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Development of a feeding strategy for high cell and PHA density fed-batch fermentation of *Ralstonia eutropha* H16 from organic acids and their salts

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ABSTRACT

The utilization of inexpensive carbon sources, including waste streams, for production of value added products has been explored for over two decades. Palm oil mill effluent (POME), a waste water stream from the palm oil industry, can be used to produce organic acids and their salts by anaerobic digestion. These organic acids are suitable as the main carbon source for growth and production of polyhydroxyalkanoates (PHAs) by organisms like *Ralstonia eutropha*. The presence of propionate/propionic acid results in the biosynthesis of the copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(HB-*co*-HV)], which is desired for its favourable thermal and mechanical properties compared to the polyhydroxybutyrate (PHB) homopolymer. A specially-designed fed-batch strategy is necessary to decrease the toxic effects of organic acids in cultures of *R. eutropha*. A pH-stat fed-batch culture technique, using organic acids as the main carbon source, was adopted in combination with an additional pO_2-dependent feed for delivering organic acid salts. The developed strategy is highly reproducible and shows a high productivity of >2 g PHA/L/h. After 44 h of fermentation, 112.4 ± 2.3 g/L cell dry weight (CDW) with 83.3 ± 1.1% P(HB-*co*-HV)/CDW containing 5.6 ± 0.4 mol% 3-hydroxyvalerate (3HV) were achieved. The described productivity, CDW and P(HB-*co*-HV)/CDW values are by far the highest reported results for *R. eutropha* cultivated on mixed organic acids as the main carbon source.

1. Introduction

The public desire for alternatives to petroleum-based products in daily life, as well as the necessity of a more environmentally friendly lifestyle, has had a major impact towards research of microbially produced polyhydroxyalkanoate (PHA). This biopolymer confers survival advantages for microorganisms during nutrient limitation, enhances stress tolerance [1,2], and is a potential source for a biodegradable, biocompatible thermoplastic with multiple potential applications [3]. Because of its hydrophobicity and biodegradability, which depends on the composition and the naturally occurring microbial species in the environment able to digest the specific PHA [4], applications in packaging have been considered [5]. Biocompatibility and biodegradability also suggest various possible applications as a matrix for controlled release of drugs, hormones, herbicides, insecticides, flavours and fragrances in medicine, pharmacy, agriculture and food industry [6]. Applications in the field of medicine were recently reviewed and discussed by Brigham and Sinskey [7].

To replace petroleum based plastics with biodegradable PHAs, one major challenge must be overcome: PHA production involves high production and recovery costs in comparison with chemically

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synthesized plastics [6]. PHA production from various carbon sources, including waste streams, by various microorganisms has been attempted at various scales and is being considered as an industrial process [8].

One of the most promising value added waste treatment methods is the conversion of agro-industrial waste streams to organic acids, using a pH-controlled anaerobic treatment and recovery of the organic acids, as described by different groups [9–11]. The organic acids can be used subsequently as carbon and energy substrates in microbial culture to produce value added, bio-based product materials.

In order to decrease PHA production costs, the replacement of refined, higher-cost carbon sources with organic acids recovered from anaerobically treated waste streams involves major changes in well-established processes. Due to highly toxic effects on cell physiology, short chain volatile fatty acid (VFA) concentrations in the culture medium must be lower than 1 g/L (acetic acid) and 0.5 g/L (propionic or butyric acids) at any point in the process [12,13].

Ralstonia eutropha, also known as *Cupriavidus necator*, was first isolated near Göttingen, Germany [14–16] and is a Gram-negative betaproteobacterium that can accumulate large amounts of intracellular PHA [17,18] under nutrient limiting conditions [15,19,20]. *R. eutropha* is capable of growing autotrophically [15] and heterotrophically on various carbon sources [21], including organic acids [13,22,23]. A good pH range (5.5–7.9), temperature conditions (25°–40 °C) and other environmental tolerances that are key for survivability [24,25], all supported by augmented research over the past 50 years make *R. eutropha* a model organism for PHA synthesis [26–29] as well as a major player for industrial production of PHA [3]. Due to presence of propionic acid, recoverable from anaerobic treated waste streams, the synthesis of P(HB-*co*-HV) in the cells is possible [30–32], potentially increasing value of the final product [3]. PHA copolymer consisting of 3-hydroxybutyrate (HB) and 3-hydroxyvalerate (HV) monomers shows better mechanical properties in comparison to PHA homopolymer consisting solely of 3-hydroxybutyrate [17]. Thus, the P(HB-*co*-HV) copolymer exhibits a greater range of applications and overall value. In addition, utilizing metabolic engineering will shape the future of cost-reduced production of PHA. This allows access to further inexpensive carbon-containing waste streams and the synthesis of PHA with a specific composition and range of applications. Even the introduction of PHA monomers that do not occur in nature, like 3-hydroxypropionate, is possible [33,34].

In this study, a fed-batch fermentation strategy was developed to overcome the toxic influence of organic acids as sole carbon source in high cell density cultures of *R. eutropha.* The cultivation methods described here enable access to a new approach of waste stream utilization.

2. Materials and methods

2.1. Bacterial strain, culture media and preculture conditions

All experiments were performed with the *R. eutropha* H16 wild type strain (ATCC 17699) [13–15]. Cells were cultured on dextrose-free tryptic soy broth (TSB) (Becton Dickinson, Sparks, MD) agar plates with 10 mg/L gentamicin sulfate at 30 °C for 48 h. One colony was used to inoculate 5 mL of TSB liquid medium with 10 mg/L gentamicin sulfate. The culture tubes were placed on a rotary shaker and incubated for 12–18 h at 30 °C.

After incubation, 50 mL of seed culture medium were inoculated to an optical density at 600 nm (OD_{600}) of 0.1 (using a 1 cm path-length cuvette) in 250-mL Erlenmeyer flasks and incubated on a rotary shaker for 24–28 h at 30 °C and 200 rpm. Seed culture medium contained 12 g/L Na_2HPO_4; 1.5 g/L KH_2PO_4; 1.7 g/L citric acid; 390 mg/L $MgSO_4$; 62 mg/L $CaCl_2$; 2 mL/L trace elements; 10 mg/L gentamicin sulfate; 30 g/L fructose; 3 g/L urea. The trace element solution consisted of 0.2 g/L $CoCl_2$ (6^*H_2O), 0.01 g/L $CuSO_4$ (5^*H_2O), 15 g/L $FeSO_4$ (7^*H_2O), 0.06 g/L NH_4Fe(III) citrate, 0.3 g/L H_3BO_4, 0.035 g/L $MnCl_2$ (4^*H_2O), 0.035 g/L $(NH_4)_6Mo_7O_{24}$, 0.031 $NiSO_4$ (7^*H_2O) and 0.1 g/L $ZnSO_4$ (7^*H_2O). Chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless noted otherwise.

Seed culture medium was centrifuged in a SORVALL®RC5C Plus centrifuge for 10 min (4 °C; 5500 rpm (4967 × *g*); SORVALL®SLA-600TC rotor). Supernatant was discarded, and pelleted cells were suspended in 0.85% NaCl and used to inoculate fermentation medium to an initial OD_{600} of 1.0–1.5. The initial fermentation medium contained 6.54 g/L Na_2HPO_4; 4.88 g/L KH_2PO_4; 390 mg/L $MgSO_4$; 62 mg/L $CaCl_2$; 1 mL/L trace elements; 0.57 g/L sodium acetate; 0.25 g/L sodium propionate; 0.18 g/L sodium butyrate; 0.5 g/L $(NH_4)_2SO_4$.

2.2. General fermentation conditions

An INFORS HT Multifors bioreactor system (Infors AG; Switzerland) with 1 L working volume was used for all experiments conducted. Growth medium temperature during fermentation was set to 30 °C and pH was kept constant at 6.9 ± 0.1. Dissolved oxygen concentration was maintained at levels around 40% by a two stage cascade at an impeller agitation speed set between 200 and 1200 rpm in the first stage and a change of oxygen mixture in inlet gas between 21% and 50% in the second stage. The flow rate of the inlet gas mix was kept at 0.5 vvm of initial culture broth volume over the whole process time.

The pH-stat feeding solution contained 187.5 g/L acetic acid, 83.4 g/L propionic acid, 60.45 g/L butyric acid, 12 mL/L trace element solution and the corresponding amount of ammonium sulfate for the C/N (Cmole/Nmole) ratios 4, 6 and 8. The high amount of ammonium sulfate made it necessary to decrease organic acid concentration in these feeding solutions to avoid precipitation of the compounds. For a C/N ratio of 10, the pH-stat feeding solution contained 295.7 g/L acetic acid, 131.6 g/L propionic acid, 95.4 g/L butyric acid and the addition mentioned above. For high C/N ratios (50–∞) the feeding solution consisted of 330.9 g/L acetic acid, 147.3 g/L propionic acid, 106.7 g/L butyric acid and the corresponding amount of ammonium sulfate, but no trace element solution. The pO_2-feeding solution contained 200 g/L sodium acetate, 89 g/L sodium propionate and 64.5 g/L sodium butyrate.

The pO_2-feeding pump was set to activate at dissolved oxygen levels of 50% and above. After an active state of 1 min the pump was programmed to deactivate itself for 5 min. Only after this time, measured dissolved oxygen concentrations of above 50% were able to activate the pO_2-feeding pump again.

2.3. Analytical methods

For PHA analysis, culture samples of 1–10 mL were placed in preweighed 15-mL polypropylene test tubes and were centrifuged (SORVALL®RC5C Plus) for 15 min (4 °C; 5500 rpm (4967 × *g*); SORVALL®SLA-600TC rotor). The supernatant was stored at −20 °C for nutrient analysis. The pellet was resuspended in 1–2 mL water and samples were frozen at −80 °C. Frozen samples were lyophilized (FreeZone Cascade Benchtop Freeze Dry System in combination with Edwards RV 5 Rotary Vane Pump) up to 2 days (50–250 mbar; −39 to −48 °C) before cell dry weight was measured.

PHA analysis was described previously [35]. The analysis of results was performed in comparison with P(HB-*co*-HV) standards from natural origin containing ~12 mol% 3HV monomer