

# Conversion of Solvent Evaporation Residues from the AB- (Acetone – Butanol) Bioprocess into Bacterial Cells Accumulating Thermoplastic Polyesters

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## Abstract

**In a bioconversion study based on utilisation of by-products from the AB- (acetone – butanol) bioprocess a new isolated Gram-negative solvent tolerant bacterium was used to convert the AB process residue after removal of the major part of the solvents. The bacterium identified as a representative of the genus *Alcaligenes* (designated as *Alcaligenes* sp. G) was capable of growth up to optical densities ranging from 8 to 20 and simultaneously of polyhydroxyalkanoate-(PHA)-accumulation up to 40% per dry weight. A standardised medium based on AB by-products containing 7 g/l of butyrate and 5 g/l of acetate at pH 7.5 was used in our studies for bioconversion into PHAs. Concentrations of 1-butanol, which is known for its membrane damaging properties in microorganisms, were tolerated in the AB by-products medium up to 4 g/l without significant inhibition of cellular growth. No inhibition of growth was observed, when the medium was adjusted to 40 g/l butyrate. Due to the toxicity of the remaining 1-butanol maintenance of sterility is of no high priority during the process. The use of acetate and butyrate from an AB process is expected to provide a higher return-on-investment than the combustion of biogas to help meet energy demands.**

## Introduction

Poly- $\beta$ -hydroxyalkanoates (PHAs) are thermoplastic, biodegradable and biocompatible polyesters produced by strains belonging to various genera of *Eubacteria* (Madison and Huisman, 1999) and by some *Archaea* (Fernandez-Castillo *et al.*, 1986; Lillo and Rodriguez-Valera, 1990). PHAs are suitable for standard techniques in polymer technology like blow-film processing and injection moulding. Due to the brittleness and other inferior physicochemical characteristics of pure crystalline PHB (poly- $\beta$ -hydroxybutyrate), different random copolymers

have been developed to improve the physico-chemical properties (Braunegg *et al.*, 1998). Copolymers are produced by feeding of specific co-substrates presenting modified precursor substrates for the PHA synthases. Most of the organisms described so far incorporate poly- $\beta$ -,  $\gamma$ - and  $\delta$ -hydroxyalkanoates with 3 to 5 C-atoms into the homo- and copolymers, when specific precursor substrates are fed (Braunegg *et al.*, 1998; Madison and Huisman, 1999; Steinbüchel and Fürchtenbusch, 1998). A copolymer of poly- $\beta$ -hydroxybutyrate and poly- $\beta$ -hydroxyvalerate has been marketed by ICI for the production of biodegradable plastics for environmentally friendly packing materials under the trade name Biopol (Byrom, 1990).

Granular PHAs are important storage compounds in microbial ecosystems promoting bacterial growth and metabolism, when the microenvironment is depleted of external substrates. PHA accumulation presents one of the next ecological steps after organic acid production in biological degradation processes. The cyclic nature of PHA metabolism in environment is indicated by the fact that several PHA-accumulating strains, for instance members of the species *A. faecalis*, are known, beside harbouring an intracellular depolymerase system, also to excrete an extracellular depolymerase capable of scavenging crystalline poly- $\beta$ -hydroxybutyrate (PHB) from remains of dead bacterial cells (Tanio *et al.*, 1982; Jendrossek *et al.*, 1996; Kita *et al.*, 1999).

Albeit many bacteria are capable to accumulate poly- $\beta$ -hydroxyalkanoates during growth in the presence of excess carbon sources with low content of nitrogen; the quest for optimised substrates in terms of price of the excess carbon source, conversion rate, yield of PHAs, growth rate and influence on the composition of the polyesters is still ongoing (Choi and Lee, 1999). For some substrates investigated, like organic acids or short chained alcohols, their intrinsic toxicity provides a major obstacle for the set-up of any technical process based on that kind of precursor. For a long period no experiments have been published comprising the use of short chained alkanolic acids for PHA accumulation in biotechnological processes. Most set-ups are based on the addition of sugars to complex artificial or technical media. Basic models comprise the use of *Ralstonia eutropha* (Rusendi and Sheppard, 1995), *Alcaligenes latus* (Braunegg *et al.* 1995; Wang and Lee, 1997a), *Azotobacter* sp. (Page *et al.*, 1995; Kim and Chang, 1998) or recombinant *E. coli* cells harbouring enzymes of the *R. eutropha* pathway on extrachromosomal elements (Kalousek and Lubitz, 1995; Wang and Lee, 1997b; Liu *et al.*, 1998; Choi *et al.*, 1998). The fermentation of medium chain length poly- $\beta$ -hydroxyalkanoates with 6 to 14 C-atoms from alkanes by *Pseudomonas* strains has been investigated by Preusting *et al.* (1992) and Schmid *et al.* (1998).

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Due to economically prohibitive costs of pure substrates, cheap carbon sources from agricultural and biotechnological by-products are of increasing interest. Cheap technical sources for PHA-production have been tested by Tan *et al.* (1997) as well as by Fürchtenbusch and Steinbüchel (1999), comprising saponified palm kernel oil and liquefaction products from low-rank coal, respectively. Low cost carbon sources might be present in waste waters from other biotechnological processes. Our basic model is the acetone butanol - (AB) fermentation, which is currently developed into a continuous process for production of 1-butanol suitable for renewable chemicals by *Clostridium beijerinckii* (Gapes *et al.*, 1996; Nimcevic and Gapes, 1999). After removal of the solvents from the fermentation broth by various techniques, the waste water typically containing butyric acid (up to 7 g/l) acetic acid (up to 5 g/l), the remainder of butanol after product removal (up to 3 g/l), partly lysed clostridial cells and occasionally also traces of formic acid and lactic acid remains. Recovery of these acids is not economic because of the low concentrations. It is also possible that in a particular AB-process run the undesired occurrence of the "acid crash" yields butyric acid for the main product, as reported by Maddox *et al.* (2000).

On the other hand the production costs of biopolymers are strongly affected by the substrates selected for cellular growth and accumulation of the polymers during the specific process (Choi and Lee, 1999). The use of acetate and butyrate from an AB-process is expected to provide a higher return-on-investment than the combustion of biogas to help meet energy demands (Gapes and Baumgartner, 2000). Since organic acids are utilised by PHA-producing bacteria, we tried to convert the residues into potentially useful polyhydroxyalkanoic acids. Our aim is to achieve maximum conversion into PHAs and to aerobically reduce the overall

biological waste load of the AB-process simultaneously. Ideally the combined process should yield no significant amount of organic waste. In the present study we describe a novel approach to treat AB-bioprocess residues after solvent evaporation procedure by bioconversion of the remainder into PHA accumulating cells using a solvent tolerant bacterium isolated recently.

## Results and Discussion

### Screening of Candidate Strains Accumulating Polyhydroxyalkanoates on AB Residues

For screening of possible candidate strains for bioconversion of AB-process residues after product removal, the acidic AB-process residues of approximately pH 4.5 were neutralised by addition of NaOH before sterilisation. Inhibition of cellular growth due to the remainants of 1-butanol proved a major problem for wild isolates and industrial strains screened for growth and accumulation properties on the pH-neutralised fermentation broth. Out of many strains tested in our screening program, one Gram-negative, motile, catalase and oxidase positive novel isolate (designated as strain *Alcaligenes* sp. G) was able to exert rapid growth in the presence of 4 g/l 1-butanol. This strain was selected for further investigations; the bacterium was identified using API 20NE (bioMérieux) according to the manufacturer's instructions and by standard tests for the characterisation of a bacterium as described by Smibert and Krieg (1994). On the basis of the results obtained, the isolated organism was recognised as a representative of the genus *Alcaligenes* most closely related to *Alcaligenes faecalis*. A detailed taxonomic description of this bacterium using the polyphasic approach will be provided in a separate publication (Schroll *et al.*, in preparation).

### Physiological Characteristics of *Alcaligenes* Strains

To evaluate the conditions, which allow bioconversion of the AB-process by-products the physiological characteristics of *Alcaligenes* sp. G have been investigated and compared with the reference strain *A. faecalis* ATCC 8750<sup>T</sup>. A pH of 7.5 turned out to be optimal for strain *Alcaligenes* sp. G for growth on AB residue. Growth temperature was set at 28°C, which is close to 30°C the standard temperature for growth of *Alcaligenes faecalis* strains (Osborne and Ehrlich, 1976; Phillips and Taylor, 1976).

In shake flask experiments strain *Alcaligenes* sp. G revealed arrest of growth at 8 g/l butanol at pH 7.5 (Figure 1) indicating relatively high tolerance to solvents. This characteristic was unique among the isolates screened, albeit *A. faecalis* ATCC 8750<sup>T</sup> revealed only slightly inferior growth characteristics on the AB residue. However, *Alcaligenes* sp. G displayed lower tolerance to sodium chloride than the type strain does. After 7 days of incubation at 28°C no lawn was detectable at 20 g/l NaCl, no colonies of strain *Alcaligenes* sp. G were detected at 70 g/l NaCl, whereas no growth of *A. faecalis* ATCC 8750<sup>T</sup> was detectable at 90 g/l NaCl. These observations indicate the occurrence of adaptive clones with higher osmotolerance in the population of *Alcaligenes* sp. G. Primarily that indicates that osmotolerance might not be the crucial requirement for growth in the medium containing AB-process residues.

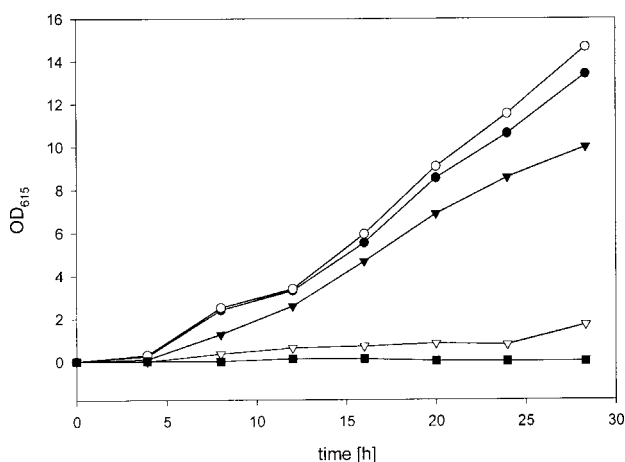


Figure 1. Growth inhibition characteristics of strain *Alcaligenes* sp. G grown in the AB residue medium containing different concentrations of 1-butanol. Following concentrations of 1-butanol were added to the AB medium in g/l: 0.0 (filled circles), 2.0 (open circles), 4.0 (filled triangles), 6.0 (open triangles), 8.0 (filled squares). Arrest of growth occurred above 6 g/l of 1-butanol in the AB residue, indicating high tolerance of the organism. The data shown represent the median of three parallel experiments in shake flasks. It is likely, that due to the complex composition of the broth providing a variety of different nutrients polyphasic growth is exerted, resulting in the linear growth curves.

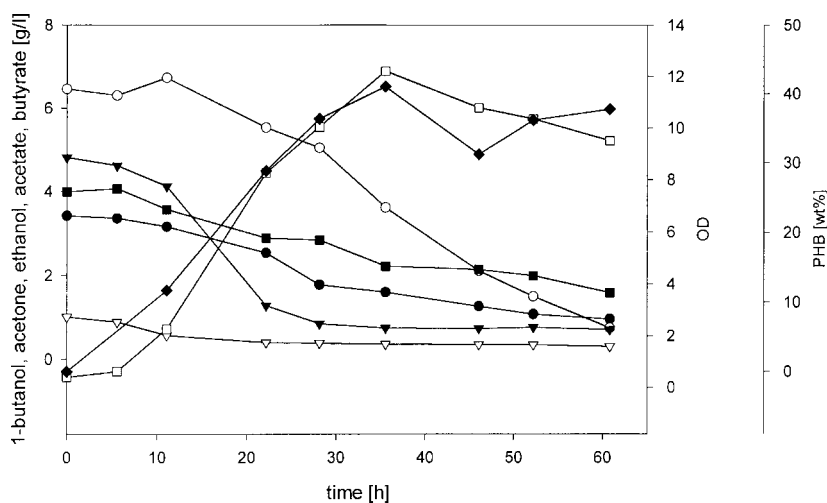


Figure 2. Catabolism of standardised by-products from the AB-process after solvent removal and accumulation of PHB. Organic compounds detected by gas chromatography were 1-butanol (filled circles), acetone (open triangles), ethanol (filled squares), acetate (filled triangles), butyrate (open circles), and PHB (filled diamonds). The optical cell density (OD) was determined at 615 nm (open squares). Preferred utilisation of acetate indicates biphasic utilisation kinetics. Ethanol, albeit being utilised for a single carbon source was metabolised at a relatively slow rate. The other solvents contained in the broth were evaporated of the broth. The PHB-content (% per dry-weight) increases during the active growth phase slightly but continuously. After depletion of nutrients, degradation of intracellular PHB is indicated. The slight decrease at the end of the bioconversion process might be due to scavenging from lysed cells, which is also backed by the graph of the optical cell density.

The primary requirement might be the tolerance to solvents and acids by the cell membrane and envelope, especially to 1-butanol, which is known to damage the cellular membranes in organisms. Butyrate did not inhibit growth of the *Alcaligenes* sp. G even when its concentration was increased up to 40 g/l at pH 7.5, similar to *A. faecalis* ATCC 8750<sup>T</sup> (data not shown). Both strains investigated present a chance to reevaluate a process based on the utilisation of organic acids.

#### Substrate Utilisation by Strain *Alcaligenes* sp. G

The substrate utilisation characteristics of strain *Alcaligenes* sp. G have been investigated to evaluate which constituents of the AB remainder are utilised for growth or conversion into PHAs and for evaluation of different precursors for the formation of PHA heteropolymers.

The utilisation of substrates for growth was tested on the mineral medium M9 with addition of 3 g/l of the respective substrate. The organism revealed growth on acetate, propionate, butyrate, valerate, heptanoate, hydroxybutyric acid, butyrolactone, (L)-lactate, citrate and ethanol as single carbon source. Significant accumulation of PHAs in mineral medium was not detectable neither by staining nor by gas chromatographic analysis, probably being a function of the low substrate concentration fed. In contrast no growth was observed when formic acid, 1,4 butandiol, 2,3 butandiol, methanol, 1-butanol, 2-butanol, 1-hexanol, glycerine, acetone or tartaric acid were used for single carbon source. Metabolic tests performed with the Biolog GN MicroPlate system revealed utilisation of tensides, non-sugar acids, several amino acids and  $\alpha$ -,  $\beta$ - and  $\gamma$ -hydroxybutyric acid (Schroll *et al.*, in preparation). With respect to PHA production, substrates are classified into growth promoting and accumulating substrates, growth promoting and non-accumulating substrates and non-growth promoting and non accumulating substrates (Lenz *et al.*, 1992).

When odd chained alkanooates were added to the standardised AB broth (5 g/l of acetate and 7 g/l of butyrate) accumulation of PHA heteropolyesters containing  $\beta$ -hydroxyvalerate (HV) was achievable in *Alcaligenes* sp. G. Accumulation of 52% of PHA per dry weight containing 37% of HV after growth for 3 d was observed when 3 g/l of

valerate were added to the medium. This indicates the possible formation of PHB/HV copolyesters in accordance to data derived by use of *A. faecalis* ATCC 8750<sup>T</sup>, when specific precursors are co-fed.

In biochemical testing using miniaturised commercial test systems (API 20NE, API 50CH, Biolog GN Microplate) as well as in flask culture experiments strain *Alcaligenes* sp. G did not utilise any sugar compound tested. Furthermore no enzymatic activity like pectinase or galacturonidase activity has been detected in *Alcaligenes* sp. G (Schroll *et al.*, in preparation) rendering it dependant on pre-treatment of the agricultural waste dedicated to serve as a substrate. By the conventional hydrolysis of casein no proteolytic activity was detected. To evaluate if complete conversion of the AB waste is feasible, also harvested clostridial cells and spores were added to the M9 medium; they were apparently not scavenged for growth by *Alcaligenes* sp. G.

In comparison of the cellular yields achievable, the still nutrient rich AB residue allows growth to higher optical densities at a higher rate than the classical standard rich medium, - Luria-Bertani medium -, does. One reason might be the non-utilisation of the sugars contributed by the yeast extract in the Luria-Bertani medium resulting in growth up to OD 3.1 in shake flask experiments instead of up to OD 20 in the AB residue. On the other hand it might be as well that remainants from the semisynthetic medium used for growth of *Clostridium beijerinckii* stimulate cell mass formation of *Alcaligenes* species. Metabolism of short chained and monomeric dissolved organic material with exception of sugars, especially of derivatives of the aliphatic fatty acid line, - and of high molecular weight PHB -, is likely to be the ecological role of *A. faecalis* and related strains in the environment. The relatively high osmotolerance observed for *Alcaligenes* sp. G and *A. faecalis* ATCC 8750<sup>T</sup> is of advantage when growing on higher concentrations of those kinds of substrates.

#### Airlift Bioreactor: Growth and PHA-Accumulation

In bioconversion experiments using strain *Alcaligenes* sp. G, it was observed that the organic acids and the rest of the nutrients were converted by the isolate to a major extent, whereas the remainants of 1-butanol and acetone

after solvent removal were not metabolised but evaporated from the broth.

The time course of a typical process run is shown in Figure 2. The graph indicates biphasic utilisation of acetate and butyrate, metabolising the acetate primarily. Utilisation of ethanol played no role in the nutrient rich environment. Conversion of AB-process by-products into cell mass of strain *Alcaligenes* sp. G containing up to 41% of PHB per dry weight yielding a maximal optical density of 12 (ca. 4.2 g/l of dry matter) at the end of the active growth phase is indicated. At this point the yield of PHB was 1.7 g/l after growth for 35 h. Approximately 25% of the organic acids were converted into PHB and 37.5% into cell mass, when other dissolved nutrients are neglected in this estimation. The slight decrease and subsequent increase in optical density when the active growth phase was finished, is likely to be caused by the depletion of substrates inducing intracellular degradation of PHA granules and by lysis of a subpopulation of cells.

*Alcaligenes* sp. G was capable of accumulating PHB at a relatively constant content of between 12 and 40% per dry weight, depending on the external conditions set, slightly increasing during its active growth phase, a characteristic, which is similar to observations from other bioprocesses using *Alcaligenes latus* strains. An explanation for significant accumulation of PHA in bacteria during the active growth phase was given by Genser *et al.* (1998), that NADH oxidase activity was absent in *A. latus* in contrast to strains yielding low PHA during balanced growth. In contrast recombinant *E. coli* cells expressing the *R. eutropha* or *A. latus* pathway accumulated only up to 3% of PHA during balanced growth in their experiments. It is likely that absence of NADH oxidase is also a characteristic of *A. faecalis* and strains of other species closely related.

In several other bioreactor experiments, final cell densities over OD 20 (ca. 7 g/l of dry matter) were achievable, yielding final accumulation of 12% to 38% of PHB per dry weight. A higher dissolved oxygen concentration seems to promote cellular growth instead of PHA accumulation in our system, which indicates some influence of nutrient limitation on the PHA accumulation characteristics of *Alcaligenes* sp. G. The alkalisising properties of the *Alcaligenes* sp. G, a general characteristic of *Alcaligenes* strains, seemed not to be in favour of the polymer yield, when in a first experiment the pH was not adjusted automatically (data not shown). The pH in the following bioreactor experiments was therefore controlled to a constant value of 7.5, which was determined to be optimal in the preceding flask experiments.

To evaluate the possibility to run the bioconversion of the AB remainder into PHAs under non-sterile conditions, several experiments were performed without sterilisation of the medium, using only a heat treatment step (100°C, 1 h). No contaminants were observed during the process run, only after 1-butanol had been evaporated from the broth completely, contaminations by *Bacillus* spp. were sporadically observed. It can be supposed that the growth conditions, especially the rest of the 1-butanol contained in the broth, inhibit contaminating micro-organisms in this process.

To investigate the possibility of the conversion of AB-process residues fermented on natural substrates, PHA formation on AB residue from potato medium (Nimcevic *et*

*al.*, 1998; Gutierrez *et al.*, 1998) was investigated. *Alcaligenes* sp. G grew to a lower optical density of OD 9 after growth for 2 d in this unbuffered but neutralised medium (pH 7.5). The PHB yield on this medium was estimated to be comparable to the yield when AB residue derived from clostridial metabolism on the semisynthetic medium was used. The fact has to be stressed, that the performance of the PHA-polymer forming bioconversion process is strongly dependent on the preceding AB-process.

## Conclusions

The PHA yield and productivity, parameters which adversely affect the process costs, observed in the experiments discussed above were somewhat inferior in comparison to other processes published for production of a pure polymer (Choi and Lee, 1999). It has to be taken into account that a low-value by-product was used as a substrate for conversion, and the process was exerted by a wild isolate, both factors limiting the performance. On the AB residue the performance of the new strain *Alcaligenes* sp. G in respect of growth rate and final cell density was better than that of the type strain *A. faecalis* ATCC 8750<sup>T</sup>.

However, due to the low concentrations of organic acids (acetate and butyrate) in the AB residue, the substrates were already metabolised at the start of the stationary phase, hence accumulation over the yield per dry weight achieved at the end of the active growth phase was not possible. Therefore, the final achievable yield of PHAs was limited by the depletion of organic acids in our system. Higher concentrations of acetate and butyrate seem to be desirable in the process, whereas sterility of the waste material after adjusting the pH value is not of high priority due to inhibition of cellular growth by 1-butanol. On the other hand the occurrence of significant PHA accumulation in the active growth phase is of interest. This property might be advantageous for future alternative continuous production facilities processing agricultural waste (Gutierrez *et al.*, 1998).

Based on the results obtained, we suggest a combined process, which starts with agricultural waste material *e.g.* potato waste (Gutierrez *et al.*, 1998), by fermenting the sugars to solvents and organic acids, the latter for conversion into PHA, finally yielding biochemicals and biodegradable polymer by simultaneous reduction of the overall amount of waste. Anyhow, a process based on this particular bioconversion of PHAs from AB waste by standard bioreactor set-ups is not likely to be economically successful, so further technical improvements will be required.

## Perspectives

Albeit many attempts to produce PHAs for environmentally compatible and totally biodegradable materials have been published and patented, no process allowing a real economic breakthrough has been presented to date (Choi and Lee, 1999), even the potential for use in medical systems (for many: Korsatko *et al.*, 1983) due to the biocompatibility properties of PHAs within living tissue remains unexploited. It has been also shown that impure PHAs can be used for both a matrix and a carbon source

supporting nitrate reducing bacterial strains for removal of nitrate from water (Langjahr and Süßmuth, 1998). Their bioreactor may be used without high technical requirements for process control allowing set-ups in rural areas. It seems technically possible to set-up a chain of bioprocesses, which start with unmarketable agricultural by-products and end with biochemicals, biopolymers and water of improved quality, if the respective conversion rates can be increased.

In general the economical usefulness of polymer accumulating processes is provided, when the substrate consists of by-products derived from other processes contributing negative costs to the specific process used for the production of PHA-polymers. Substrate metabolism to PHA at a sufficient conversion rate and formation of cell mass at a high rate yielding high cell density is desired. In contrast complex waste substrates contain lots of growth inhibiting substances, and their usefulness is limited by a pre-given C/N ratio. Another limitation to our binary process proposed yielding biochemicals and PHA is, that the concentration of the organic acids required for accumulation substrate is not sufficient for accumulation of PHA over 45% of dry weight maximum when grown on the AB by-products. The organic acids contribute to formation of (D-) $\beta$ -OH-butyrylCoA to a major degree, which is used for precursor substrate by the PHA-synthases. Usually a high PHA content per dry weight is desired because the substrate, which is the major cost factor in model systems, appears to be wasted simultaneously increasing the costs of cellular breakage (Choi and Lee, 1999). In our process the still low yield could be overcome by addition of another growth substrate or increasing the concentration of organic acids fed.

On the other hand bacteria like *Alcaligenes* strains, which accumulate significant amounts of PHAs during the active growth phase, also harbour an intracellular PHB-depolymerase system for utilisation of granules under starvation conditions. Mutant strains characterised by deletions in these genes are likely to improve the yield and productivity dramatically. This strategy should allow for high yield continuous processes.

## Experimental Procedures

### Strains and Chemicals

The strain *Alcaligenes* sp. G was originally isolated from the surface layer of an eutrophic garden pond in Lower Austria. The reference strain *A. faecalis* ATCC 8750<sup>T</sup> (= DSM 30030<sup>T</sup>) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) GmbH, Braunschweig, Germany and the strain *Clostridium beijerinckii* NRRL B592 was obtained from the Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, Illinois. *Clostridium beijerinckii* NRRL B592 was stored according to Nimcevic *et al.* (1998). *Alcaligenes* sp. G and *A. faecalis* ATCC 8750<sup>T</sup> were stored at -70°C in Luria-Bertani broth containing 15% glycerol. Inocula for all experiments were taken from these glycerol stocks, streaked onto Luria-Bertani agar (Atlas, 1993), and incubated for 48 h at 28°C. For preparation of Luria-Bertani media peptone from casein extract (Difco), instead of peptone from meat was used. Acetic, valeric, heptanoic acid were purchased from Merck, propionic acid from Sigma, all other substrates and chemicals from Fluka. All chemicals were p. A. grade.

### Preparation of the AB By-products used for Bioconversion Studies

The AB-process residues were prepared according to the method of Gapes *et al.* (1996) by growth of *Clostridium beijerinckii* NRRL B592 in a two-stage chemostat with on-line solvent removal containing a semisynthetic complex mixture with 60 g/l of glucose being the main carbon source. In addition to the main carbon source (glucose) the medium used for bioprocessing of AB by *Clostridium beijerinckii* NRRL B592 was composed of (g/l in distilled water): 5.0 yeast extract, 1.0 K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O, 1.0 KH<sub>2</sub>PO<sub>4</sub>,

1.0 MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5 FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.01 *p*-aminobenzoic acid, 3.0 ammonium acetate. After AB fermentation and application of the solvent removal processes the remainder was centrifuged for 10 min at 4100 rpm (Sigma 4K15). The supernatant was used for bioprocessing of PHA accumulating cells. After centrifugation approximately 0.25 g/l of solid residues were contained in the supernatant. To provide repeatable experiments, due to normal deviations in concentrations of by-products after the evaporation process, organic acids were added to assure the standard concentration of butyrate 7 g/l and 5 g/l of acetate. After adjustment of the pH to 7.5 the broth was sterilised without any further concentrating step applied. Non-sterile experiments were performed in an identical manner except that heat treatment (100°C, 1 h) of the medium was applied instead of autoclaving.

AB-process residue based on potato medium was fermented according to Nimcevic *et al.* (1998), and processed as described above.

### Shake Flask Experiments

Cultures dedicated to substrate utilisation and PHA pattern analysis were grown in 100 ml Erlenmeyer flasks sealed by cellulose caps, equipped with a magnetic stirrer containing 50 ml of the medium and of the respective substrate at 28°C and 600 rpm for 3 up to 7 days. The mineral medium M9 was prepared according to Maniatis *et al.* (1982), for provision of trace elements solution SL-6 (DSM catalogue) was added. Propionate, valerate and heptanoate were utilised for production of copolyesters containing 3-OH valerate. The substrates were added to the broth at the concentration of 3 g/l and neutralised to pH 7.5 in the AB residue, or to pH 7.0 in M9 experiments, respectively, before autoclaving for 20 min at 121°C. Clostridial cells and spores were suspended in M9 medium after being washed twice with saline and autoclaving. All experiments were performed in triplicate.

### Esterification of PHAs and Gas Chromatographic Analyses

For determination of the PHA content lyophilized cell mass derived from a sample of 10 ml washed with distilled water prior to freezing was prepared applying whole cell methanolysis according to Brauneegg *et al.* (1978), with addition of benzoic acid for internal standard. For separation of the methyl esters the GC system was used as is described in Schroll *et al.* (1996). Samples suspected of containing  $\gamma$ -hydroxybutyrate incorporated into the polymers were analysed by Prof. Brauneegg, TU Graz.

The gas chromatographic analysis of ethanol, acetate, butyrate and 1-butanol produced by clostridia was performed as described previously (Gapes *et al.*, 1996).

### Bioreactor Experiments

For the production of PHB at bioreactor scale a 2.5 l airlift bioreactor system was used. The operating temperature was set at 28°C. The system was equipped with online monitoring of pH and redox potential. For measurement of the pH an electrode 405-DPAS-SC-K8S/225 (Mettler Toledo) was used, the redox potential was measured by a redox electrode Pt4865-20-K9 (Ingold) monitored by a Redox/pH controller Knick Process unit 73 pH. In the reactor the pH was adjusted to 7.5  $\pm$  0.1 throughout the process run by automatic addition of sulphuric acid (2 N). Protection from airborne contaminations was obtained by application of air membrane filters to the air flow. An airflow of 1.5 l/min was provided. During the process the oxygen saturation was monitored by a dissolved oxygen electrode (Mettler Toledo) controlled by a 4500 pO<sub>2</sub> transmitter (Mettler Toledo). The data processing measured online was facilitated by the LIME Process Control software (Advanced Technical Software). After autoclaving the bioreactor (121°C, 45 min) was inoculated with 50 ml from a shake flask culture of strain *Alcaligenes* sp. G grown over night at 28°C in AB by-products medium. Samples were drawn periodically to determine the decrease of the content of solvents by gas chromatography, to estimate the content of PHB and the optical density. Prior to harvesting at single points during the process the pH was double-checked by measurement with an external pH electrode pH Meter MP 220 (Mettler Toledo).

The optical density (OD) was measured at 615 nm by a CE2343D Digital Grating Photometer, Alpha series, CECIL. For the qualitative detection of PHA-granules cells were stained using Sudan black as described by Burdon (1946) and examined by bright field light microscopy (Leica DMLS) at a magnification of 1000 x. Sterility and identity of the strain was determined by light microscopy and streaking out on LB agar.

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