Microbial community engineering for biopolymer production from glycerol

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Abstract In this work, the potential of using microbial community engineering for production of polyhydroxyalkanoates (PHA) from glycerol was explored. Crude glycerol is a by-product of the biofuel (biodiesel and bioethanol) industry and potentially a good substrate for bioplastic production. A PHA-producing microbial community was enriched based on cultivation in a feast–famine regime as successfully applied before for fatty acids-based biopolymer production. A glycerol-fed sequencing batch reactor operated at a 2-day liquid and biomass residence time and with feast–famine cycles of 24 h was used to enrich a mixed community of PHA producers. In a subsequent fed-batch PHA production step under growth-limiting conditions, the enriched mixed community produced PHA up to a dry weight content of 80 wt.%. The conversion efficiency of substrate to PHA on electron basis was 53%. Since glycerol is entering the metabolic pathways of the cell in the glycolytic pathway, it was anticipated that besides PHA, polyglucose could be formed as storage polymer as well. Indeed, polyglucose was produced in low amounts (∼10 wt.%). The results indicated that the feast–famine-based enrichment strategy was comparably successful to obtain a microbial community compared to fatty acids-based enrichment described before.

Keywords Glycerol · Biodiesel · Mixed community · Polyhydroxyalkanoate · Bioplastic · Storage polymer

Introduction

Biological wastewater treatment processes are characterized by the exposure of microorganisms to transient conditions, where biomass is submitted to alternating periods of high and low substrate concentrations, and aerobic and anaerobic environments. In these unbalanced conditions, it has been found that microorganisms respond by the production of storage polymers (van Loosdrecht et al. 1997). Different types of organic storage polymers have been reported (Zevenhuizen and Ebbink 1974). Among them, polyhydroxyalkanoates (PHA) and polyglucose-like substances are the most frequently encountered (Beun et al. 2002; Karahan et al. 2008).

Besides its role as a carbon and energy storage reserve for the microorganisms, PHA may represent an environmental friendly alternative to petrochemical plastics (Steinbüchel and Lütke-Eversloh 2003). However, high production costs are associated with current industrial biotechnology-based PHA production processes. We are investigating the use of open mixed microbial communities (MMC) and waste streams or by-products as raw material for the PHA production process in order to valorise organic waste and reduce the PHA production costs (Kleerebezem and van Loosdrecht 2007; Reis et al. 2003).

In general, MMC research on PHA production is focused on conversion of the waste organic carbon into
volatile fatty acids (VFA) which are subsequently used for PHA production. Crude glycerol is a waste product rich in organic compounds generated during biodiesel production. Also during bioethanol production, a glycerol containing waste stream is generated. The glycerol weight percentage in crude glycerol depends on the biodiesel manufacturing process and varies between 62 and 90 wt.% (González-Pajuelo et al. 2005; Mothes et al. 2007; Thompson and He 2006). Considering that biodiesel production generates 10 wt.% crude glycerol (Johnson and Taconi 2007), the amount of crude glycerol produced in Europe in 2009 amounts approximately 900 ktons (European Biodiesel Board, 2011, http://www.ebb-eu.org/stats.php). The potential of this waste stream as substrate for several pure culture microbial conversion processes acquired importance during the last years as reviewed by da Silva et al. (2009). Crude glycerol could be an interesting substrate for mixed microbial community PHA production that has not received much attention yet, and may not require fermentation of the substrate to VFA first.

The aim of this work was to investigate and demonstrate the feasibility of PHA production by a MMC using glycerol as substrate. As in the work of Johnson et al. (2009), a two-step process is proposed. Firstly, a PHA-producing community should be enriched on glycerol from a natural inoculum. Secondly, the PHA cellular content of the biomass harvested from the first step needs to be maximized. For the biomass enrichment step, a sequencing batch reactor (SBR) was used to favour growth of PHA-accumulating microorganisms. Due to the alternating presence and absence of substrate in a SBR system, a feast–famine regime was established which has shown to be very effective for enrichment of PHA-producing microorganisms. For the PHA content maximization step, growth was prevented by exclusion of a growth nutrient from the medium in order to channel the conversion of all the substrate into storage polymer. The kinetics and stoichiometry of the conversion process have been evaluated.

Materials and methods

Sequencing batch reactor for biomass enrichment

For the enrichment of a PHA-producing culture, a double jacket glass reactor with a working volume of 2 L (Applikon, The Netherlands) was used. The reactor has been continuously operating since October 2008 as an open (non-sterile) sequencing batch reactor. The inoculum was activated sludge obtained from the municipal wastewater treatment plant of Dokhaven in Rotterdam, The Netherlands.

Operation of the reactor was based on a 24-h cycle according to the following scheme (time in minutes): (1) idle phase (0–5); (2) feeding phase (5–17), in which 1,000 mL of fresh medium was supplied to the reactor; (3) reaction phase (17–1,423); (4) effluent withdrawal phase (1,423–1,435), in which 1,000 mL of reactor medium was removed and (5) idle phase (1,433–1,440). Throughout the whole cycle, the medium was stirred and aerated. The end of the batch was immediately followed by the start phase of the next batch, resulting in a hydraulic and solid retention time (HRT and SRT) of 48 h. The amount of withdrawn biomass in one cycle equalled the amount of newly formed biomass.

Mixing was provided by two standard geometry six-blade turbines (Applikon, The Netherlands).

The air flow rate was maintained at 1 L/min using a mass flow controller (Brooks Instrumental, USA). The reactor temperature was controlled at 30 °C by means of a water jacket and a thermostat bath (Lauda, Germany). To prevent temperature changes in the reactor, the medium was heated to 30 °C with a thermostat bath (Lauda, Germany) prior to being added to the reactor. The pH of the reactor liquid was maintained at 7.0±0.1 using 0.5 M NaOH and 0.5 M HCl. Off-gas cooling (4 °C) was applied in order to avoid water evaporation. The pumps and the pH were controlled by the MFCS/win software. On-line measurements (dissolved oxygen, pH, temperature, acid dosage and base dosage) were acquired with the same software.

The medium solution contained 14.19 g/L of glycerol (154 mM). The composition of the nutrients solution was NH4Cl 3.61 g/L, KH2PO4 3.39 g/L, MgSO4 1.37 g/L, KCl 0.53 g/L and 15 mL/L of trace element solution (Vishniac and Santer 1957). After sterilization of the mineral medium, 1.5 mL/L of a 33-g/L allyl thiourea solution (ATU) was added to prevent nitrification activity. In the influent phase of each batch cycle, 100 mL of carbon source, 100 mL of nutrients source and 800 mL of dilution water were pumped into the reactor. The reactor was cleaned about once per week to prevent excessive biofilm formation on the glass walls, metal parts and electrodes.

Fed-batch reactor for the determination of the maximum storage capacity

For the determination of the maximum storage capacity, biomass collected at the end of a SBR cycle was used. The accumulation experiment was conducted under the same conditions as used for the enrichment reactor except for the medium composition: no nitrogen source was supplied during these experiments in order to prevent growth. The same set-up described above was used, but in a fed-batch
mode instead of SBR mode. The accumulation experiment lasted for 28 h, and concentrated glycerol (3.2 M) was dosed at certain time intervals, resulting in 20.5 g of glycerol dosage during the whole experiment.

Analytical methods

The dissolved oxygen (DO) was measured with a DO electrode (Mettler Toledo, USA) as percentage of air saturation, and pH was monitored with a pH electrode (Mettler Toledo, USA). Carbon dioxide and oxygen partial pressures in the gas leaving the reactor were analyzed in dried gas with a gas analyzer (NGA 2000, Rosemount, USA). Registered values were corrected for the actual air pressure.

Samples taken for glycerol and ammonium measurement were filtrated with 0.45-μm pore size filter (PVDF membrane, Millipore, Ireland). Glycerol concentration was determined by high performance liquid chromatography (HPLC) using an Aminex HPX-87H column from BioRad (T=60 °C) coupled to an UV and RI detector using phosphoric acid (0.01 M) as eluent. Ammonium concentration was measured using Lachat’s Quickchem 8500 flow injection analysis (FIA) system. The total suspended solids (TSS) concentration was determined by filtration according to standard methods (American Public Health 1971). The elemental composition of the organic fraction of biomass was assumed to be CH1.8O0.5N0.2. For PHA and polyglucose analysis, the methods proposed by Smolders et al. (1994) were used. Pure PHB (Sigma) was used as external standard. Freeze-dried biomass samples and PHB standards were weighed using an analytical balance and transferred into tubes with screw caps. One milligramme of benzoic acid in 1-propanol was used as internal standard. Of a mixture of concentrated HCl and 1-propanol (1:4), 1.5 mL and 1.5 mL of dichloroethane were added and heated for 2 h at 100 °C. After cooling, free acids were extracted from the organic phase with 3 mL water. One millilitre of the organic phase was filtered over water-free sodium sulphate into GC vials. The propylesters in the organic phase were analyzed by gas chromatography (model 6890N, Agilent, USA) equipped with a FID, on an HP Innowax column. For polyglucose, freeze-dried biomass samples were weighed using an analytical balance and transferred into tubes with screw caps. Six molars HCl was directly added to a final concentration of 0.6 M, and tubes were heated at 100 °C. A minor alteration compared to the cited reference was introduced: the time for heating was increased from 1 to 3 h. Glucose was measured in the supernatant by HPLC using an Aminex HPX-87H column from BioRad (T=60 °C) coupled to a UV and RI detector using phosphoric acid (0.01 M) as eluent. Nile blue A staining was performed according to the method proposed by Ostle and Holt (1982).

Results

Enrichment of a mixed community with high storage capacity

The reactor was inoculated with sludge from an aerobic wastewater treatment plant. The evolution of the dissolved oxygen in the reactor during several cycles is plotted in Fig. 1. After six to 15 cycles, a clear feast–famine sequence became visible in the oxygen concentration. During the initial period, bacteria significantly grew in the feast phase. This led to the decrease of oxygen concentration in the feast phase during adaptation of the community. Once a community with a high storage capacity was obtained, the oxygen concentration was stable during the feast phase (e.g. cycle 40, Fig. 1). The end of the feast period, when the substrate (glycerol) was depleted, corresponded to the rapid increase in dissolved oxygen, and a lower oxygen uptake in the remainder of cycle 40 (indicated by the relatively high dissolved oxygen) was related to growth of the bacteria on the storage polymer. From day 40 onwards, the dynamic pattern of the dissolved oxygen became constant. The establishment of a storage-oriented community could also be inferred from the ammonium consumption in the reactor. The fraction of ammonium that was consumed during the feast phase decreased to approximately 15–25% of the total ammonium uptake. Consequently, most ammonium was taken up after the substrate was consumed, demonstrating the shift from growth on glycerol to growth on a storage polymer (Fig. 2).

Another useful parameter for monitoring the performance of the enrichment reactor was the length of the feast phase.
phase. The evolution of this parameter is plotted in Fig. 3. The THRIVING of microorganisms capable of quickly consuming the substrate was related with the shortening of the length of feast phase. After 40 days of operation, the length of the feast phase was stable for the remaining experimental time at approximately 2.0–2.5 h.

Initially, the selective pressure in this system was based on the specific substrate uptake rate. This parameter is directly related with the length of the feast phase, and it is defined as mass of consumed substrate per mass of biomass per hour. The specific uptake rate increased during the start-up period, from 0.28 to 0.58 g/g h. This fact suggested the outcompeting of other bacteria by a highly efficient storage polymer-producing organism.

Once the length of the feast phase was stable, the selective pressure was based on the advantage of microorganisms that produced storage compounds over those that used substrate for growth while the substrate was present. This observation is justified by the decreased ammonium uptake during the feast phase, indicating a shift towards a higher flux of the substrate towards storage relative to the flux towards cell growth (Fig. 2).

The system reached a steady operational performance within 100 days after start-up. The dynamic pattern of the dissolved oxygen and pH remained identical from cycle to cycle, and the TSS concentration at the end of the cycle remained constant already after 40 cycles. For the relative distribution of ammonium uptake between feast and famine, a longer period was needed to stabilize. Obviously, this is a more sensitive parameter to evaluate stationary reactor behaviour.

Cycle characterization

The conversions of several important compounds during a cycle under steady operational conditions are given in Fig. 4. The dissolved oxygen profile (Fig. 4a) clearly depicts the same characteristic feast–famine regime pattern as in cycle 40 (Fig. 1), indicating that after 40 cycles (or 20 times the biomass retention time), a PHA-producing microbial community has been selected. Dissolved oxygen concentration sharply decreased, and the oxygen uptake rate (OUR) increased during the first hours of the cycle (Fig. 4b). Oxygen consumption was associated with glycerol uptake. Glycerol was present in the reactor only during the first 2 h, i.e. the feast phase. Substrate consumption was accompanied by a rapid production of both a polymer of glucose (referred to as polyglucose) and PHA, as polyhydroxybutyrate (PHB), and little ammonium consumption (Fig. 4c). This justifies the assumption that during the feast phase substrate is mainly converted to storage compounds rather than used for growth. The famine phase started when the glycerol was depleted. Storage polymers were the only carbon and energy source during the rest of the cycle. Growth on those storage polymers was reflected by the uptake of ammonium as indicated in Fig. 4d. The variation in the inorganic carbon production rate and the oxygen consumption rate, as shown by Fig. 4b, also confirms the transition of the feast to famine phase.

The biomass concentration at the end of the cycle was 0.57±0.07 g/L. The observed yields during the feast phase were 0.35 gPHB/g glycerol and 0.14 gpolyglucose/g glycerol. Considering that both PHB and polyglucose were used for growth, a yield of 0.62 gbiomass/g storage polymer was observed during the famine phase. The effective biomass yield on glycerol was 0.30 gbiomass/g glycerol. The observed kinetic and stoichiometric parameters for the enrichment reactor are summarized in Table 1. The possible presence of other storage compounds was tested from evaluating the carbon and electron balances from the experimental data. The recovery was on average 94.8±7.8% and 101.7±8.9% for C-mmol and e-mmol balance, respectively. This indicates that, likely, all carbon-containing compounds were measured in this experiment, and no extra storage compound was present.
Determination of the maximum storage capacity

In a fed-batch accumulation experiment, the maximal PHA storage capacity of the mixed community was determined (Fig. 5).

Within 6 h, 67 wt.% of the cell dry weight corresponded to accumulated PHB. After 22 h, the community had stored up to 77 wt.% of PHB, and the maximum content attained was 80 wt.% after 28 h. This PHB content was reached while glycerol was still present in the liquid. It seems this is therefore the highest PHB content attainable by the current mixed community. In contrast, polyglucose remained at a low value during the accumulation experiment.

The biomass used for the accumulation experiment was harvested at the end of the cycle of the enrichment reactor. After the remaining nitrogen source from the SBR cycle was depleted, growth could not occur, and all the glycerol was used for storage polymers synthesis or respiration. Within 6 h, 67 wt.% of the cell dry weight corresponded to accumulated PHB. After 22 h, the community had stored up to 77 wt.% of PHB, and the maximum content attained was 80 wt.% after 28 h. This PHB content was reached while glycerol was still present in the liquid. It seems this is therefore the highest PHB content attainable by the current mixed community. In contrast, polyglucose remained at a low value during the accumulation experiment.

Carbon and electron balances were evaluated considering two different time ranges. During the first 6 h, 100% of the dosed glycerol was recovered in PHB, biomass growth, inorganic carbon production and oxygen consumption. In contrast, when the balance was calculated between the beginning of the experiment and the end (28 h), the recovery percentage was lower. Only 87% of the dosed carbon and 82% of the electrons (or COD) dosed to the system were found back in the measured products. After reaching a certain accumulation percentage, the substrate...
uptake seemed to get uncoupled from the storage polymer formation. This can be due to the production of other cellular components or excretion products from the cells that were not taken into account in the material balances. The amount of polymer produced per substrate consumed in the first 6 h was 0.40 gPHB/g glycerol, which is equivalent to an electron yield of 0.53 e\textsuperscript{−}mol/e\textsuperscript{−}mol.

Microbial community analysis

Sludge samples were microscopically analyzed when the steady operation of the reactor was reached. These examinations revealed, based on the distinctive morphology described by Seviour et al. (2000), that the dominant microorganism is similar to the group of G-bacteria and has not been identified yet. G-bacteria are grouped in clusters of large cocci shaped in sizes of 2–4 μm forming tetrads. They are non-motile, and there were no indications of spore formation. A picture of the mixed community is shown in Fig. 6. The presence of PHA was further confirmed by specific staining on PHA (Fig. 7).

Discussion

In this study, we have demonstrated that glycerol is a good substrate for PHB production by mixed microbial communities. The use of a feast–famine SBR for community selection proved again a very successful approach to give a stable community with high PHA-accumulating capacity and a high PHA formation rate with no need of metabolically or genetically engineered PHA-producer strains (Jiang et al. 2011b; Johnson et al. 2009; Lemos et al. 2006). We compared the findings of our study with literature data (see Supplementary Table S1). A detailed overview of many of the different bacterial strains producing PHA from glycerol can be found in Ibrahim and Steinbüchel (2009). Critical parameters such as media composition, cultivation time, experimental set-up, temperature and cell dry weight substantially differed between references. One common feature is the use of pure cultures of microorganisms whereas our work was based on a non-defined mixed microbial community. According to Kleerebezem and van
Loosdrecht (2007), the use of MMC allows for saving energy, since no sterilization is required, reduces fermentation equipment costs and less process control is needed. Glycerol-fed MMC for biopolymer production has been also studied by Dobroth et al. (2011). A maximum accumulation of 67 wt.% of PHA was attained using crude glycerol from a biodiesel manufacturing facility. However, it was the methanol fraction present in the fed stream which was utilized as primary carbon source while glycerol was not consumed according to their results. Nevertheless, the main body of research conducted on the use of crude glycerol as substrate for PHA production reports the preferable use of glycerol compared to methanol (Ashby et al. 2004; Bormann and Roth 1999; Cavalheiro et al. 2009; Koller et al. 2005; Mothes et al. 2007; Zhu et al. 2010). According to these studies, it would be likely the case that the glycerol fraction will be used for PHB synthesis instead of methanol. In most of the studies with glycerol as substrate, the type of PHA found was, as in our study, PHB. Only three of 19 references included in Table S1 reported a different type of PHA. Ashby et al. (2004) obtained medium-chain-length-PHA (mcl-PHA) using a pure culture of Pseudomonas corrugate 388. The same authors, in 2005, using a mixture of Pseudomonas oleovorans NRRL B-14682 and P. corrugate 388, found a blend of short-chain-length and mcl-PHA. Koller et al. (2005) obtained PHB with 8–10% of hydroxyvalerate, synthesized by an unidentified osmophilic organism. None of these studies reported the presence of polyglucose; from the literature, it is unclear whether these cultures do not store polyglucose or whether it was not measured.

Reported yields vary between 0.08 and 0.37 gPHA/g substrate. The observed yield in this study, 0.40 gPHA/g substrate, is the highest reported when glycerol is used as substrate for PHA production. The maximum theoretical yield that can be obtained in the conversion of glycerol to PHB based on the PHB production pathway via acetyl-CoA amounts 0.47 gPHB/g glycerol (0.67 Cmol PHB/Cmol glycerol). The simultaneous occurrence of a polyglucose polymer and growth, due to the residual ammonium present in the reactor, explain the gap between the theoretical and the observed yield of PHB over glycerol in this study.

When comparing our findings with pure culture results, an outstanding fact is the specific PHA production rate, $q_{PHA}$, of our community. Specific PHA production rate is, at least, one order of magnitude higher in our work than in the results reported for pure cultures. The selection strategy employed, based on firm ecological principles, was proven successful: not only the PHA synthesis rate was the highest reported when using glycerol as substrate but also the polymer content was among the highest values reported. Moreover, according to Johnson et al. (2009), the longer the community is cultivated in the feast–famine regime, its PHA-producing capacity and rate improve due to the continuous selective pressure and competition. Consequently, MMC performance for PHA production can be further improved by the applied selection strategy since natural selection will lead automatically to faster and more efficient communities.

To date, the highest PHA content reached by a mixed community was 89 wt.% (Johnson et al. 2009). This value was reached with acetate as carbon source, after community selection during years. Other processes using MMC reached 54 wt.% using fermented olive mills effluent (Dionisi et al. 2005), 48 wt.% using fermented paper mill effluent (Bengtsson et al. 2008) and with fermented molasses, 78 wt.% has been reported (Albuquerque et al. 2010). Recently, Jiang et al. (2011b) enriched a community using lactate as carbon source. The enrichment could accumulate over 90 wt.% PHB within 6 h, which is currently the highest value reported in mixed community process. The polymer concentration reached in this study is comparable to the values attained with other mixed community processes. Also, the specific conversion rates found in this study are similar to those reported in literature for the extensively studied fatty acid-based communities (Table 2).

Glycerol enters the metabolic pathway of the cell in the glycolytic pathway. Since gluconeogenesis and PHB synthesis compete for C-3 intermediates, studying the regulation between these two processes may lead to a process optimization with higher PHA formation efficiency.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>PHA (wt.%)</th>
<th>$Y_{PHA}$ (g PHA/g substrate)</th>
<th>$q_{PHA}$ (g PHA/g biomass h)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>89</td>
<td>0.43</td>
<td>1.20</td>
<td>Johnson et al. 2009</td>
</tr>
<tr>
<td>Lactate</td>
<td>90</td>
<td>0.46</td>
<td>1.76</td>
<td>Jiang et al. 2011b</td>
</tr>
<tr>
<td>Acetate</td>
<td>67</td>
<td>0.50</td>
<td>0.35</td>
<td>Serafim et al. 2004</td>
</tr>
<tr>
<td>Fermented molasses</td>
<td>75</td>
<td>0.65</td>
<td>0.37</td>
<td>Albuquerque et al. 2010</td>
</tr>
<tr>
<td>Valerate</td>
<td>11</td>
<td>0.37</td>
<td>0.07</td>
<td>Lemos et al. 2006</td>
</tr>
<tr>
<td>Propionate</td>
<td>60</td>
<td>0.35</td>
<td>0.30</td>
<td>Jiang et al. 2011a</td>
</tr>
<tr>
<td>Glycerol</td>
<td>67</td>
<td>0.40</td>
<td>0.34</td>
<td>This study</td>
</tr>
</tbody>
</table>

Table 2  PHA production by MMC submitted to feast–famine regime
Based on PHA staining (Fig. 7), it can be concluded that the community is producing PHA. Since staining techniques do not give reliable results on polyglucose detection (Serafim et al. 2002), it was not possible to determine by visual methods the presence of both polymers.

Based on the literature about MMC, PHA is directly formed out of the central metabolite acetyl-CoA while polyglucose is formed when sugars are present in the feed (van Loosdrecht et al. 1997). Consequently, sugar-rich substrates are not suitable carbon sources for direct production of PHA with MMC. An anaerobic fermentation step prior to the enrichment phase is needed to convert sugars to VFA (Albuquerque et al. 2007; Bengtsson et al. 2008; Reis et al. 2003; Temudo et al. 2007). For intermediates in the glycolysis, the natural storage polymer was until now unclear. This study showed that at least for glycerol, no fermentation will be needed for producing PHA.

Choi and Lee (1997) reported that the price of PHAs strongly depends on substrate cost, accounting for about 40% of the total production costs. Also, the need for axenic operation with a pure culture contributes significantly to equipment costs and operational costs. For newer processes employing genetically modified organisms, this will be even higher. As a result of the increasing demand for renewable fuels, biodiesel production has tremendously increased in recent years, leading to an excess of crude glycerol. This waste stream is impure and has a low economic value (Chi et al. 2007). Here with crude glycerol is an economically attractive raw material for industrial PHA production by mixed microbial communities. Two of the major drawbacks associated to current PHA production can be overcome using microbial community engineering: elevated costs for substrate and sterile operation. In this study, we have demonstrated that glycerol is a very good substrate for MMC-based PHA production. Future research will focus the use of a more complex substrate, i.e. crude glycerol from a biodiesel production plant for PHA production with MMC.

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