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ORIGINAL ARTICLE

Biotechnological production of poly(3-hydroxybutyrate) with *Wautersia eutropha* by application of green grass juice and silage juice as additional complex substrates

M. KOLLER, R. BONA, C. HERMANN, P. HORVAT, J. MARTINZ, J. NETO, L. PEREIRA, P. VARILA, & G. BRAUNEGG

Institute of Biotechnology & Bioprocess Engineering, Graz University of Technology, Petersgasse 12, A-8010 Graz, Austria

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Abstract
Alternative inexpensive complex nitrogen- and phosphate sources from agriculture, green grass juice (GGJ) and silage juice (SJ), were added to cultivation medium in order to investigate their impact on growth of the well-known polyhydroxyalkanoate (PHA) accumulating strain *Wautersia eutropha*. The influence of these additives was directly compared with cultivations on defined minimal mineral medium (M) as well as on the same medium supplemented with more expensive complex additives: corn steep liquor (CSL) and casamino acids (CA). It turned out that the supplementation with most complex additives results in shortening of lag-phases of bacterial growth and in higher end-concentrations of residual biomass compared with M-medium. Finally, higher volumetric productivities for poly(3-hydroxybutyrate) (3-PHB) were achieved. The effect of the inexpensive additive SJ on volumetric productivity was similar to the result for the expensive CA (0.653 vs. 0.619 g L^{-1} h^{-1}). The same was found for the biomass concentration (7.00 vs. 7.44 g L^{-1} respectively). Together with an economic appraisal presented in this study, the results suggest it is possible to make the sustainable process of microbial PHA-production more economically feasible. A survey of the thermal characteristics and molecular mass properties of the isolated polymers completes this work.

Keywords: *Complex additives, green grass juice, polyhydroxyalkanoates,* Wautersia eutropha, *silage juice*

Introduction

Polyhydroxyalkanoates (PHAs) are biodegradable polyesters with physical properties of thermoplastics and/or elastomers. Therefore, they can be applied as alternatives to common plastic materials produced from mineral oils (Holmes 1988; Brandl et al. 1990; Steinbüchel & Valentin 1995; Lee et al. 1996). PHAs are synthesized in the cytoplasm of various prokaryotic strains usually from carbohydrates, but also from other renewable resources (Hocking & Marchessault 1994; Bourque et al. 1995; Ramsay 1995). Under unfavourable growth conditions (surplus of carbon source plus limitation of an essential substrate, e.g. nitrogen, phosphate or oxygen), some strains are able to divert the usual carbon flux (Acetyl-CoA synthesized in the central metabolic pathways of the microorganism) from biosynthesis of protein (biomass) constituents to the formation of compounds acting as precursors for the production of PHA, mainly poly-3-hydroxybutyric acid (3-PHB). Due to a high degree of polymerization, their molecular weights can reach several million (Wang & Lee 1997; Steinbüchel & Lütke-Eversloh 2003). These polymers serve as an intracellular reserve carbon- and energy source which normally will be degraded if the external carbon source is depleted. Because of their outstanding property of complete degradation to water and CO_2, they are embedded into natures carbon cycle. If applied instead of fossil oil derived polymers, the mass balance of carbon from biomass will be closed, but its durability will be prolonged compared with the usual biological life cycle of PHA-carbon. The fossil fuel energy demand during the life span of PHAs will not exceed the amount necessary for the industrial production and

Correspondence: M. Koller, Institute of Biotechnology & Bioprocess Engineering, Graz University of Technology, Petersgasse 12, A-8010 Graz, Austria. Tel: +43-316-8738411. Fax: +43-316-8738434. E-mail: martin.koller@tugraz.at

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processing of PHAs itself. The negative effects of CO_2 accumulation in the atmosphere, e.g. the green house effect, will be related only to the amount derived from energy production and not to the carbon in the biopolymer itself. Therefore, PHA production significantly lowers the amount of fossil-oil derived carbon in the cycles of nature, compared with petrochemical polymer synthesis. Hence, there are sound ecological reasons for using PHAs instead of mineral oil-derived plastics.

In order to compete with common plastics, the production costs for PHAs have to be minimized. Numerous studies consider economic factors of PHA production (Choi & Lee 1990; Zhang et al. 1994). An important field of investigation is the microbial production of PHAs based on cheap surplus- and waste materials. Among these substrates, not only carbon sources e.g. molasses (Zhang et al. 1994), starch (Kim 2000), whey from the dairy industry (Koller et al. 2005; Pavolo & Casella 2003), surplus glycerol from biodiesel production (Ashby et al. 2004; Koller et al. 2005), xylose (Lee 1998; Silva et al. 2004) and plant oils (Fukui & Doi 1998) are available, but also sources for nitrogen and phosphate: fish peptone (Page & Cornish 1993), meat extract, casamino acids (CA), corn steep liquor (CSL), soybean hydrolysate (Lee 1998) and cotton seed hydrolysate (Lee 1998). It has to be expected that, in respect to future limitations of fossil fuels, there will be an enhanced need for alternative raw materials, e.g. surplus- and waste materials from agricultural production.

It was previously found that supplementation with a small amount of complex nitrogen source such as tryptone could enhance 3-PHB production by recombinant *Escherichia coli* in a defined medium containing glucose as sole carbon source (Lee & Chang 1994). In the northern hemisphere a remarkable agricultural area of green grass land is available. The green biomass is a convenient source of green grass juice as a primary product from bio-refinery processes. Because of current changes in the structure of Austrian agriculture, characterized by a decrease of grassland utilization for production of cattle feed, the bio-refinery process deals with innovative utilization pathways for green biomass, not only for grass fibres, but also for grass juices (information from: "Fabrik der Zukunft", www.nachhaltigwirtschaften.at).

Aim of this study

Within this bio-refinery process, it was reasonable to investigate microbial growth and PHA production by *Wautersia eutropha*, a stable organism that is known to accumulate PHA with high productivity (Choi et al. 1990) by supplementing common defined mineral medium with such inexpensive agricultural by-products (green grass juice, GGJ; and its fermentation product silage juice, SJ). These results were compared with the PHA production using well-known additives which do not constitute surplus products in Europe (corn steep liquor, CSL), or contribute significantly to the production costs (casamino acids, CA). CA in technical quality is sold today at a price of 85 –119.6 US-$ per kg (information from: www.krackler.com).

Materials and methods

Microorganism

Wautersia eutropha DSM 545 (formerly known as *Ralstonia eutropha* and *Alcaligenes eutrophus*) was obtained from DSMZ culture collection (Braunschweig, Germany). The facultative chemolithotroph cells form gram-negative straight rods and are peritrichously flagellated (Holding & Shewan 1974).

Cultivation of microorganism and media formulations

A chemically defined minimal mineral medium (M) according to Küng (Küng 1982) was used for cultivations in shaking flasks and a laboratory bioreactor containing (per liter): $Na_2HPO_4 \cdot 2H_2O$, 4.5 g; KH_2PO_4, 1.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $(NH_4)_2SO_4$, 2 g; $CaCl_2 \cdot 2H_2O$, 0.02 g; NH_4Fe(III) Citrate, 0.05 g; trace element solution SL6,[1] 1 mL; glucose, 10 g.

Shaking flask experiments

Two series of shaking flask experiments were performed as follows:
300 mL baffled shaking flasks containing 100 mL of the M-medium were supplemented with the following concentrations of GGJ or SJ (%, v/v):

- GGJ: 0; 0.5; 1.0; 2.5; 5.0; 10.0 and 13.5
- SJ: 0; 0.1; 0.5; 1.0; 2.5; 5; 10; 15; 20 (pH value of SJ was adjusted to 7.0)

In order not to destroy thermo labile components in the GGJ and SJ, they were sterilized by filtration using absolute filters (*Nalgene syringe filters*) with a pore size of 0.20 μm. The cultivation flasks were inoculated with 5% (v/v) of a dense preculture from the late exponential phase cultivated on M-medium (optical density at $\lambda = 420$ nm: ≈ 20). All experimental set ups in shaking flasks were performed in duplicate. Growth was monitored via the optical density.

Bioreactor experiments

Five fermentations were accomplished as follows: One of the complex nitrogen sources listed below was supplemented to M-medium as an aqueous solution at the beginning of the fermentations: saturated solution of casamino acids (CA; Difco), saturated solution of corn steep liquor (CSL; Merck, Germany), green grass juice (GGJ; local producer, Styria, Austria), silage juice (SJ; local producer, Styria, Austria). No complex additives were added to the control fermentation (Figure 2a, b). The pH values were adjusted to 7.0 and cultivations were carried out at $T = 30^{\circ}C$. The volumes of added solutions of CA and CSL as well as the volumes of GGJ and SJ always amounted to 5% (v/v). Table I depicts the composition of GGJ and SJ in comparison with CA and CS. Glucose was added at the beginning of the fermentations at a concentration of $10\ g\ L^{-1}$; additional feedings with glucose were done by pulse feeding if necessary, using a 50% (w/w) glucose solution.

For all bioreactor fermentation experiments, a 2 L laboratory bioreactor (Bioengineering, Uster, Switzerland, Type KLF 2000) equipped with three axial-propeller stirrers was used. Temperature (30°C), pH value (7.0, Hamilton) and oxygen partial pressure (Ingold) were controlled automatically. The concentration of dissolved oxygen was controlled by adjustment of the agitation speed and kept at about 40% of the saturation concentration of oxygen in water; oxygen was supplied at $150\ L\ h^{-1}$ through an absolute filter (Sartorius Midisart 2000). The bioreactor was filled with 1.75 L of medium (depends on experiment) and inoculated with 80 mL of a preculture from the late exponential phase.

Analytical procedures

Determination of optical density. As a routine quick analysis during the process the biomass concentration was followed on a Hitachi U-1100 spectrophotometer by monitoring the optical density (OD), measured at $\lambda = 420$ nm against deionized water as zero-reference.

Determination of glucose. An HPLC consisting of a thermostated Aminex HPX 87H column, a HP 7673 Controller, a JASCO 880-PU intelligent HPLC pump, and a BISCHOFF RI-Detector 8110 was used. The analytes were eluted with 0.005 M H_2SO_4 ($0.60\ mL\ min^{-1}$).

Determination of NH_4^+. NH_4^+ was determined electrochemically with an Orion ion-selective electrode.

Determination of cell dry mass. About 5 mL of fermentation broth was centrifuged in pre-weighed glass tubes. The supernatant was used for substrate analysis, the remaining biomass pellet was frozen and lyophilized overnight. Gravimetric difference against empty tubes was calculated after weighing, the pellets were later used for determination of PHA. The determinations were done in duplicate.

Determination of poly-3-hydroxybutyric acid (3-PHB). Intracellular PHA in lyophilized biomass samples was transesterified by acidic methanolysis. Analysis was carried out with a HP 5890 Series II gas chromatograph (30 m HP5 column, protected by a 5 m HP 1 capillary pre-column). The analytes were detected by flame ionization detector (FID), the carrier gas was helium and a split ratio of 1:10 was used (Braunegg et al. 1978). Pure 3-PHB from Biopol™ was used for calibration. The 3-PHB content (%) was defined as the percentage of the ratio of 3-PHB concentration to dry cell mass. Residual biomass ($g\ L^{-1}$) was defined as the difference of cell dry mass ($g\ L^{-1}$) and 3-PHB concentration ($g\ L^{-1}$).

Table I. Composition of green grass juice (GGJ), silage juice (SJ), casamino acids (CA) and corn steep liquor (CSL)

	GGJ	SJ	CA	CSL
pH value of liquid additive	5.89–5.95	4.26–4.32	6.2	4.12
Dry matter of liquid additive [$g\ L^{-1}$]	44.2	19.55	19.55	19.55
Protein content [$g\ L^{-1}$]	7.2	6.50	13.9[2]	17
Lactic acid [$g\ L^{-1}$]	10.3	57.25	–	7
Acetic acid [$g\ L^{-1}$]	0.80	29.80	–	–
Propionic acid [$g\ L^{-1}$]	0.00	8.40	–	–
NH_4^+ [$g\ L^{-1}$]	0.12	0.38	0.60	0.19
Glucose [$g\ L^{-1}$]	3.43	32.50	–	20 [20]
Fructose [$g\ L^{-1}$]	5.69	39.25	–	–
Lactose [$g\ L^{-1}$]	1.29	5.38	–	–
Arabinose [$g\ L^{-1}$]	0.93	3.05	–	–
Xylose [$g\ L^{-1}$]	0.39	0.00	–	–
Ash/dry matter [%]	27.4	26.20	19.4	16

Isolation of polyhydroxyalkanoates. Cells cultivated in the bioreactor were pasteurized *in situ* (80°C, 30 min), harvested by centrifugation, frozen and lyophilized for 24 h. After degreasing the biomass by overnight Soxlet extraction with ethanol, PHA was Soxlet extracted overnight with $CHCl_3$ and finally precipitated from the $CHCl_3$ solution by addition of a 10-fold amount of cold ethanol. The purity of the extracted material as well as the completeness of the extraction was determined by GC.

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 and equipped with two PLgel 5 μm mixed-C columns. $CHCl_3$ was used as solvent (flow rate 1.0 mL min^{-1}). Monodisperse polystyrene standards were used for calibration.

Thermal analysis characterization was performed on a Mettler TA 4000 System instrument consisting of DSC-30 Differential Scanning Calorimeter, TGA-50 furnace with M3 microbalance, and TA72 GraphWare software. DSC samples of approximately 5 mg were weighed in 40 μL aluminium pans; an empty pan was used as reference. Measurements were carried out under 80 mL min^{-1} nitrogen 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}. Data for melting point, degree of crystallinity and cold crystallization were reported from the first heating scan, data for glass transition from the second heating scan. The degree of crystallinity (X_c) was determined by considering the value of the melting enthalpy of 146 J g^{-1} for the 100% crystalline 3-PHB (Barham et al. 1984).

Economic appraisal. The basis for the calculations was the number of production cycles per year (n_c). This value included typically 8640 annual working h. The cycle time (t_c) was calculated by addition of the laboratory scale fermentation time plus 12 h for preparation and follow-up treatment of the bioreactor. The following equations were used for all calculations:

Production cycles per year:

$$n_c^{(4)} = \frac{8640[h]^{(5)}}{t_c[h]^{(6)}}$$

Annual production of 3-PHB in 100 m^3 bioreactor [t]:

$$m_{PHB}[t] \Big/ 100m^{3(7)} = \frac{100[m^3]^{(8)} \times n_c^{(4)} \times c^{(9)}}{1.3^{(10)} \times 1000^{(11)}}$$

Necessary bioreactor volume for annual production of 1 ton 3-PHB:

$$Vol[L]^{(12)} = \frac{1[t]^{(13)} \times 1.3^{(10)} \times 100000^{(14)}}{c^{(9)} \times n_c^{(4)}}$$

Annual production of 3-PHB on a laboratory scale:

$$m_{PHB}[\mathrm{kg}]^{(15)} = 1.75[L]^{(16)} \times c^{(9)} \times n_c^{(4)} \times 0.001^{(17)}$$

Results and discussion

Evaluation of ideal concentrations of green grass juice (GGJ) and silage juice (SJ)

Investigations of green grass juice (GGJ), showed that, within the same time period (15 h), additions of 2.5% and 5.0% (v/v) produced a higher increase in biomass compared with pure minimal medium. Both 2.5% and 5.0% (v/v) (1.1 and 2.2 g L^{-1} dry matter) also shortened the lag-phase of bacterial growth from 6.5 h (minimal medium) to 4.5 h. The best results were achieved with 5.0% (v/v) GGJ, so this concentration was chosen for the bioreactor fermentation. Higher additions than 5% (v/v) were not advantageous.

With silage juice (SJ) it was found that more biomass was generated after 15 h, when 0.1, 0.5, 1.0, 2.5, 5.0 or 10.0% (v/v) of SJ were supplemented to the minimal medium. Higher additions did not increase biomass production, but some showed inhibitory effects. The impact of 5.0 and 10% (v/v) was nearby the same; therefore a concentration of 5% (v/v) was chosen for the bioreactor experiment.

Growth of Wautersia eutropha *and PHA-synthesis in the bioreactor experiments*

Five fermentations were analyzed in order to get detailed yields and kinetic data. Table II summarizes the most significant results (maximal specific growth rate μ_{max}, volumetric productivity of 3-PHB for the entire process[18], specific product formation rate q_p[19], maximum specific product formation rate $q_{p\,max}$ (the highest calculated value for q_p during the cultivation), the 3-PHB content in CDM and yield coefficients for biomass from inorganic nitrogen. In order to allow a comparison of results from different fermentations, the final concentrations of residual biomass (expressed as the difference between total biomass and 3-PHB) and 3-PHB concentrations (based on broth volume) are shown together for all fermentations (Figure 1). All other results are presented in Figures 2–6. These figures

Table II. Main results from five fermentations: *W. eutropha* on defined minimal media with and without supplementations (minimal medium M, corn steep liquor CSL, casamino acids CA, green grass juice GGJ, silage juice SJ)[21]

Additive	max. μ [h^{-1}]	3-PHB/CDM [% w/w]	Vol. productivity [3-PHB; g L^{-1} h^{-1}]	Specif. productivity q_p [g g^{-1} h^{-1}]	max. specific productivity $q_{p\ max}$ [g g^{-1} h^{-1}]	*Y* [g Residual biomass/g $NH_4(SO_4)_2$]
M	0.084	76.7	0.287	0.074	0.15	1.37
CSL	0.19	84.2	0.477	0.095	0.18	1.47
CA	0.14	84.0	0.619	0.083	0.16	2.51
GGJ	0.11	77.2	0.283	0.071	0.18	1.24
SJ	0.12	77.3	0.653	0.093	0.16	1.46

illustrate the time courses (a) for substrates (glucose and inorganic nitrogen) and (b) for products (cell dry mass, residual biomass and 3-PHB).

Each of the investigated additives resulted in higher concentrations of residual biomass compared with minimal medium (M) (see Figure 2a, 3a, 4a, 5a, 6a). The best results were obtained for media supplemented with SJ or CA (7.00 and 7.44 g L^{-1} respectively; Figure 4a, 6a), and were significantly better than those achieved in other media, i.e. GGJ, CSL and M-medium (4.0; 5.02 and 3.86 g L^{-1}, respectively; Figure 5a, 3a, 2a). In addition, these results are supported by the calculated maximum specific growth rates (μ_{max}, Table II). The highest specific growth rate was achieved in medium supplemented with CSL (0.19 h^{-1}) which is similar to data from Marangoni et al. (2001), the other results were lower: 0.14 h^{-1} (CA), 0.12 h^{-1} (SJ), 0.11 h^{-1} (GGJ) and 0.084 h^{-1} for M-medium particularly when the medium was supplemented with CA or SJ, there was virtually no lag phase for the formation of residual biomass (see Figure 4a and 6a).

The 3-PHB content in biomass differed only slightly (Table II) and fluctuated from 84.2 (CSL) to 76.7 (M-medium)% (w/w), values which are typical for this strain (Sudesh et al. 2001). Thus, the PHB content in the biomass was not influenced by the type of complex substrate added, and the total quantity of 3-PHB in fermentations was determined by the biomass yield. This is also reflected by similar values of specific production rates q_p (Table II) which are all in the range of 0.15–0.18 (g g^{-1} h^{-1}).

In contrast to the growth-associated PHA-producer *Alcaligenes latus*, only negligible amounts of 3-PHB are accumulated during balanced growth of *W. eutropha* (Han & Lee 2003). Therefore in the cultivations described in this study, production of 3-PHB was initiated by depletion of the nitrogen source (ammonium sulphate). Figure 2a, b–Figure 6a, b allow direct comparison of the time curves of inorganic nitrogen source with the corresponding concentration of residual biomass and 3-PHB. They also show that due to the lack of nitrogen source, the

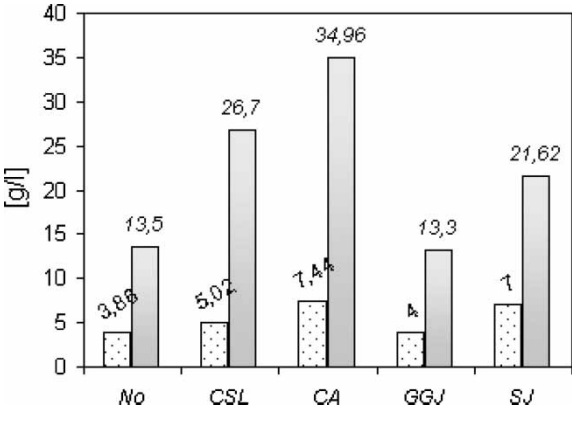

Figure 1. Final concentrations of protein and 3-PHB after cultivation of *W. eutropha* on minimal media with and without supplementation with complex additives. ⬚ Protein, ■ 3-PHB.

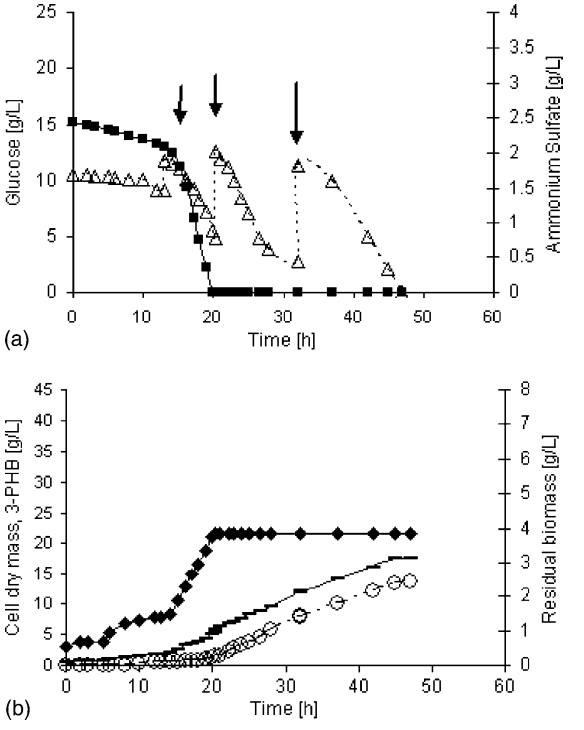

Figure 2. Fermentation patterns for (a) substrates (glucose, ammonium sulphate) and (b) products (residual biomass, cell dry mass, 3-PHB): *W. eutropha* on mineral medium (M). Arrows indicate the supplementation with glucose. —■— ammonium sulphate, · · ·△· · · glucose, · · ·○· · · 3-PHB, —▬— cell dry mass, —◆— residual biomass.

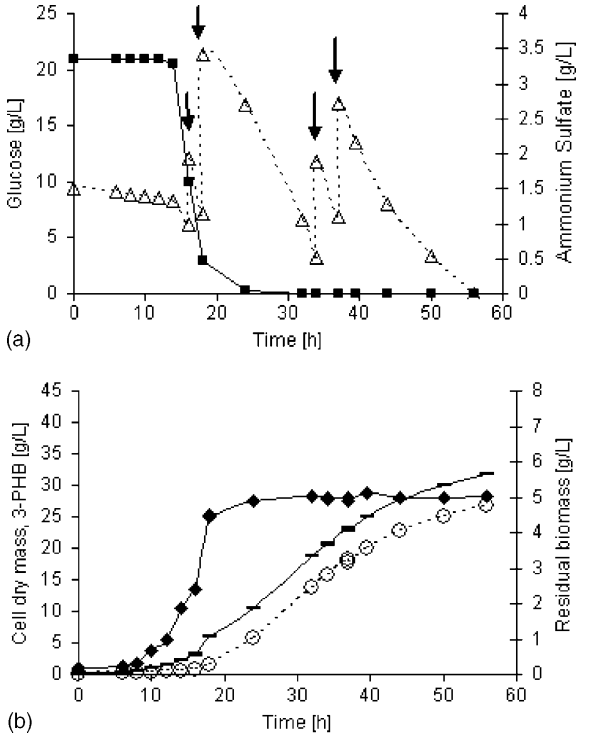

Figure 3. Fermentation patterns for (a) substrates (glucose, ammonium sulphate) and (b) products (residual biomass, cell dry mass, 3-PHB): *W. eutropha* on mineral medium supplemented with 5% (v/v) corn step liquor (CSL, saturated solution). Arrows indicate the supplementation with glucose. ammonium sulphate, glucose, 3-PHB, cell dry mass, residual biomass.

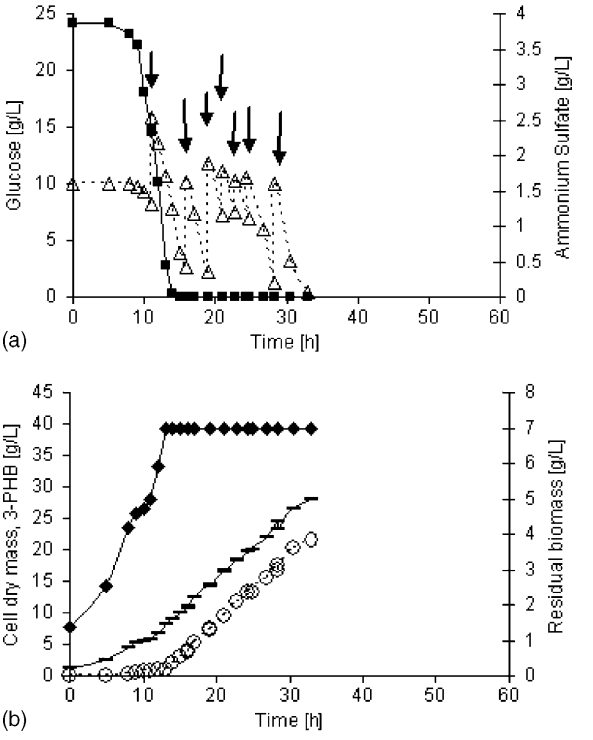

Figure 4. Fermentation patterns for (a) substrates (glucose, ammonium sulphate) and (b) products (residual biomass, cell dry mass, 3-PHB): *W. eutropha* on mineral medium supplemented with 5% (v/v) casamino acids (CA, saturated solution). Arrows indicate the supplementation with glucose. ammonium sulphate, glucose, 3-PHB, cell dry mass, residual biomass.

formation of protein (residual biomass) stops after ammonium sulphate becomes limited.

The highest volumetric productivities for 3-PHB were achieved when CA and SJ were added to the medium (0.619 and 0.653 $g\,L^{-1}\,h^{-1}$, see Table II), corresponding to more than the double the productivity without application of complex additives (i.e. 0.287 $g\,L^{-1}\,h^{-1}$). The 3-PHB productivity of 0.283 ($g\,L^{-1}\,h^{-1}$) in the experiment with GGJ indicates that SJ was a better supplement compared to GGJ. In order to make these productivities comparable at different concentrations of residual biomass, they were normalized (see Table II). This resulted in specific productivities q_p[18]. These values can be understood as average values for the entire process. They were comparably high for SJ and CSL (0.093 $g\,g^{-1}\,h^{-1}$ and 0.095 $g\,g^{-1}\,h^{-1}$, respectively), followed by CA (0.083 $g\,g^{-1}\,h^{-1}$). The lowest values were calculated for pure mineral medium M and GGJ (0.074 and 0.071 $g\,g^{-1}\,h^{-1}$). In addition, the yields of residual biomass $Y_{X/AS}$ (expressed as difference between total biomass and PHB per mass of inorganic nitrogen source) were similar in magnitude (range 1.24–1.47 $g\,g^{-1}$), except for biomass grown on medium supplemented with CA (2.51 $g\,g^{-1}$). This exception was caused by the higher content of convertible nitrogen in CA compared to the other sources.

Economic appraisal

The enhancements of the process described in the previous section can be visualized by calculations for an intended annual production of 1 ton of 3-PHB under the same conditions as in the laboratory fermentations. The results (Table III) show that the minimal medium as well as green grass juice needs more than double the bioreactor volume for an annual synthesis of 1 *t* of PHB compared with production based on supplementation with CA. Under the same conditions, the necessary reactor volume for production on CSL and SJ media will be $\approx$30 and 6% larger than the reactor volume for the CA medium. The annual production in a 100 m^3 (total volume) reactor (a common industrial scale) was also calculated (Table III), revealing that production on CSL and SJ media will be 76.6 and 93.5% of the annual production on CA medium, while production on minimal medium and GGJ medium would be only 44.3 and 44.1% of this value. This economic appraisal clearly shows that the easily

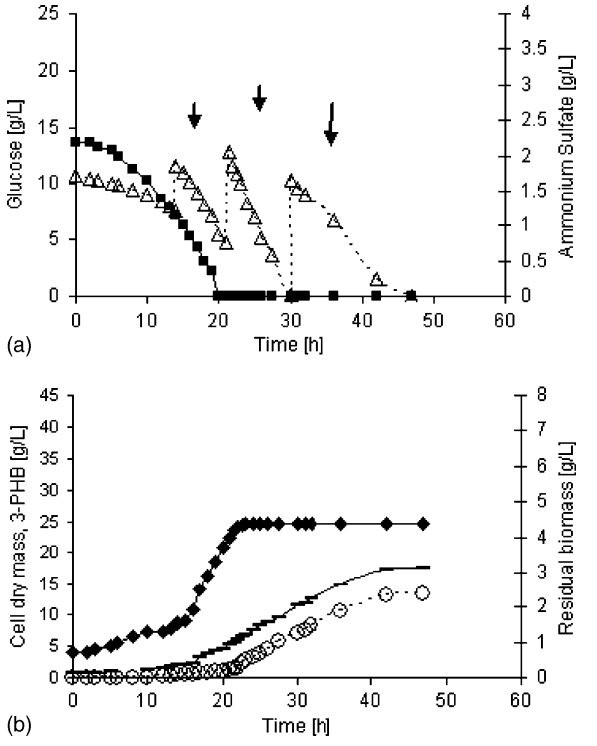

Figure 5. Fermentation patterns for (a) substrates (glucose, ammonium sulphate) and (b) products (residual biomass, cell dry mass, 3-PHB): *W. eutropha* on mineral medium supplemented with 5% (v/v) green grass juice (GGJ). Arrows indicate the supplementation with glucose. —■— ammonium sulphate, · · ·▵· · · glucose, · · ·⊙· · · 3-PHB, —▬— cell dry mass, —◆— residual biomass.

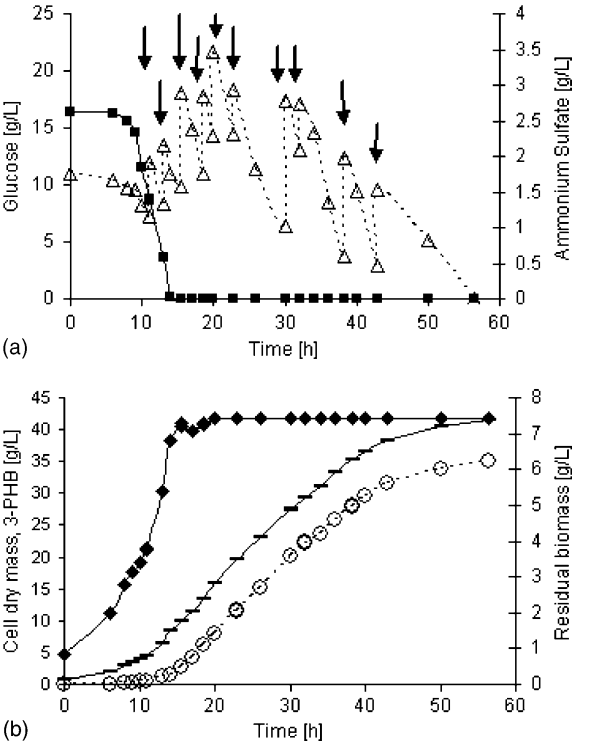

Figure 6. Fermentation patterns for (a) substrates (glucose, ammonium sulphate) and (b) products (residual biomass, cell dry mass, 3-PHB): *W. eutropha* on mineral medium supplemented with 5% (v/v) silage juice (SJ). Arrows indicate the supplementation with glucose. —■— ammonium sulphate, · · ·▵· · · glucose, · · ·⊙· · · 3-PHB, —▬— cell dry mass, —◆— residual biomass.

available and cheap SJ results in productivities very similar to more expensive CA.

Polymer characterization

Table IV shows the results for molecular mass analysis and thermodynamic parameters. The data were taken from polymer samples at the end of the five fermentations. Due to the fact that the complex additives only influence growth phase, but not the intracellular production of PHA, the results from the different fermentations presented in Table IV were very similar. The molecular mass data (M_w in the narrow range of 413,000–434,000, see Table IV) correspond well to those reported (Doi 1990a). Using the same isolation method as applied in this study, Taniguchi et al. obtained 3-PHB from *W. eutropha* of $M_w = 330{,}000$ (Taniguchi et al. 2003); Kahar also reported M_w values of 380,000 for 3-PHB from *W. eutropha* (Kahar et al. 2003). Polydispersity indices of the samples examined were quite high (around 4), but similar to published data (Kahar et al. 2003). For each of the investigated samples, the degree of crystallinity X_c (lowest value $64.6 \pm 0.4\%$ polymer from GGJ, highest value $66.3 \pm 2.7\%$ polymer from CA) as well as the high melting temperature T_m (180°C for each polymer) are in a typical range for pure 3-PHB homopolymer (Doi 1990a). The value for the glass transition point T_g (always at 6°C) is also typical for this type of

Table III. Calculated values for necessary bioreactor scales.

Additive	Annual production of 3-PHB in laboratory scale bioreactor [kg]	Annual production cycles n_c	Duration of production cycle t_c	Total reactor volume needed for annual production of 1 ton 3-PHB [L]	Annual production of 3-PHB in 100 m^3 scale [t]
M	3.4	145	59.5	665	150.5
CSL	5.9	127	68.0	384	260.0
CA	7.7	126	68.5	295	339.0
GGJ	3.4	146	59.0	667	149.8
SJ	7.2	192	45.1	314	318.7

Table IV. Molecular weight data and thermal characterization for 3-PHB at the end of the fermentations.[22]

Additive	Weight average molecular weight M_w	Polydispersity index P_i ($=M_w/M_n$[23])	Melting point[24] T_m [°C]	Glass transition[25] T_g [°C]	Cold crystallization[22] T_{cc} [°C]	Degree of crystallinity[26] X_c [%]
M	429000±17000	4.13±0.08	180±1	6 ±1	150±2	65.4±1.1
CA	431000±6000	4.20±0.34	180±1	6±0	149±1	66.3±2.7
CSL	413000±30000	4.30±0.20	180±1	6±0	150±1	66.2±1.4
GGJ	432000±52000	4.02±0.80	180±0	6±1	151±1	64.6±0.4
SJ	434000±27000	4.01±0.64	180±1	6±0	151±2	65.9±1.4

polyester (Abe & Doi 2001). Because of the high melting points, the investigated polymers might be susceptible to thermal degradation during melt processing due to pyrolysis of aliphatic secondary esters of the repeating units. This problem can be overcome by co-feeding of propionic acid (Taniguchi et al. 2003) or valeric acid (Doi 1990b). These compounds act as precursors for formation of 3-hydroxyvalerate units (3-HV) that significantly lower the products melting point. The resulting material should be more suitable for thermal processing (Doi 1990b).

Conclusion

In this study, alternative additives from agriculture, green grass juice (GGJ) and silage juice (SJ), were supplemented to common cultivation medium for growth and poly(3-hydroxybutyrate) production by *W. eutropha*.

In laboratory scale bioreactor cultivations, SJ turned out to be a very promising co-substrate with respect to price, product quantity and moderate positive impact on growth (μ_{max} similar to CA medium but significantly lower than in the CSL medium, similar PHB productivity and formation of residual biomass as for the CA medium). All investigated cultivation parameters show that SJ has more positive impacts on the process than GGJ.

The added complex sources were applied for improvement of the growth phase, and therefore did not significantly influence the specific product formation rates nor the polymer properties. The positive impact of the additives resulted from higher concentrations of residual biomass achieved by addition of SJ, CA and CSL, increasing the volumetric productivity, but not the specific productivity of PHA.

The economic attractiveness of SJ, which is available in large quantities in central Europe, depends on the price difference between CA or CSL and SJ. CSL, for example, is produced in many countries, but, if needed in central Europe, has to be imported. Calculations for economic appraisal done in this study clearly demonstrate that, on an industrial scale, SJ gives productivities very similar to the more expensive CA and therefore can constitute a viable alternative.

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Notes

1 SL6: Trace elements solution (per liter): $ZnSO_4$. $7H_2O$, 100 mg; H_3BO_3, 300 mg; $CoCl_2$. 6 H_2O, 200 mg; $CuSO_4$, 6 mg; $NiCl_2$. $6H_2O$, 20 mg; Na_2MoO_4. $2H_2O$, 30 mg; $MnCl_2$. 2 H_2O, 25 mg.
2 Calculated from a known content of amino nitrogen of 9.4% (w/w)
3 Arrows indicate the refeeding with glucose solution (50% w/w)
4 Production cycles per year
5 Production hours per year
6 Duration of production cycle plus 12 h (typical value for preparation and follow-up treatment of the bioreactor)
7 Annual production of 3-PHB in 100 m bioreactor [t]
8 Bioreactor scale
9 Final concentration of 3-PHB (depending on experiment)
10 Factor fermentation broth – total volume of bioreactor
11 Factor for calculation of tons
12 Intended annual production of 3-PHB
13 Necessary bioreactor volume for annual production of 1 ton 3-PHB
14 Factor for calculation of liters
15 Annual production of 3-PHB on a laboratory scale
16 Laboratory bioreactor scale
17 Conversion factor t → kg
18 Volumetric productivity: $r_p = (\triangle$ PHB [g L^{-1}]/$\triangle$ t [h])
19 Specific productivity: $q_p = (r_p$ [g L^{-1} h]/residual biomass [g L^{-1}])
20 Value represents sum of all sugars present
21 Minimal medium
22 Standard deviations refer to triplicate determination of all parameters
23 M_n = Number average molecular weight
24 Reported from the first DSC–heating scan
25 Reported from the first DSC–heating scan
26 Degree of crystallinity.

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