Time curves of 3-HB, 3-HV, and PHA from whey sugar.

3. Results and Discussion

3.1. Production of Poly[3-(HB-*co*-HV)] from Whey. The fermentation pattern (Figure 1) shows the time courses of the main substrates glucose and galactose from hydrolyzed whey as well as the concentrations of residual biomass (monitored by protein determination) and PHA. The organism is able to convert both hexoses from hydrolysis of whey lactose, but for glucose, higher conversion rates were calculated than for galactose (see also time curves for substrates in Figure 1). At the end of the cultivation, a maximal PHA concentration of 5.5 g/L was achieved; the protein concentration reached a maximum value of 5.5 g/L. Therefore, a maximum content of PHA in biomass of 50% was calculated (ratio of PHA/protein = 1:1, for the time curve of the ratio see Figure 2).

As can be seen from Figure 3, the investigated organism possesses a rare and precious property: it produces 3-hydroxyvalerate (3-HV) units directly from hexoses. Normally the biotechnological production of 3-HV demands precursors (propionic acid, valeric acid) that on the other hand contribute significantly to the production costs. During the production phase, the share of 3-HV in the polymer remained at a constant level of 8−10% (w/w). Together with the ratio PHA/protein, this time curve is shown in Figure 2. For most applications, a 3-HV share of about 20% is necessary to lower the crystallinity of the product sufficiently. Because approximately half of this percentage is directly produced from surplus whey, the needed amount of precursors decreases to only 50%.

Table 1. Polymer Characterization

	whey	GLP
1st melting endotherm (T_{g1}) [°C]	149.7	128.7
2nd melting endotherm (T_{g2}) [°C]	160.7	138.8
cold crystallization peak [°C]	63.4	64.5
glass transition temperature [°C]	7.0	7.0
3-HV/PHA [% w/w]	8−10	8−10
M_w [kDa]	696	253
polydispersity index (M_w/M_n)	2.2	2.7

Table 2. Comparison of Results from Cultivations on Whey, GLP, and GLP plus MBM

	whey	GLP	GLP + MBM
max μ [1/h]	0.11	0.06	0.11
π_{max} [1/h]	0.077	0.081	0.098
Y (PHA/whey sugars) [g/g]	0.33		
Y (PHA/GLP) [g/g]		0.23	0.18
max protein concentration [g/L]	5.5	5.5	1.75
max PHA concentration [g/L]	5.5	16.2	5.91
PHA/CDM [%] (end)	49.6	76.0	75.0
vol. productivity [g/(L·h)] (PHA; total duration)	0.05	0.14	0.039

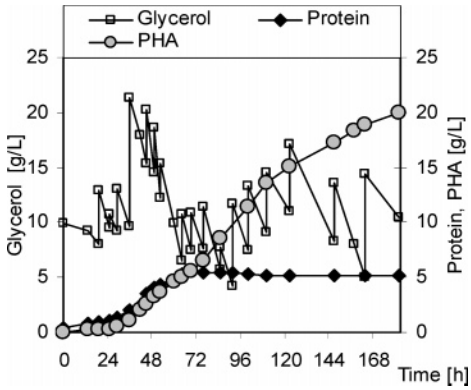

Figure 4. PHA production on GLP from biodiesel production: time curves of substrates and products during a fed-batch fermentation. Increases in glycerol concentrations are due to refeeding of GLP.

The molecular weight distribution was determined with a high weight-averaged molecular weight M_w = 696 kDa and a polydispersity index of Pi = M_w/M_n = 2.2 (M_n is the number-averaged molecular weight) at the end of the fermentation. Thermoanalysis of the polymer resulted in a glass transition point T_g of 7.0 °C, a cold crystallization point T_c at 63.4 °C, and two melting endotherms T_{m1} and T_{m2} at 149.7 and 160.7 °C (Table 1). Compared with homopolymer poly(3-hydroxybutyrate) (3-PHB), these low melting temperatures

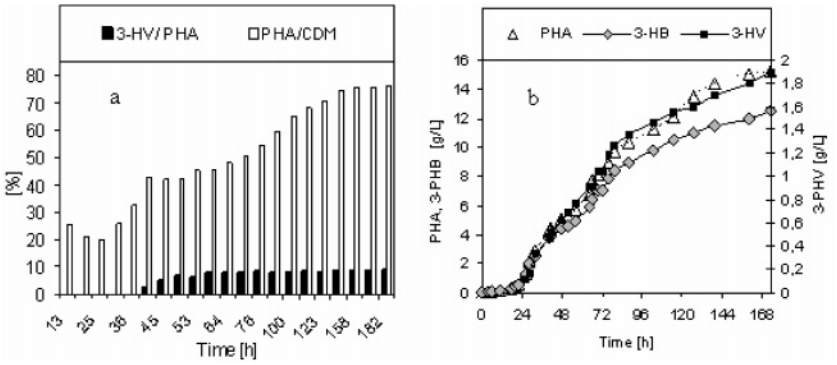

Figure 5. Percentages of 3-HV in polymer and of PHA in CDM (a) and time curves of 3-HB, 3-HV, and PHA (b).

are advantageous for further processing of the material; the low melting points might be caused by the presence of 3-HV units, leading to a disruption of the high crystalline 3-PHB matrix. [16] On the other hand, the discovery of more than one melting endotherm indicates a not-random distribution of 3-HV in the 3-PHB matrix, so that the material is of heterogeneous nature. A formation of blocks of 3-HV or the formation of polymer blends might have taken place. According to this analysis, the quality of produced copolyester is certainly sufficient for applications in polymer extrusion technology.

Table 2 shows the most important results of the fermentation.

3.2. Production of Poly[3(HB-*co*-HV)] from GLP from Biodiesel Production. Similar to the first experiment, the concentration of the substrate was kept approximately constant in the range of 10 g/L. Pre-experiments demonstrated that exceeding the substrate concentration beyond 20 g/L does not show any negative impact (data not shown). Figure 4 shows the fermentation pattern of the experiment, including time curves for glycerol, PHA, and protein.

The composition of the polymer and the units of 3-HV (8−10% 3-HV/PHA) produced from GLP were similar to the ones from whey sugars, without supplementation of precursors. At the end of the fermentation, the molecular weight distribution was determined with a weight-averaged molecular weight $M_w = 253$ kDa and a polydispersity index of Pi = 2.7. This significantly lower molecular weight data (compared with the polymer produced from whey) correspond very well to results from the literature.[17] It has been demonstrated that molecular weights decrease when glycerol or glycols are present in the medium. These substances cause termination of chain propagation by covalent linking at the carboxyl terminus of PHA.[17] On the other hand, there might be several special fields of application in future for low molecular mass PHAs; for example, they could act as softeners. Similar to the PHA from whey, two melting endotherms occurred. The maxima of these melting peaks are about 20 °C lower than those of the polyester from whey. Also the values for cold crystallization (63.4 °C for whey and 64.5 °C for GLP, respectively) and the glass transition temperature (7.0 °C in both cases) are very similar for both polymers. To be compared with results from the fermentation with whey sugars, data from polymer characterization are shown in Table 1.

Figure 5a shows the percentages of 3-HV in the polyester and of PHA in the cell dry mass (CDM), and time curves of 3-hydroxybutyrate (3-HB), 3-HV, and PHA are depicted in Figure 5b.

Comparison of the results of this fermentation on GLP with the experiments on hydrolyzed whey sugars (Table 2) showed that the highest calculated value for the specific growth rate (max μ) was higher on hydrolyzed whey sugars. Nevertheless, the same final protein concentration (5.5 g/L) could be achieved in both cases. Problems with galactose uptake occurring with this organism caused a lower end concentration of PHA in the case of hydrolyzed whey sugars (5.5 g/L from whey compared with 16.2 g/L from GLP). Galactose consumption only occurred together with glucose consumption. This also explains the differences of volumetric productivities and the lower end content of PHA in CDM as well as the differences in the maximum specific production rates (max π), when hydrolyzed whey was used as the substrate. An additional feeding with hydrolyzed whey sugars would, therefore, be necessary to continue PHA production. On the other hand, similar yields were calculated for PHA formation from hydrolyzed whey lactose and from GLP ($Y_{PHA/whey}$ sugars, 33%; $Y_{PHA/GLP}$, 23%).

3.3. Hydrolysis of MBM: Production of a Cheap and Safe Complex Nitrogen and Phosphorus Source. MBM was hydrolyzed under both acid and alkaline conditions. According to the degrees of hydrolysis (after 24 h, 97.0% after acidic hydrolysis and 93.5% after alkaline hydrolysis), both hydrolysis methods turned out to be suitable. After neutralization, the hydrolysis products were tested in shaking flask cultivations using the same osmophilic organism as in sections 3.1 and 3.2. Figure 6 depicts the growth curves on both hydrolysates, acid and alkaline, monitored via the OD. The growth on acidic hydrolysate was faster than on alkaline hydrolysate. Values for maximum specific growth rates μ were 0.17 h^{-1} with acidic hydrolysate and 0.07 h^{-1} on alkaline hydrolysate, respectively. As a conclusion, the hydrolysis product from MBM can be used as a substitute of at least parts of expensive complex substrates such as yeast extract. Because of the osmophilic behavior of the investigated organism, the high salt content of the hydrolysate and later neutralized product does not cause problems for the process.

3.4. Production of Poly-3(hydroxybutyrate-*co*-hydroxyvalerate) from GLP and MBM. After acidic hydrolysis,

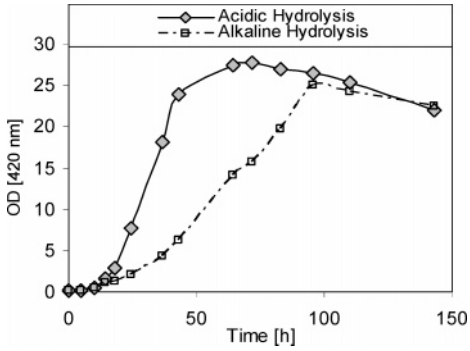

Figure 6. Application of hydrolyzed MBM growth curves.

MBM was supplemented in the fermentation batch to obtain the same amount of nitrogen compared with mixtures of yeast extract and casein peptone at the two fermentations described before. Also GLP was supplemented to keep the concentration constant at approximately 10 g/L. Table 2 shows the most significant kinetic data.

Table 2 shows that values for maximum specific productivity, percentage of PHA in biomass, and yield of PHA from GLP are comparable with results from the fermentation on GLP with expensive nitrogen sources. The highest specific growth rate was calculated to be 0.11 g/(g·h), the same value that was found for growth on whey sugars. The final protein concentration was only 32% compared with the fermentation on GLP described before; therefore, lower volumetric productivity was achieved. However, the results show that a part of the complex nitrogen and phosphorus source can be substituted by hydrolyzed MBM.

4. Conclusion and Future Work

According to the results, the biotechnological utilization of both agricultural surplus products, whey and GLP, as cheap carbon sources for production of the high-value product PHA is possible. Together with the application of hydrolyzed MBM hereby demonstrated to be a suitable complex nitrogen and phosphorus source, production costs for PHA production can be minimized considerably. Depending on the substrate (high M_w on whey hexoses, low M_w on GLP), totally different molecular masses of PHAs were produced indicating that a broad spectrum of biopolyesters with different properties are available when using carbon and nitrogen sources from surplus products as raw fermentation feedstock materials. The production of a constant amount of 3-HV from cheap substrates makes the process economically even more interesting and, depending on the carbon source, results in different product properties. Future activities will focus on improvement of the organism for direct conversion of whey lactose without prior hydrolysis to further minimize production costs. This strain improvement could be done by cloning of β-galactosidase into the bacterial cells.

Acknowledgment. This work was supported by Wheypol Growth EU-Project GRD2-2000-30385, BIODIEPRO EU-Project NNE5/2001/832, and Zukunftsfonds Steiermark − "Zukunftsfähige Technologien auf Basis Tiermehl und anderer biogener Rohstoffe". The authors thank for molecular mass analysis and thermal analysis characterization done by University of Pisa, Department of Chem. & Ind. Chem. at the laboratories of Prof. E. Chiellini by Elizabeth Grillo (polymer from whey) and at TU Graz, Institute of Organic Chemical Technologies, at the laboratories of Prof. F. Stelzer (polymer from GLP).

References and Notes

(1) Brandl, H.; Gross, R. A.; Lenz, R. W.; Fuller; R. C. *Adv. Biochem. Eng. Biotechnol.* **1990**, *41*, 77−93.
(2) Wang, F.; Lee, S. Y. *Appl. Environ. Microbiol.* **1997**, *63*, 3703−3706.
(3) Choi, J.; Lee, S. Y. *Appl. Microbiol.* **1999**, *51*, 13-21.
(4) Zhang, H.; Obias, V.; Gonyer, K.; Dennis, D. *Appl. Environ. Microbiol.* **1994**, *60*, 1198−1205.
(5) Bundesamt für Statistik (Swiss Federal Statistic Office), Neuchâtel, Switzerland, 2000.
(6) Hocking, P. J.; Marchessault, R. H. In *Chemistry and technology of biodegradable polymers*, Griffin, G. J. L., Ed.; Chapman & Hall: New York, 1994; pp 48−96.
(7) Ahn, W. S.; Park, S. J.; Lee, S. Y. *Appl. Environ. Microbiol.* **2000**, *66*, 3624−3627.
(8) Ahn, W. S.; Park, S. J.; Lee, S. Y. *Biotechnol. Lett.* **2001**, *23*, 235−240.
(9) *Oleoline Glycerine Market report*, 2003 (www.oleline.com).
(10) Biodiesel BDI Anlagenbau Ges.m.b.H., Graz, Austria, 2004.
(11) http://www.med4you.at/bse/bse_rind.htm (accessed May 2004).
(12) *Safe technical utilization of meat and bone meal*; Task Force; Graz Technical University: Graz, Austria, 2001 (press release).
(13) Braunegg, G.; Sonnleitner; B, Lafferty, R. *Eur. J. Appl. Microbiol.* **1978**, *6*, 29−37.
(14) www.dsm.com/dfs/diary/products/enzymes (accessed Feb 2004).
(15) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *19*, 265.
(16) Doi, Y. *Microbial polyesters*; VHC Publishers: New York, 1990.
(17) Madden, L. A.; Anderson, A. J.; Shah, D. T.; Asrar, J. *Int. J. Biol. Macromol.* **1999**, *25*, 43−53.

BM049478B