PHA Production in Aerobic Mixed Microbial Cultures

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PROEFSCHRIFT

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Katja JOHNSON (geb. SCHMID)

Diplom-Ingenieurin, processtechnologie, Hamburg University of Technology, Duitsland

geboren te Peine, Duitsland

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To Nick & Sarah

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Summary

PHA production in aerobic mixed microbial cultures

Polyhydroxyalkanoate (PHA) is a common intracellular energy and carbon storage material in bacteria, which is considered as a bioplastic due to its plastic like properties. PHAs are versatile materials which are biodegradable and made from renewable resources. Commercial production of PHAs is currently based on pure culture processes employing either natural PHA producers or genetically modified bacteria. Pure culture processes use generally pure sterile substrates and axenic reactors, leading to high production costs and thus relatively expensive products.

An alternative approach for the production of PHAs is the use of mixed culture biotechnology, using non-sterile waste streams as a substrate and open reactors. The use of cheaper substrates, less energy (no sterilization of substrate or reactors) and cheaper equipment could reduce the production costs compared to pure culture processes. However, the mixed culture PHA production process requires optimization for higher cellular PHA contents to be competitive with pure culture processes. The research described in this thesis aimed at improving the cellular PHA contents that can be achieved in open mixed cultures.

A two-step process consisting of (i) a culture enrichment and growth step and (ii) a PHA production step was used. For the enrichment of a mixed culture with PHA producing bacteria a selective pressure in the form of alternating periods of short presence of the carbon substrate (feast phase) and long absence of the carbon substrate (famine phase) under fully aerobic conditions was employed. PHA storing bacteria generally outcompete other bacteria in such a feast-famine system due to their very high substrate uptake rate (which is not limited by the growth rate) and due to the ability to grow in a more balanced way throughout feast and famine phase.

A sequencing batch reactor (SBR) was used to establish the feast-famine regime. The cultures enriched in the first step under different operational conditions were tested for their ability to produce PHA in the second step, the PHA production step. For this purpose the cultures were supplied with an excess of carbon source (fed-batch reactor) while withholding a suitable nitrogen source in order to avoid growth and direct as much carbon as possible into PHA storage. To simplify the system for the optimization studies a mineral medium with acetate as the sole carbon substrate was used in all experiments rather than real wastewater. Acetate yielded pure polyhydroxybutyrate (PHB) as the storage polymer.

In order to compare different operational conditions, specific reaction rates and observed yields had to be calculated for the key compounds acetate, biomass, PHB, carbon dioxide, oxygen and the nitrogen source ammonia from measurements performed during a stable SBR cycle or fed-batch experiment. Both SBR and fed-batch reactor were highly dynamic systems with changing reaction rates and liquid volumes, making the evaluation of experimental data a complex task. A very detailed data analysis was carried out for each SBR cycle measurement and fed-batch experiment. The data analysis included for example the correction of

measurements for sampling effects and liquid volume changes, the computation of oxygen consumption and carbon dioxide evolution, and the calculation of the best estimates for all reaction rates and total conversions at each time point with the help of a metabolic model (Chapter 2). The metabolic model was used in order to be able to describe the dynamics of the system and in order to ensure that material balances would close. The metabolic model described the measurements generally very well. The reaction rates computed with the metabolic model showed clearer trends than those calculated without the help of the model.

Different operational conditions were tested for the biomass enrichment step (SBR). The first two process parameters investigated were low sludge residence times (SRTs) of 4 d, 1 d and 0.5 d and the impact of different degrees of nitrogen versus carbon limitation (Chapter 3). Low SRTs are required for a high biomass productivity in the first step. The impact of nitrogen limitation was investigated, because many waste streams that are suitable substrates for mixed culture PHA production are nutrient limited. Enrichment of a PHA storing community was successful at 4 d and 1 d SRT, but less successful at 0.5 d SRT. Nitrogen limitation in the SBR generally led to competition for nitrogen and consequently to a selective pressure for high growth rates. Carbon limitation in the SBR led to a PHB storage strategy (high acetate uptake rate) and usually to higher PHB contents (about 70 wt%) in subsequent fed-batch experiments compared to cultures enriched under nitrogen limitation. Carbon limitation in the SBR allowed PHB storing bacteria to benefit more from their ability to store PHB by being able to grow throughout the famine phase. Carbon limitation and SRTs higher than 0.5 d were identified as favourable conditions for the biomass enrichment step in the SBR. Nutrient limited wastewaters may require supplementation with nutrients for this step.

Another parameter that was investigated was the reactor temperature (Chapter 4). The reactor temperature will influence the reaction rates, but also the selective pressure in the SBR. The influence on the reaction rates can be investigated by applying short-term temperature changes (i.e. one SBR cycle) while the combined effect on reaction rates and selective pressure can be studied in long-term temperature change experiments. In short-term temperature change experiments the reactor temperature of a stable SBR operated at 20°C was changed for one cycle to 15, 25, 30 or 35°C. It was found that reaction rate changes in the famine phase could be described over the whole temperature range with the Arrhenius equation with one temperature coefficient. For the feast phase different temperature coefficients were estimated for acetate uptake, PHB production and growth. These were only valid for temperatures 5°C higher or lower than the steady state temperature. After long-term changes to either 15 or 30°C the reactor performance changed considerably: At lower temperatures the feast phase was long and a growth strategy prevailed. This culture had a very low PHB storage capacity (about 35 wt%). At 30°C the feast phase was short and a PHB storage strategy dominated. This culture was able to store 84 wt% PHB. Higher SBR temperatures appear to be a good strategy to support the enrichment of PHB storing bacteria.

In Chapter 5 we report the most successful operating strategy applied during this thesis. A SBR culture was enriched that was able to store 89 wt% PHB within only 7.6 h in a fed-batch

experiment. This culture had been enriched with a longer cycle length of 12 h as compared to our previous studies (4 h cycle length), at 1 d SRT, 30°C and carbon limitation. Another key to the high PHB content was the long operating time under these conditions of over a year. The maximum PHB storage capacity of this culture had improved with time. The long cycle length combined with a low SRT was found to favour growth of bacteria that can store a high amount of PHB at a high rate, since this is needed in order to continue to grow throughout the much longer famine phase.

After the operating conditions in the SBR had been optimized, also the PHA production step in the fed-batch reactor was investigated. The temperature in fed-batch experiments did not influence the maximum PHB storage capacity, but only the reaction rates (Chapter 4). Fed-batch experiments were typically conducted using fed-batch systems without nitrogen source in the feed. With the aim of using waste streams as a substrate for PHA production, nutrient limitation or starvation may not always be feasible. We therefore investigated the influence of nitrogen starvation, nitrogen limitation and nitrogen excess on the maximum PHB content obtained in fed-batch experiments (Chapter 6). Under nitrogen starvation conditions the biomass reached a maximum PHB content of 89 wt%, under nitrogen limitation 77 wt% and under nitrogen excess 69 wt%. In the latter two experiments PHB contents decreased after these maxima were reached, because growth led to a dilution of the PHB pool. Nutrient starvation seems thus to be the best strategy for maximal PHB production in the fed-batch step.

Chapter 7 summarizes and integrates the findings from all individual studies. In this chapter also some remaining issues are discussed and recommendations for future research are provided. With the aim of using real waste streams in the future and producing other PHAs apart from PHB, the next steps would be the use of more diverse carbon source mixtures and eventually a scale-up of the system.

In conclusion, mixed culture PHB production has been successfully optimized in this thesis. A mixed culture was established with the capacity to produce PHB levels as high as in pure culture production processes, and at very high PHB production rates. Cultivation conditions have been identified that lead to a selection of a stable mixed microbial culture with a superior PHA production capacity. Compared to previous work with mixed cultures, a more than four times higher cellular PHB content was obtained. Herewith a highly competitive process has been established that may contribute to the development of a more sustainable and renewable biopolymer production in a future bio-based economy.

Katja Johnson

Samenvatting

PHA productie door aerobe gemengde culturen

Polyhydroxyalkanoaat (PHA) is een door micro-organismen gemaakt intracellulair opslagmateriaal van koolstof en energie. PHA wordt beschouwd als een bioplastic door haar plasticachtige eigenschappen. Commerciële productie van PHA is momenteel gebaseerd op reincultuur processen waarbij in vele gevallen gebruik wordt gemaakt van genetisch gemodificeerde micro-organismen. Reincultuur processen behoeven steriele substraten en bioreactoren, met als gevolg hoge productiekosten en daardoor relatief dure producten.

Een alternatieve benadering voor PHA productie is het toepassen van mengcultuur biotechnologie, waarbij niet-steriele afvalstromen en niet-gesteriliseerde bioreactoren worden gebruikt. Het gebruik van goedkopere substraten, een lager energieverbruik (door afwezigheid van sterilisatie van media en bioreactoren), en goedkopere bioreactoren en apparatuur kunnen zorgen voor gereduceerde PHA-productiekosten. Teneinde echter een volwaardig alternatief te zijn voor reincultuur processen dient het cellulaire PHA-gehalte in mengcultuur processen geoptimaliseerd te worden. Het in dit proefschrift beschreven werk was gericht op verbetering van het cellulaire PHA-gehalte in mengcultuur processen.

Een tweetraps process bestaande uit (i) een biomassa verrijkings- en groeifase, en (ii) een productie fase werd toegepast. Voor de verrijking van een mengpopulatie met de capaciteit om PHA te produceren dient selectiedruk op de mengpopulatie te worden aangebracht. Bij het hier beschreven onderzoek is selectiedruk aangebracht door de mengpopulatie in aanwezigheid van zuurstof afwisselend bloot te stellen aan korte perioden met substraat (feestfase) en lange perioden zonder substraat (hongerfase). In een dergelijk afwisselend feest-honger systeem hebben bacteriën die PHA kunnen opslaan een competitief voordeel doordat ze gedurende de feest fase zeer snel substraat op kunnen nemen, en doordat ze kunnen groeien op het opslagpolymeer in de honger fase.

Een opeenvolgende batch reactor (SBR) werd gebruikt om een feest-honger regiem te verkrijgen. De capaciteit om PHA op te slaan van de gekweekte biomassa werd vervolgens getest in een tweede stap, de PHA productie stap. Voor dit doel werd de biomassa continue gevoed met een overmaat aan substraat in afwezigheid van een essentiële stikstofbron om biomassagroei te voorkomen en zoveel mogelijk substraat richting PHA te dirigeren. In de hier beschreven experimenten is acetaat als enige koolstof en energiebron gebruikt. Door gebruik van acetaat wordt polyhydroxybutyraat als enig opslagpolymeer gevormd.

Teneinde de resultaten van de verschillende experimenten te kunnen vergelijken werden biomassa specifieke reactiesnelheden en geobserveerde yields berekend voor de belangrijkste componenten in het systeem; acetaat, biomass, PHB, kooldioxide, zuurstof, en ammonium. Zowel de verrijkingsexperimenten als de productie-experimenten zijn zeer dynamisch met variërende reactiesnelheden en vloeistofvolumes, waardoor evaluatie van de experimenten een gecompliceerde taak bleken. Een eenduidige methode is ontwikkeld en toegepast voor data-analyse van alle cyclusmetingen tijdens verrijkingsexperimenten en voor alle PHA-

productie experimenten. Data-analyse omvatte bijvoorbeeld de correctie van metingen door monstername en veranderingen in vloeistofvolume, berekening van zuurstofopname en kooldioxide productie, en de schatting van alle reactiesnelheden en totale omzettingen met behulp van een metabool model (Hoofdstuk 2). Het metabole model werd gebruikt om de dynamiek van het proces adequaat te kunnen beschrijven en om massabalansen te controleren. In de meeste gevallen beschreef het model de gemeten concentraties zeer goed. Door de met het model geschatte reactiesnelheden tussen verschillende experimenten te vergelijken konden duidelijke trends worden waargenomen.

De invloed van verschillende operationele variabelen op de verrijkingsstap van het proces is onderzocht. De eerste twee onderzochte variabelen betreffen de slibverblijftijd (SRT - 4, 1, of 0.5 dag) en de invloed van stikstof- versus koolstoflimitatie (Hoofdstuk 3). De in dit onderzoek toegepaste lage SRT-waarden zijn noodzakelijk om een hoge biomassaproductiviteit te verkrijgen in de verrijkingsstap. De invloed van stikstoflimitatie is onderzocht omdat vele industriële afvalwaters nutriënten gelimiteerd zijn. Verrijking van PHA producerende biomassa was succesvol bij SRT-waarden van 4 en 1 dag, maar niet bij 0.5 dag. Stikstoflimitatie leidde tot competitie om stikstof en dus tot selectie van micro-organismen met hoge groeisnelheden. Koolstoflimitatie leidde tot selectie van micro-organismen met als strategie om PHA op te slaan (gekoppeld aan een hoge specifieke acetaat opname snelheid) en tot hogere cellulaire PHA-concentraties (ongeveer 70% g/g) tijdens accumulatie-experimenten. Koolstoflimitatie in de verrijkingsstap maakte het mogelijk voor PHA accumulerende bacteriën te groeien tijdens de hongerfase op opgeslagen PHA. De conclusie was dat koolstoflimitatie gecombineerd met SRT-waarden hoger dan 0.5 dag gunstig is voor de verrijkingsstap van het PHA productieproces. Nutriëntengelimiteerde afvalwaters behoeven nutriëntendosering voor de verrijkingsstap.

Een andere procesvariabele die is onderzocht betreft de reactortemperatuur (Hoofdstuk 4). De temperatuur beïnvloedt reactiesnelheden, maar ook de competitie tussen verschillende micro-organismen in de reactor. De korte termijn invloed van de temperatuur op de reactiesnelheden is onderzocht door de gekweekte biomassa gedurende een operationele cyclus bloot te stellen aan een andere temperatuur. Lange termijn effecten zijn onderzocht door biomassa in de verrijkingsstap meerdere slibleeftijden lang bloot te stellen aan een andere temperatuur. Korte termijn experimenten zijn uitgevoerd door met biomassa gekweekt bij 20°C een cyclusmeting te doen bij 15, 25, 30, en 35°C. Het kon worden aangetoond dat veranderingen in reactie-snelheden tijdens de hongerfase konden worden beschreven met behulp van de Arrhenius vergelijking en een constante temperatuurscoëfficiënt. Voor de feestfase werden verschillende temperatuurscoëfficiënten geschat voor acetaat opname, PHB productie en microbiële groei. Bovendien bleken deze coëfficiënten alleen geldig als de temperatuur niet meer dan 5°C afweek van de kweektemperatuur. Langdurige groei van biomassa in de verrijkingsstap bij 15 of 30°C had een grote invloed op de verrijkingsstap: bij 15°C was de feestfase erg lang en domineerden bacteriën die een groeistrategie hanteerden over bacteriën met een PHA-opslag strategie. Bij 30°C was de feestfase erg kort en bacteriën met een PHA-opslag strategie domineerden het system. De bij 30°C gekweekte biomassa bleek in staat tot 84% (g/g) PHB-

opslag in accumulatie-experimenten. De hogere temperatuur van de verrijkingsreactor bleek bij te dragen aan de ophoping van bacteriën met een zeer hoge PHA-opslag capaciteit.

In hoofdstuk 5 wordt de meest succesvolle bedrijfsvoering voor de verrijkingsreactor beschreven. De in dit hoofdstuk beschreven biomassa bleek in staat tot 89% (g/g) opslag van PHB binnen 7.6 uur in een accumulatie-experiment. Teneinde deze biomassa te verkrijgen werd de verrijkingsreactor bedreven bij een cycluslengte van 12 uur in plaats van 4 uur bij voorgaande experimenten. Een andere voorwaarde om de superieure eigenschappen van deze biomassa te krijgen was de zeer lange kweektijd van meer dan een jaar. Het kon worden aangetoond dat de PHB accumulatie capaciteit toenam in de tijd. De langere cyclustijd gecombineerd met een lage SRT geven een competitief voordeel aan bacteriën die hoge percentages PHA op kunnen slaan met een hoge snelheid, teneinde langzaam te kunnen groeien tijdens de veel langere honger-fase.

Nadat de operationele omstandigheden in verrijkingsstap waren geoptimaliseerd, diende ook de accumulatiestap te worden geoptimaliseerd. De temperatuur in de accumulatiestap bleek geen invloed te hebben op de maximale PHB opslag capaciteit, maar wel op de reactiesnelheden (Hoofdstuk 4). Accumulatie-experimenten werden in de meeste gevallen uitgevoerd zonder stikstofbron in het influent. Omdat in vele afvalwaters nutriënten niet limiterend zijn, is de invloed van een stikstofbron op de PHB accumulatie capaciteit onderzocht. Accumulatie-experimenten uitgevoerd in afwezigheid van stikstofbron zijn vergeleken met experimenten met beperkte stikstofdosering, en met een overmaat aan stikstof in de voeding (Hoofdstuk 6). In de drie experimenten werd een afname in PHA-opslag capaciteit waargenomen bij een toename in N-dosering: 89% zonder stikstof, 77% met beperkt stikstof, en 69% bij overmaat stikstof. De experimenten waarbij stikstof aanwezig was in het influent nam het PHB-gehalte af nadat de maximale waarden waren bereikt als gevolg van groei. Conclusie is dat nutriëntenlimitatie de beste strategie is voor de accumulatiestap.

Hoofdstuk 7 geeft een samenvatting van het onderzoek en integreert het werk beschreven in de verschillende hoofdstukken. Ook worden een aantal overgebleven zaken bediscussieerd en aanbevelingen voor verder onderzoek beschreven. Teneinde in de toekomst industriële afvalstromen te kunnen gebruiken en andere PHA's dan PHB te kunnen produceren, is het van belang dat de invloed van verschillende substraten op het proces wordt onderzocht.

Samenvattend kan gesteld worden dat het PHB-productieproces succesvol is geoptimaliseerd. Uit een ongedefinieerd inoculum is een verrijkte mengcultuur verkregen met de capaciteit om PHB-gehaltes te produceren die vergelijkbaar zijn met in reinculturen verkregen PHB-gehaltes, en bovendien met aanzienlijk hogere snelheid. De operationele omstandigheden zijn gedefinieerd waarbij in de verrijkingsstap een stabiele mengpopulatie van bacteriën wordt verkregen met een superieure PHB-productiecapaciteit. Hierdoor is een zeer competitief proces ontwikkeld dat een bijdrage kan leveren aan de ontwikkeling van een duurzamere productie van biopolymeren in een toekomstige bio-gebaseerde economie.

Katja Johnson

CHAPTER 1

General Introduction

1

Biorefineries

In the last century petroleum-based products have gradually replaced products derived from renewable materials. Now, there is a strong trend back from the oil-based economy to a more bio-based economy. This change is driven by the concern about climate change caused by green house gas emissions and also by the predicted depletion of petroleum resources and the desire for a secure and independent energy supply (Ragauskas et al., 2006; Goldemberg, 2007; Himmel et al., 2007; Lange, 2007).

In a bio-based economy factories called biorefineries would produce intermediates and end products from renewable resources such as biomass. Equivalent to petrochemical refineries, biorefineries are envisaged to use all basic compounds of biomass (cellulose, starch, sugars, lignin, oils etc.) and to convert them into biofuels, bioenergy, biomaterials (e.g. bioplastics) and biochemicals (Kamm et al., 2007; Ragauskas et al., 2006). Chemical, enzymatic and microbial processes can be employed to transform carbohydrates, the major fraction of biomass, into building-block chemicals, which form the basis for the production of bio-based fuels, chemicals and materials (Ragauskas et al., 2006; Kamm et al., 2007). Examples of key building block chemicals include alcohols and carboxylic acids (Ragauskas et al., 2006). Many of these building blocks can be produced by microbial fermentation, but also final products such as bioplastics can be directly produced with microorganisms. Usually these processes employ pure cultures of microorganisms and therefore require well-defined feedstocks and aseptic process conditions. Moreover these processes often have to be performed as batch processes. These conditions result in expensive equipment and high energy consumption, making industrial biotechnology based on pure cultures unfavourable for the large scale production of relatively cheap bulk biochemicals and biomaterials.

Mixed culture biotechnology

An alternative approach for the sustainable production of some biochemicals and biomaterials could be the application of mixed culture biotechnology. Mixed culture biotechnology aims to produce products with processes employing open undefined mixed cultures and ecological selection principles, thus combining the methodology of environmental biotechnology with the goals of industrial biotechnology. Mixed cultures have so far only been applied for waste treatment (e.g. biological wastewater treatment plants, composting facilities, anaerobic digesters) and partly for the production of bioenergy (biogas), metals (biohydrometallurgy) or traditional fermented foods.

The principle of mixed culture biotechnology is based on natural selection and competition rather than on genetic or metabolic engineering (Kleerebezem et al., 2007). Selective pressure for a desired metabolism is applied on a diverse inoculum by choosing the substrate and operating conditions of the bioreactor in an appropriate way, i.e. to engineer the ecosystem rather than the organisms. Mixed culture biotechnology offers several advantages compared to

pure culture biotechnology: reactors are operated under unsterile (non-axenic) conditions leading to lower energy and equipment costs, a risk of strain degeneration and contamination does basically not exist, waste streams could be used as a substrate rather than pure substrates, the technology is not protected by patents, and reactors could be operated in a continuous fashion.

The Netherlands Organization for Scientific Research (NWO), Division for Technical Sciences (STW), funded a project with the title "Directed evolution of product formation in mixed microbial cultures" that was aiming at evaluating the potential of mixed culture biotechnology to produce biochemicals and bioplastics. The first part of this project was dealing with the anaerobic mixed culture fermentation of carbohydrate containing streams into a stable mixture of smaller molecules such as fatty acids and alcohols and is described in detail in the thesis of M. Temudo (2008). The second part of this project was to investigate the feasibility of converting such fermentation products with open mixed cultures into a bioplastic – this is the subject of this thesis.

Bioplastics

About 260 million tons of plastics are produced worldwide each year and the per capita consumption of plastics in North America and Western Europe is estimated at about 100 kg per year (www.plasticseurope.org). Some plastics are reused, recycled or burned for energy recovery, but a large amount accumulates in landfills, in oceans, and elsewhere in the environment. The 'Great Pacific Garbage Patch', a gyre of marine litter in the central North Pacific Ocean, is believed to be in size somewhere between twice the size of Texas to twice the size of continental United States and contains probably around 100 million tons of debris (Marks et al., 2008; www.wikipedia.org). In 2004 the then U.N. Secretary-General Kofi Annan drew attention to some of the problems caused by plastics in oceans by stating: "Marine trash, mainly plastic, is killing more than a million seabirds and 100 000 mammals and sea turtles each year". Conventional plastics do not biodegrade but break down into smaller and smaller fragments. Microscopic plastic fragments and fibres have been shown to be present in oceans and sediments, and the amount of plastic in plankton samples has tripled between the 1960s and 1990s (Thompson et al., 2004). Plastics (and chemical pollutants absorbed by the plastics) eventually enter the food chain and the consequences of this are not clear, yet.

Plastics are so widely used because they offer advantages over alternative materials such as metal, wood and paper: Plastics are very versatile, very light, durable, cheap, water resistant and transparent to name just some of the advantages. However, the durability is also a disadvantage as the 'Great Pacific Garbage Patch' shows. Moreover conventional plastics are made form fossil fuels and are therefore not sustainable.

A major part of plastics is used for packaging materials due to their lightness and transparency. In the case of for example food packaging some plastics will only be used for a few days before they are disposed, but if not recycled or burned, these packaging materials may still exist in the

environment 100 years later. Especially for short-term applications the use of biodegradable materials such as bioplastics would clearly be beneficial.

Bioplastics are defined for the purpose of this thesis as plastic-like materials that are biodegradable/compostable and made from renewable materials. Some biodegradable plastics are made from non-renewable materials (e.g. polycaprolactone (PCL), Ecoflex® from BASF), while some plastics derived from renewable materials are not biodegradable (e.g. polyamide). These are not considered as bioplastics here.

Bioplastics currently on the market are for example starch-based materials (e.g. produced by Novamont, Italy), cellulose-based materials (e.g. cellophane), polylactic acid (e.g. by NatureWorks, USA; Purac, the Netherlands; Toyota, Japan) and polyhydroxyalkanoates (e.g. Metabolix, USA; PHB Industrial SA, Brazil; Tianan Biologic Material, China; Biomer, Germany). These materials differ greatly in their properties such as moisture permeability, crystallinity, glass transition temperature, melting point etc. They are therefore not necessarily competing with each other but may be used for different applications. In the Netherlands bioplastics are becoming more and more common as food packaging materials for organic fruits and vegetables in supermarkets (Figure 1.1 A). The packaging is marked as compostable in several European countries by a seedling logo (Figure 1.1 B).

Even though polyhydroxyalkanoates (PHAs) have unique properties compared to the other bioplastics like for example high heat and moisture resistance, their market penetration is currently very small. This will probably change once the large scale production facility (110 million pounds PHA per year) of the Metabolix and ADM joint venture 'Telles' will be starting up in the second half of 2009 (www.mirelplastics.com). Also Tianan Biologic Material is planning to increase its production capacity from currently about 2000 tons per year to about 10 000 tons per year (Lunt, 2008). The price of PHA is currently around 4.9 $/kg but is expected to drop once larger scale production facilities are running (Lunt, 2008).

Figure 1.1. A: Examples of bioplastic packaging (pictures from www.naturapackaging.com). **B**: Logo of the European Bioplastics Association for compostable packaging.

Polyhydroxyalkanoate (PHA)

PHA is a polyester of hydroxy fatty acids which is naturally produced by many different bacteria as an intracellular carbon and energy reserve material (Wältermann et al., 2005). Figure 1.2 shows the general structure of PHAs. The most abundant PHA is the homopolymer poly(3-hydroxybutyrate) (PHB) (Anderson et al., 1990). The monomer composition of the polymer, the chain length of the polymer and the length of the side chains influence the properties of the polymer. PHAs are interesting as bioplastics as they can exhibit thermoplastic and/or elastomeric properties, are enantiomerically pure chemicals (only *R*-stereoisomer), non-toxic, biocompatible, made from renewable resources and biodegradable (Steinbüchel, 2001). Moreover, the polymer can be hydrolyzed and the hydroxy fatty acid monomers could serve as chiral building block chemicals for the production of biochemicals (Ren et al., 2005).

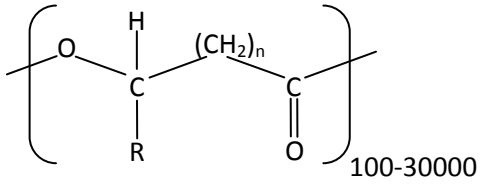

Figure 1.2. General structure of polyhydroxyalkanoates (Lee, 1996). Examples: n = 1, R = CH_3 is poly(3-hydroxybutyrate); n = 1, R = CH_2-CH_3 is poly(3-hydroxyvalerate).

Some bacteria can accumulate PHAs up to a content of 70 to almost 90 wt% of their dry weight (Steinbüchel et al., 1998). For commercial production of PHA, Metabolix is employing genetically engineered bacteria which have been reported to produce 100 g/l PHA in 40 h or less from pure substrates like sugars, resulting in up to 90 wt% cellular PHA content (Anon., 2002). Tianan Biologic Material is using a natural PHA producer (*Ralstonia eutropha*[1]) with the produced polymer being a copolymer of hydroxybutyrate and hydroxyvalerate monomers (PHBV) (Lunt, 2008).

The bacterial metabolism for the production and degradation of PHB has mostly been studied in *Ralstonia eutropha* (Figure 1.3) and is probably similar in other species (Steinbüchel et al., 1991). Starting from acetyl-CoA only three enzyme catalyzed steps are required to produce PHB: a condensation of two molecules of acetyl-CoA to acetoacetyl-CoA, a subsequent reduction to hydroxybutyryl-CoA and finally the polymerization to PHB. The degradation most likely follows a different pathway via hydroxybutyrate and acetoacetate (Figure 1.3). Other PHA monomers can be produced via the fatty acid β-oxidation pathway or via the de novo fatty acid synthesis pathway (see Luengo et al. (2003) for a graphic summary of pathways). PHA is typically produced by bacteria under imbalanced growth conditions or under nutrient limiting

[1] also named *Hydrogenomonas eutropha*, *Alcaligenus eutrophus*, *Wautersia eutropha*, *Cupriavidus necator*

conditions. The regulation of PHB production in *R. eutropha* appears not be regulated on the transcriptional or translational level, but rather at the enzyme level with all three enzymes involved in PHB production being constitutively synthesized (Steinbüchel et al., 1991). The first step catalyzed by the ketothiolase seems to be a key in the regulation of PHB synthesis: the enzyme is inhibited by the presence of free CoA, which presumably occurs under unrestricted growth conditions when the levels of free CoA are high (Steinbüchel et al., 1991). If growth is restricted due to an external limitation (nutrient or oxygen limitation) or due to an internal limitation (insufficient presence of enzymes required for growth), levels of free CoA presumably will be low and PHB production is favoured. The regulation and mechanisms of intracellular PHB degradation are still poorly understood (Jendrossek et al., 2002).

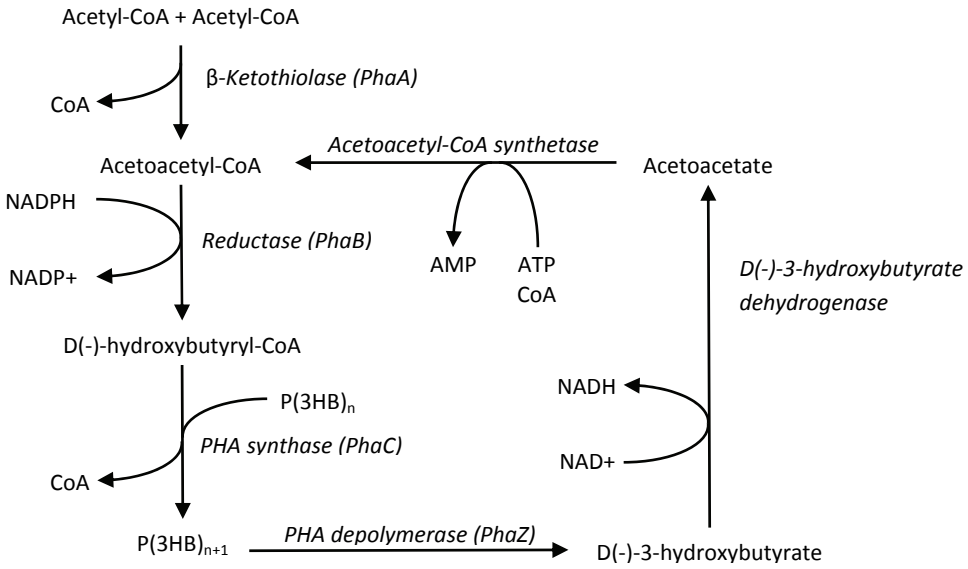

Figure 1.3. Metabolic pathway of synthesis and degradation of polyhydroxybutyrate in *Ralstonia eutropha*, adapted from Lee (1996).

PHA is stored inside the cells in its amorphous state in a number of granules. The name 'carbonosomes' has been proposed by Jendrossek (2009) for the granules as they appear to be complex organized subcellular structures. The granules are covered by proteins such as the PHA synthase, PHA depolymerase, so called phasin proteins and regulatory proteins, and possibly also by a phospholipid monolayer (Jendrossek, 2009; Grage et al., 2009). Phasins are the most abundant protein on the granule surface and their role seems to be related to regulating the size and number of PHA granules as well as stabilizing them (Grage et al., 2009).

PHA needs to be extracted from the bacterial cells for bioplastic production. In the past solvents were used to extract the polymer, now Tianan Biologic Material claims to use a water-based extraction process (Lunt, 2008).

PHA production with mixed cultures

PHA production in mixed cultures has been studied in the past mostly in relation to its relevance in wastewater treatment rather than as a potential production process (van Loosdrecht et al., 1997). Only in recent years optimization of the process has led to improved PHA contents of up to 65 wt% (Serafim et al., 2008a; Dias et al., 2006). This is still low compared to the best axenic cultures of natural producers and recombinant bacteria and requires further optimization for efficient downstream processing of the product.

The subject of mixed culture PHA production has been reviewed in several publications (Reis et al., 2003; Dias et al., 2006; Serafim et al., 2008a). The key for a successful mixed culture PHA production process is the enrichment of a mixed culture with microorganisms that have a superior ability to produce PHA in high amounts (high storage capacity). Two main strategies need to be distinguished for the enrichment of PHA storing bacteria from a mixed culture inoculum such as for example activated sludge: 1) enrichment under alternating anaerobic and aerobic conditions; and 2) enrichment under alternating feast (presence of carbon source) and famine (absence of carbon source) conditions. Both strategies make use of the ecological role of PHA as a storage polymer by applying continuously dynamic conditions that will favour the storage of PHA in one phase and the degradation of PHA in another phase.

In the first strategy substrate containing for example acetate as a carbon source will be supplied to a reactor during an anaerobic phase. Specialized bacteria such as polyphosphate accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs) are able to take up the acetate but cannot grow without the presence of oxygen as a final electron acceptor. However, PAOs and GAOs can convert acetate into PHA. The energy (ATP) and reducing power (NADH or NADPH) required for this process is supplied through degradation of internally stored glycogen/polyglucose and in the case of PAOs also through hydrolysis of internally stored polyphosphate (see Reis et al. (2003) for a graphic representation of the metabolism). After all carbon source is depleted an aerobic phase follows in which the internally stored PHA is used by PAOs and GAOs to grow and to replenish the glycogen/polyglucose and polyphosphate (only PAOs) pools. This process is continuously repeated with a removal of excess biomass at the end of each anaerobic-aerobic cycle, resulting in a very strong selective pressure for organisms best adapted to these conditions such as GAOs and PAOs.

In the second enrichment strategy substrate containing for example acetate as a carbon source will be supplied to a reactor during an aerobic phase. Bacteria present in the reactor can thus grow and/or store PHA during this feast phase. After the feast phase an aerobic phase of carbon starvation follows (famine), in which bacteria that previously stored (part of) the carbon source as PHA will be able to continue growing while bacteria that did not store any carbon source will starve. If this process is continuously repeated with a removal of excess biomass at the end of each feast-famine cycle, microorganisms that are able to store PHA will be enriched since they have a competitive advantage in this system: PHA storing bacteria can take up the carbon source very fast and they can continue to grow in the long famine phase (Johnson et al.,

2009a). The most successful bacterial behaviour in such a feast-famine system will be the behaviour that results in the most new biomass produced over the whole cycle. If PHA storing bacteria take up the substrate very fast and store it as PHA, they secure the greatest amount of substrate and can thus produce the greatest amount of biomass. Competition in the feast-famine system is thus generally not based on growth rate but rather on substrate uptake rate.

After PHA storing bacteria have been enriched with any of the two strategies, the enrichment step would be continued as a biomass production step (with continued enrichment for PHA producing bacteria). The biomass harvested from this step would then be used in a second step for the actual PHA production. For PHA production cultures would be supplied with an excess of carbon source until maximum PHA contents are reached. PHA production can be performed under conditions that prevent growth (e.g. nitrogen limitation) in order to prevent dilution of PHA through formation of new biomass.

Maximum PHA contents reported for cultures enriched with the anaerobic-aerobic strategy are lower than for the feast-famine strategy (Serafim et al., 2008a; Reis et al., 2003). For this reason the feast-famine strategy was chosen for a further optimization of the mixed culture PHA production process in the framework of this thesis.

PHA production with mixed cultures is aiming at using waste streams as a substrate. Waste streams containing mainly carbohydrates would be fermented first through a mixed culture anaerobic fermentation to yield organic acids and alcohols which can then be used as a substrate for PHA production (Temudo, 2008). Waste streams that have been successfully used in this way are for example molasses (Albuquerque et al., 2007), paper mill waste water (Bengtsson et al., 2008a; Bengtsson et al., 2008b), and olive oil mill effluent (Beccari et al., 2009). For this thesis, however, artificial wastewater was used in order to have a high degree of control over the composition of the substrate.

The reactor type used for the biomass enrichment and production step was a sequencing batch reactor (SBR), as this type of reactor is ideal to achieve the dynamic conditions of a feast-famine regime in a continuous way. Each SBR cycle consisted usually of several phases split up over either 4 or 12 hours: (i) a short start phase in which the 2 litre reactor was half full and was stirred and aerated, (ii) a very short filling phase in which the reactor was filled up with fresh medium (stirred and aerated); the beginning of the filling phase was also the beginning of the feast phase, (iii) a long reaction phase consisting of the remaining feast phase (until carbon source depletion) and the famine phase (stirred and aerated), (iv) a short biomass withdrawal phase in which a certain fraction of the reactor liquor was withdrawn to set the sludge residence time (SRT) of the reactor (stirred, not aerated), (v) a short settling phase (not stirred, not aerated), and (vi) a short effluent withdrawal phase in which supernatant was withdrawn until the reactor was only half full (stirred, not aerated). The last phase was immediately followed by the first phase of the next cycle resulting in a continuous sequence of batches with the biomass from the previous cycle being the inoculum for the next cycle.

When a stable (steady state) SBR culture was obtained, i.e. when no significant changes occurred in the reactor behaviour over a period of at least 5 days, the SBR culture was used for a PHA production experiment to evaluate its PHA storage capacity. The PHA production experiments were performed in the same kind of reactor vessel but operated as a fed-batch reactor, usually without the addition of a nitrogen source in order to maximize cellular PHA contents.

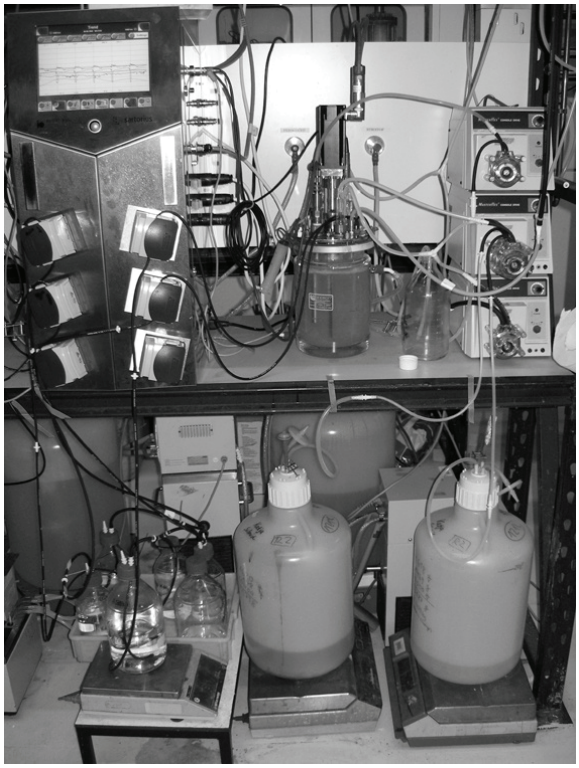

Figure 1.4. Reactor set-up.

Figure 1.4 shows a picture of the SBR set-up. The big bottles in the foreground contained the concentrated carbon source (sodium acetate) and nutrient source, the big bottle in the background dilution water. Water, carbon source and nutrient source were mixed and automatically pumped together in the reactor by the two lower pumps on the right side. The third pump was used for effluent/biomass withdrawal. The smaller glass bottles under the bench contained acid and base for pH control, both pumped via the biocontroller standing on the left side of the reactor.

Figure 1.5 shows the typical conversions of the relevant compounds occurring in a PHA producing feast-famine SBR culture. For simplicity it is assumed that all new medium is pumped instantaneously into the reactor at the beginning of the feast phase. In the feast phase acetate is taken up by the culture along with ammonia (both amounts decrease) and oxygen (Figure 1.5 shows the cumulative amount of oxygen taken up as positive values). Part of the acetate and all ammonia are used to produce new active biomass (amount of active biomass increases). Active biomass is defined as total biomass without PHA. Acetate is also converted into PHB (PHB contents increase). The homopolymer PHB is typically produced from acetate by feast-famine cultures. Oxygen is required as the final electron acceptor for the generation of energy (ATP) during acetate oxidation, while carbon dioxide is produced as the final product remaining after acetate oxidation (together with water). At the end of the feast phase acetate is depleted and the PHB content of the biomass peaks. In the famine phase the previously stored PHB is used as a carbon and energy source for continued growth – PHB contents and amounts of ammonia decrease while the amount of biomass increases. PHB is now oxidized for energy generation which consumes oxygen and releases carbon dioxide.

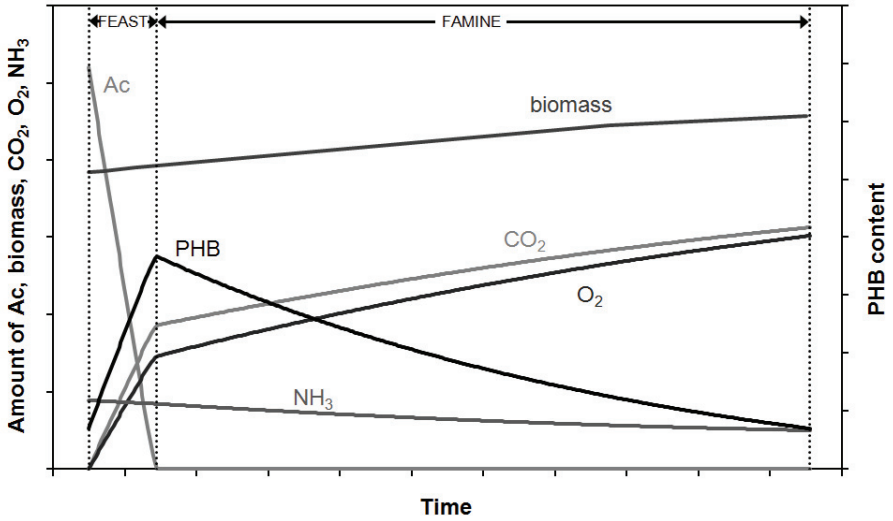

Figure 1.5. Typical SBR cycle of a PHB storing culture. Biomass is active biomass, i.e. without PHB. Carbon dioxide is displayed as cumulative production and oxygen as cumulative consumption. Acetate (Ac), biomass and ammonia are total amounts present in the reactor, PHB contents are presented as amount of PHB relative to the amount of biomass.

The key reactions taking place in the feast and famine phase can be summarized in a metabolic model (Figure 1.6). All reactions represent chains of reactions lumped together into one reaction to keep the model simple. In this way only 5 reaction equations are needed to describe the feast phase and only 4 for the famine phase. The stoichiometry of most of these reactions is quite well known (van Aalst-van Leeuwen et al., 1997). The three cell internal metabolites acetyl-CoA, ATP and NADH are treated as conserved moieties, i.e. all acetyl-CoA, ATP and NADH produced in any of the reactions needs to be consumed in any of the other reactions so that no net accumulation of these compounds occurs. The 3 conserved moieties couple the 6 reactions, leaving only 3 degrees of freedom for the system. The kinetic model, which complements the stoichiometric model and makes the metabolic model complete, therefore only requires the definition of 3 reaction rates (2 for the feast and one for the famine phase) and the remaining rates will follow from stoichiometry. The metabolic model is described in more detail in Chapter 2 of this thesis.

The metabolic model can be used to model conversions of acetate, PHB, ammonia, active biomass, carbon dioxide and oxygen in the SBR or in fed-batch experiments and to compare them with actual measurements. In this way model parameters can be estimated and the quality of the measurements can be evaluated. The model can also be used to find better estimates for all reaction rates, conversions and observed yields for measured data sets. The model is thus a very useful tool when optimizing the PHA production process and comparing different experiments.

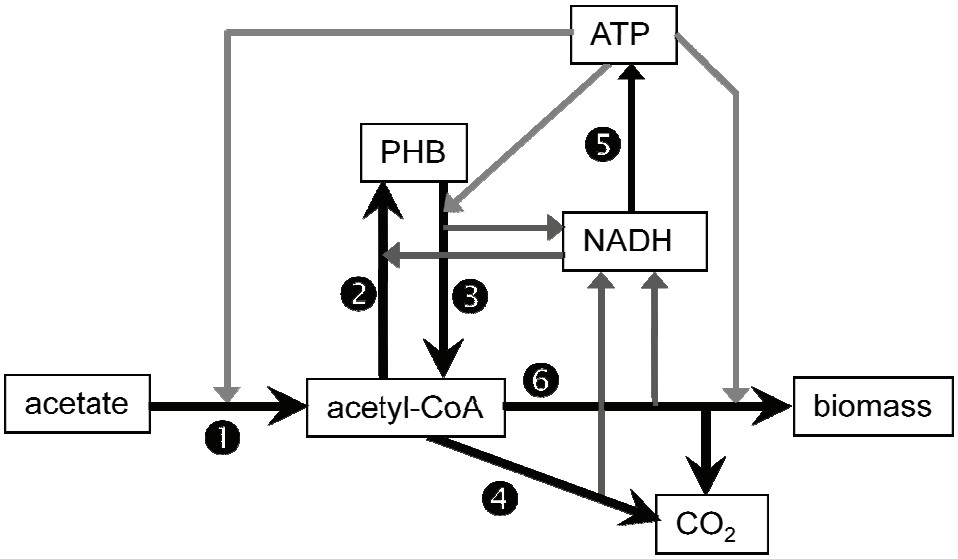

Figure 1.6. Graphic representation of the metabolic model for PHB producing feast-famine cultures. ❶ acetate uptake and activation (only in feast phase), ❷ PHB production (only in feast phase), ❸ PHB consumption (only in famine phase), ❹ catabolism, ❺ oxidative phosphorylation, ❻ anabolism.

Scope and outline of this thesis

The aim of this thesis was to study and optimize PHA production of mixed microbial cultures enriched in feast-famine SBRs. Cultures were enriched in feast-famine SBRs under different conditions such as different sludge residence times (SRT), different cycle lengths, carbon and nitrogen limitation, and different temperatures in order to investigate the influence of these parameters on the PHA metabolism. The PHA storage capacity, as one of the key constraints for process optimization, was evaluated in fed-batch experiments for each culture.

For comparison of the different conditions employed in the SBR it was necessary to calculate all reaction rates and observed yields for each SBR steady state. **Chapter 2** describes the very detailed data analysis that was carried out for each SBR cycle measurement and fed-batch experiment. The data analysis included for example the correction of measurements for sampling effects, the computation of oxygen consumption and carbon dioxide evolution, and the calculation of the best estimates for all reaction rates and total conversions at each time point with the help of a metabolic model.

Chapter 3 deals with the first two process parameters that were investigated for their effect on the SBR behaviour and PHA storage capacity: low SRTs (0.5 to 4 d) and the carbon to nitrogen ratio of the medium supplied to the SBR. Low SRTs are unusual for a wastewater based process since biomass production is usually to be avoided during wastewater treatment. However, for a commercial PHA production process the production of PHA producing biomass at high rates is essential and low SRTs will be required for high productivities. The carbon to nitrogen ratio of the medium is relevant since many suitable wastewaters for PHA production are nitrogen limited. Nitrogen limitation is beneficial for reaching high PHA contents in the fed-batch PHA

production stage, but it was unclear how nitrogen limitation versus carbon limitation would effect the enrichment of PHA storing bacteria in the SBR.

The influence of temperature is described in **Chapter 4**. The reactor temperature may have several effects in a feast-famine SBR. Generally biological reaction rates increase and decrease with a small increase or decrease in temperature, respectively. Different reaction rates may have different sensitivities to temperature changes and the reactor behaviour can thus change with a temperature change. This would be an effect that can be observed in short-term experiments. Additionally, there will be a long-term temperature effect as the mixed culture adapts to the changed temperature and the selective pressure changes in favour of organisms with a different optimum temperature. The short- and long-term effects of temperature changes on the SBR behaviour and on maximum PHA storage capacities were investigated in this chapter.

In **Chapter 5** results are reported from a culture for which the results from previously investigated parameters (including also cycle length) were considered in order to optimize the culture for a high PHA storage capacity. This culture was operated for over a year under the same conditions and the effect of time on the storage capacity of this culture was also studied.

Chapter 6 deals with the PHA production stage of the process. The fed-batch experiments for PHA production in previous experiments were successfully executed by just feeding a carbon source and therefore avoiding growth and the associated dilution of PHA through biomass. Also this stage is envisaged to be supplied with real wastewater in a commercial PHA production process. Although these wastewaters are often nitrogen limited, they are usually not free of nitrogen and may even contain an excess of nitrogen in some cases. It was therefore relevant to study the influence of the carbon to nitrogen ratio on PHA accumulation (rate and maximum storage capacity) in fed-batch experiments.

Chapter 7 summarizes and integrates the findings from all individual studies. In this chapter also some remaining issues are discussed and recommendations for future research are provided.

CHAPTER 2

Model-Based Data Evaluation of Polyhydroxybutyrate Producing Mixed Microbial Cultures in Aerobic Sequencing Batch and Fed-Batch Reactors

Katja Johnson, Robbert Kleerebezem, Mark C. M. van Loosdrecht

Biotechnology and Bioengineering (2009) 104(1):50-67

ABSTRACT

The production of polyhydroxyalkanoates (PHA) with mixed microbial cultures is a promising approach for the sustainable production of bioplastics. Usually a two-step process is employed consisting of (i) the enrichment of a PHA producing mixed culture in a sequencing batch reactor (SBR) and (ii) the subsequent PHA production in a fed-batch reactor. Both reactors are highly dynamic systems, particularly if the SBR is working at low sludge residence times (SRT) or if growth is (partly) permitted in fed-batch systems. Under these conditions the concentrations of substrate, PHA and biomass change rapidly, complicating the identification of biomass specific conversion rates as required for process characterization. We developed a structured approach for the evaluation of such SBR and fed-batch experiments consisting of five steps: 1. Measurement of a sufficiently large set of parameters including off-gas concentrations, 2. Corrections of measurements for effects of sampling and addition of liquids (pH control, substrate), 3. Calculation of oxygen uptake and carbon dioxide evolution rates, the latter including inorganic carbon dissolved in the liquid phase, 4. Balancing of the measured conversions, 5. Evaluation of the measurements by means of a metabolic model. This approach has been successfully applied to a large number of data sets. Step 1-4 ensured that data sets of high quality were obtained. Step 5 allowed to find the best estimates for all conversions and biomass specific rates for the measured data sets, while complying with material balances. Conversions of the substrate acetate, the nitrogen source ammonia and of the storage polymer PHA (here polyhydroxybutyrate (PHB)) were described very accurately by the model. Modelled off-gas conversions often deviated somewhat from measured conversions, which might be partly due to an inaccurate model stoichiometry. Nonetheless, the described approach proved to be a very useful tool for the evaluation and comparison of PHB producing cultures.

Introduction

Polyhydroxyalkanoates (PHAs) are biopolymers of hydroxy fatty acids which are naturally produced by many different bacteria as an intracellular carbon and energy reserve material (Wältermann et al., 2005). The properties of PHAs resemble those of some polyolefins with the added benefit of being biodegradable and made from renewable resources (Braunegg et al., 1998). Industrial processes for the production of PHAs as a bioplastic employ generally pure cultures of natural PHA producers or recombinant bacteria, but the production costs are still too high for PHAs to become a competitive commodity plastic (Dias et al., 2006). Production costs could potentially be reduced by using open undefined mixed culture processes for the production of PHA and waste streams rather than pure chemicals as substrates. In open mixed culture systems sterilization of the bioreactors and media would not be required, leading to lower energy and equipment costs (Reis et al., 2003). A considerable amount of research is currently directed towards mixed culture PHA production (Albuquerque et al., 2007; Bengtsson et al., 2008a; Dai et al., 2007; Dionisi et al., 2006; Serafim et al., 2008b). For a review see Dias et al. (2006).

A promising approach for producing PHAs with mixed cultures is the use of a two-step process. In the first step a PHA producing culture is enriched using alternating presence and absence of the carbon source (feast and famine periods) as a selective pressure, hereby making use of the ecological role of PHAs as a storage polymer. This feast-famine regime can be implemented using a sequencing batch reactor (SBR). In the second step the culture enriched in the first step is subjected to continuous presence of the carbon source, usually under growth limiting conditions, in order to maximize the cellular PHA content (fed-batch reactor). This process has been reported to result in up to 65 wt% cellular polyhydroxybutyrate (PHB) content (Dias et al., 2006), while recombinant *E.coli* has been reported to reach up to 80 - 90 wt% (Slater et al., 1988).

In order to be competitive with commercial PHA production processes, the mixed culture process requires further optimization for higher PHA contents and rates. For process optimization studies it is crucial to calculate and compare biomass specific rates, observed yields and maximum PHA contents. In SBRs operated at high sludge residence times (SRT) and short cycles (e.g. SRT ≥ 4 d, 4 h cycles) averaged biomass specific rates can be easily measured and calculated by assuming that the concentration of active biomass (i.e. not considering intracellular PHA) does not change throughout the cycle (Beun et al., 2002). However, SBRs operated at low SRTs as in our experiments are highly dynamic systems where rate and concentration changes (including biomass concentration) occur rapidly. Therefore it is very difficult to measure rates and yields accurately and a substantial amount of sampling is required. Especially changes in the concentration of active biomass are difficult to measure, but are essential for the calculation of biomass specific rates. Conversely, if a large amount of samples is taken from the reactor, a significant fraction of biomass is removed which will influence measurements such as off-gas measurements or substrate requirements in fed-batch experiments. Additional errors can occur if the diluting effect of acid and/or base dosed for pH control on concentrations measured in the reactor broth is neglected.

In order to manage the described problems, we have developed a structured approach to measure and evaluate PHB producing SBR and fed-batch cultures: Firstly it is important to measure a sufficiently large set of the relevant state variables in the reactor liquid and off-gas throughout a SBR cycle or fed-batch experiment. Secondly a correction needs to be made at each time point due to the variable reactor liquid volume caused by sampling, pH control and substrate addition. Thirdly oxygen and carbon dioxide transfer rates can be calculated from the off-gas measurements. These are used to compute oxygen uptake rates and carbon dioxide evolution rates, the latter considering inorganic carbon dissolved in the reactor liquid phase. Fourthly carbon, nitrogen and electron / chemical oxygen demand (COD) balances are computed for each sampling point to check for inconsistencies and the quality of the measurements. In a final step a generalized metabolic model is used to calculate biomass specific rates and conversions, while ensuring that all balances close. 'Redundant' measurements (like measuring growth over one SBR cycle with total suspended solids (TSS)

samples, ammonia samples and SRT) can be included in the procedure to find the best estimate for the state variables (e.g. biomass concentration).

This paper aims to provide a tool for the evaluation of measurements of PHB producing mixed cultures which are increasingly operated under highly dynamic conditions. The methodology we describe here will be particularly useful for SBRs operated at short SRTs (< 4 days) but also for SBRs operated at longer SRTs, if for example a significant fraction of the reactor volume is removed through sampling or if material or electron balances do not close. Additionally our approach can be applied for fed-batch experiments for PHA production in order to calculate the continuously changing PHA production rate.

The described approach is not limited to these cultures, but can be used for any dynamic fermentation process where a metabolic model is available.

Materials and Methods

Sequencing batch reactor (SBR) for culture enrichment

A double-jacket glass bioreactor with a working volume of 2 litres (Applikon, The Netherlands) was used for the cultivation of PHB producing bacterial cultures. Operation of the SBR was based on either 4 or 12 h batch cycles consisting of a start phase, an influent phase (fresh medium was supplied), a reaction phase and a biomass withdrawal phase. If the sludge residence time (SRT) required was longer than the hydraulic residence time (HRT) a settling phase and effluent withdrawal phase were introduced after the biomass withdrawal phase. The amount of biomass withdrawn was adjusted according to the SRT required.

The reactor was equipped with a stirrer with two standard geometry six-blade turbines. The flow of air to the reactor was controlled with a mass flow controller (Brooks Instrument, USA). In experiments without off-gas recycling an air flow between 1.3 – 1.8 l_N/min was used. In experiments with off-gas recycling the flow of air was reduced to 0.2 l_N/min and off-gas was recirculated about six to seven times through the reactor. All gas flows were checked with a gas flow analyzer before conducting experiments. Tubing between the reactor and the analyzer was kept as short and thin as possible in order to avoid delays in the measurement. The reactor was temperature controlled at either 15, 20 or 30°C by means of a water jacket and a thermostat bath (Lauda, Germany). The pH of the reactor liquid was maintained at 7.0 $\pm$ 0.1 using 1 M HCl and 1 M NaOH. The pumps, the stirrer, the airflow and the pH were controlled by a biocontroller (BIOSTAT B plus, Sartorius or ADI 1030, Applikon, NL) which in turn was controlled by a PC using the software MFCS/win (Sartorius Stedim Systems, USA) or BIODACS (Applikon, NL). MFCS/win or BIODACS were also used for data acquisition of the online measurements (DO, pH, temperature, acid dosage, base dosage, off-gas oxygen and carbon dioxide).

The amount of biomass removed from the reactor during both the biomass and effluent withdrawal phase determined the SRT (see Appendix H). The reactor was considered to be in

steady state when for at least five days (i) the concentration of total suspended solids (TSS) at the end of the cycle, (ii) the measured SRT and (iii) the length of the feast phase as indicated by the DO changes were constant. The SRT was varied from 0.5 d to 4 d. The HRT was 8 h in experiments with 4 h cycles and 24 h in experiments with 12 h cycles. When the reactor was in steady state, a cycle of the SBR was monitored additionally to the online measurements by offline samples (acetate, TSS, PHA, ammonia).

The initial inoculum of the SBR was aerobic activated sludge from the second aerobic stage of the Dokhaven wastewater treatment plant in Rotterdam, the Netherlands (September 2004). For each new experiment the sludge from the previous experiment was used as inoculum. Over the months the sludge was subjected to different conditions. Biomass was harvested from the reactor at different time points in order to study the PHA production capacity of the biomass in fed-batch experiments.

The medium for the SBR consisted of $NaAc \cdot 3H_2O$, NH_4Cl, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, KCl, trace elements solution according to Vishniac and Santer (1957) and allylthiourea (to prevent nitrification). Concentrations were varied for each experiment. The medium was preheated or cooled to the reactor temperature before being pumped into the reactor.

Fed-batch reactor for PHB production

Steady state biomass from the SBRs was used for PHB production in fed-batch experiments. For this purpose the same set-up as for the culture selection was used, but in a fed-batch mode. 1 l of culture from the end of a SBR cycle was mixed with 1 l of acetate- und ammonia-free medium (same composition as for the SBR, but no NaAc and NH_4Cl). To start the PHB production, a pulse of about 40 - 60 mmol sodium acetate was fed to the reactor. Further carbon source was either supplied manually as pulses of sodium acetate (when DO signal increased) or automatically with 1 or 1.5 M acetic acid via the pH control (set to pH 7). Growth was limited in these experiments as no nitrogen source was supplied and only a small amount (if any) remaining nitrogen source from the previous SBR cycle was available. The progress of the experiments was monitored via online (DO, pH, acid and base dosage, off-gas CO_2 and O_2) and offline (acetate, TSS, PHA, ammonia) measurements.

Analytical Methods

The concentration of dissolved oxygen (DO) in the reactor was measured with a DO electrode (Mettler Toledo, USA) as percentage of air saturation and the pH was monitored with a pH electrode (Mettler Toledo, USA). The temperature of the reactor broth was measured with a thermo element. The amount of acid or base dosed for pH control was measured online. Carbon dioxide and oxygen partial pressures in the gas entering and leaving the reactor were analyzed in dried gas with a gas analyzer (NGA 2000, Rosemount, USA). Gas measurements and calibrations were corrected with the actual atmospheric air pressure for standard conditions.

Samples taken from the reactor for analysis of acetate and ammonia were immediately filtered with a 0.45 µm pore size filter (PVDF membrane, Millipore, Ireland). The acetate concentration in the supernatant was measured with a Chrompack CP 9001 gas chromatograph (Chrompack, The Netherlands) equipped with a FID, on a HP Innowax column. The ammonia concentration was determined spectrophotometrically as ammonium-nitrogen with a cuvette test (Lange, Germany). The biomass concentration was measured as total suspended solids (TSS) by filtration according to standard methods (Taras et al., 1971). The amount of PHB was subtracted from the TSS to calculate the concentration of active biomass. The active biomass concentration was converted from g/l into carbon moles per litre (Cmol/l) assuming a composition of $CH_{1.8}O_{0.5}N_{0.2}$ with a molecular weight including ash of 25.1 g/Cmol (Beun et al., 2002).

Samples taken for PHA analysis were added to 15 ml tubes containing 5 drops of formaldehyde in order to stop all biological activity. Samples were subsequently washed with tap water and freeze-dried. Pure PHB (Sigma) was used as a standard in the analysis and treated alongside with the samples. Freeze-dried biomass samples and 3 standards were weighed with an analytical balance and transferred into tubes with screw caps. 1 mg of benzoic acid in 1-propanol was added as the internal standard. 1.5 ml of a mixture of concentrated HCl and 1-propanol (1:4) and 1.5 ml of dichloroethane was added. The closed tubes were heated for 2 h at 100°C. After cooling, free acids were extracted from the organic phase with 3 ml water. 1 ml of the organic phase was filtered over water-free sodium sulphate into GC vials. The propylesters in the organic phase were analyzed by gaschromatography (model 6890N, Agilent, USA) equipped with a FID, on a HP Innowax column. Results were expressed as weight percentage of PHB of the total solids.

Modelling

The software Microsoft Excel was used for all data evaluations and modelling. The solver tool implemented in Microsoft Excel was used for the parameter estimation based fitting of the model to the measurements. The Microsoft Excel solver tool uses the Generalized Reduced Gradient (GRG2) nonlinear optimization code.

Data treatment and modelling

Measurement corrections for sampling and pH control

When a number of samples are taken from a SBR or fed-batch reactor during an experiment, the amount of biomass removed from the reactor with the samples can be significant with lab-scale reactors (typically around 5 - 20% in our experiments). Sampling will result in a decrease of oxygen uptake and carbon dioxide production and in fed-batch experiments with pH controlled feeding also in a decrease in substrate dosing. If neglected, this loss of biomass can lead to gaps in the balances and misinterpretation of measured conversion rates.

The addition of acid and base for pH control has a diluting effect on the measured concentrations in the liquid phase (TSS, acetate, ammonia). The extent of the dilution effect depends on many factors including the concentrations of the acid and base solution, the buffering capacity of the medium and the amount of substrate fed to the reactor. This dilution effect may be negligible in SBR cycles; in fed-batch experiments, however, it can have a considerable impact if liquid substrate is supplied to the reactor. Both the sampling and the dilution effect can be accounted for by correcting the measured conversions and concentrations back to a standard volume of a reactor without sampling and liquid addition. This is described in detail in Appendix B.

Oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER)

Carbon and electron balances are important tools for the evaluation and assessment of experiments and the correct measurement of OUR and CER is therefore essential. OUR is often measured using a DO electrode in a respirometer outside the reactor (e.g. Serafim et al., 2004) or by interrupting aeration in the main reactor (e.g. Dionisi et al., 2006), however, this brings in extra uncertainty and does not allow for a continuous measurement of the OUR and there is no equivalent measurement available for CER.

Alternatively, carbon dioxide and oxygen measurements in the reactor off-gas can be used to calculate the OUR and CER at any time point during an experiment (Pratt et al., 2003). For a high accuracy of this method it is important that the concentration differences between the air entering the system and the off-gas leaving the system is much larger than the inaccuracy of the measurement. This is generally not a problem for the carbon dioxide measurement as the change from e.g. 0.03% to 0.30% is a relatively large change. In contrast, a corresponding change from 20.95% oxygen in air to 20.68% in off-gas is a relatively small change and difficult to measure accurately enough. In order to achieve a greater depletion of oxygen in the off-gas, the air flow rate can be reduced, but a reduced air flow rate will also lead to a lower gas-liquid mass transfer rate and therefore potentially oxygen limitation. A better method to increase oxygen depletion is the use of off-gas recycling. The flow of fresh air can be decreased almost down to the minimum amount required by the off-gas analyzer, while the recycling stream can be adjusted to yield the same gas-liquid mass transfer as without off-gas recycling. Figure 2.1 shows a schematic representation of the off-gas recycling system.

The oxygen transfer rate (OTR) and carbon dioxide transfer rate (CTR) in the whole system were calculated from the mass balance over the gas phase of the whole system (neglecting the accumulation term) as shown in Table 2.1, equations (I-1) and (I-2). $x_{CO_2}^{S,out}$, $x_{CO_2}^{S,in}$, $x_{O_2}^{S,out}$ and $x_{O_2}^{S,in}$ are the molar fractions of carbon dioxide and oxygen, respectively, in the gas entering the system (“in”, air) and leaving the system (“out”, as measured by the analyzer) and V_M is the molar gas volume. Changes in the gas flow rate $\dot{Q}_G^{in}$ due to oxygen consumption and carbon dioxide production were neglected as the difference in transfer rates of oxygen and carbon

dioxide is very small in comparison to the gas flow rate (error < 1%). The transfer rates were corrected with the correction factor f_G for the influence of sampling (see Appendix B).

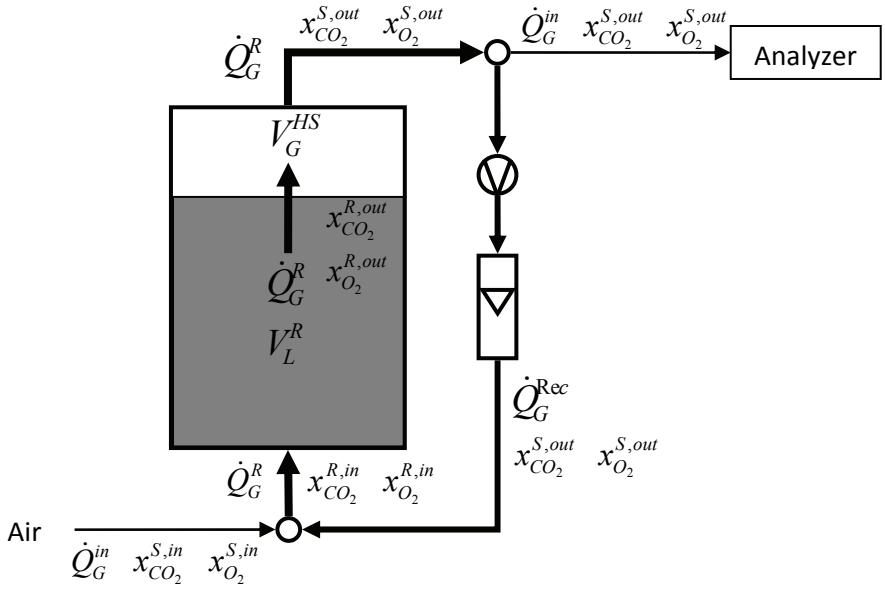

Figure 2.1. Scheme of the off-gas recycling set-up. S and R indicate boundaries for mass balances for calculating oxygen and carbon dioxide transfer rates.

Table 2.1. Oxygen and carbon dioxide transfer rates (OTR, CTR) and oxygen uptake and carbon dioxide evolution rates (OUR, CER). Superscript S indicates the whole system, R indicates the reactor. See also Figure 2.1.

Oxygen	**Carbon dioxide**
$OTR^S(t_i) = (x_{O_2}^{S,out}(t_i) - x_{O_2}^{S,in}) \cdot \frac{\dot{Q}_G^{in}}{V_M} \cdot f_G(t_i)$ (I-1)	$CTR^S(t_i) = (x_{CO_2}^{S,out}(t_i) - x_{CO_2}^{S,in}) \cdot \frac{\dot{Q}_G^{in}}{V_M} \cdot f_G(t_i)$ (I-2)
$OTR^R(t_i) = (x_{O_2}^{R,out}(t_i) - x_{O_2}^{R,in}(t_i)) \cdot \frac{\dot{Q}_G^{R}}{V_M} \cdot f_G(t_i)$ (I-3)	$CTR^R(t_i) = (x_{CO_2}^{R,out}(t_i) - x_{CO_2}^{R,in}(t_i)) \cdot \frac{\dot{Q}_G^{R}}{V_M} \cdot f_G(t_i)$ (I-4)
$OUR(t_i) = OTR^R(t_i) + \frac{dDO(t_i)}{dt_i \cdot 100\%} \cdot V_L^R \cdot P(t_i) \cdot H_{O_2} \cdot x_{O_2}^{S,in}$ (I-5)	$CER(t_i) = CTR^R(t_i) + \frac{dIC_{aq}(t_i)}{dt_i}$ (I-6)
$cumOU(t_i) = \int_0^{t_i} OUR(t_j)\, dt_j$ (I-7)	$cumCE(t_i) = \int_0^{t_i} CER(t_j)\, dt_j$ (I-8)

As the reactor had a relatively large headspace of about 1 l and because the off-gas was recycled through the reactor several times, fast changes in the oxygen consumption and carbon dioxide production rates were not well reflected in the transfer rates calculated with balances over the whole system. Therefore independent mass balances over the gas phase in the reactor liquid were used to calculate the oxygen and carbon dioxide transfer rates more accurately (equations (I-3) and (I-4)). The molar fractions of carbon dioxide and oxygen in the gas entering

the liquid phase of the reactor ($x_{CO_2}^{R,in}$, $x_{O_2}^{R,in}$) were derived from a mass balance over the "mixer" of air and recycled gas (see Appendix C). The molar fractions of carbon dioxide and oxygen in the gas leaving the liquid phase of the reactor and entering the headspace ($x_{CO_2}^{R,out}$, $x_{O_2}^{R,out}$) were derived from the mass balance over the headspace gas volume (see Appendix C). $\dot{Q}_G^R$ is the gas flow rate through the reactor liquid phase which is equal to the sum of $\dot{Q}_G^{in}$ and the recycle stream $\dot{Q}_G^{Rec}$.

The oxygen uptake rate (OUR) of the microorganisms was calculated from the OTR with a balance over the reactor liquid phase (equation (I-5)) as the sum of the transferred oxygen and the DO change in the liquid.

In principal, the carbon dioxide evolution rate (CER) can be calculated in the same way from the CTR, however, the dissolved carbon dioxide concentrations were not measured. While the changes of the amount of dissolved oxygen are negligible in the calculation of the OUR, the changes in the amount of inorganic carbon (IC) dissolved in the liquid are significant and can not be neglected, as carbon dioxide reacts with water to form carbonic acid, which further reacts to bicarbonate and carbonate:

$$CO_2^G \xleftrightarrow{k_{L,CO_2}a} \left\{CO_2^{aq} + H_2O \leftrightarrow H_2CO_3\right\} \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+ \qquad (2.1)$$

Carbon dioxide in the liquid phase and carbonic acid are usually lumped together as carbonic acid. The acid/base reactions involving carbonic acid, bicarbonate and carbonate are faster than the hydration of carbon dioxide, but the mass transfer from the liquid to the gas phase is considered to be even slower and was thus assumed to be the rate limiting step for carbon dioxide stripping (Hill, 2006; Pratt et al., 2003). The concentration of inorganic carbon dissolved in the liquid phase IC_{aq} can then be calculated from the mass transfer and acid/base equilibria (see Appendix E).

The carbon dioxide evolution rate (CER) of the microorganisms was consequently calculated from a balance over the reactor liquid phase as shown in Table 2.1, equation (I-6).

Finally, the cumulative amount of carbon dioxide released and oxygen taken up by the microorganisms ($cumCE$, $cumOU$) at a certain time point were calculated by integrating the time dependent evolution and uptake rates over time (equations (I-7) and (I-8)).

Metabolic model

Stoichiometry

Van Aalst-van Leeuwen et al. (1997) proposed a metabolic model for acetate-fed PHB producing cultures of *Paracoccus pantotrophus,* which involves seven metabolic reactions: (i) acetate uptake and activation to acetyl-CoA, (ii) synthesis of biomass monomers from acetyl-CoA, (iii) polymerization of biomass monomers and maintenance, (iv) catabolism, (v) oxidative

phosphorylation, (vi) PHB production from acetyl-CoA, and (vii) PHB degradation to acetyl-CoA. Subsequently this model has been applied by Beun et al. (2002) and Dias et al. (2005) for mixed feast-famine cultures of PHB producing microorganisms and was found to describe experimental results very well. The same model was applied in this work for our acetate-fed PHB producing mixed cultures, however, a few alterations were made (see Table 2.2 for the exact reaction equations): (i) all reactions were defined on a carbon-mole base where applicable, (ii) compounds that were not considered in balances (e.g. H_2O, CoA) were omitted from the equations, (iii) biomass monomer production and polymerization were combined in one equation, (iv) the biomass composition employed by Beun et al. (2002) was used, and (v) maintenance was taken out of the equations, as the main purpose of the reaction equations was to derive the maximum stoichiometric yields and maintenance has no influence on these. ATP requirements for maintenance were included in the kinetics.

Table 2.2. Reactions considered in the metabolic model on a carbon-mole base where applicable (adapted from van Aalst-van Leeuwen et al. (1997)). δ is the efficiency of oxidative phosphorylation.

1	Acetate uptake, activation	$1\,HAc + 1\,ATP \rightarrow 1\,Ac\text{-}CoA$
2	PHB production	$1\,Ac\text{-}CoA + 0.25\,NADH \rightarrow 1\,PHB$
3	PHB consumption	$1\,PHB + 0.25\,ATP \rightarrow 1\,Ac\text{-}CoA + 0.25\,NADH$
4	Catabolism	$1\,Ac\text{-}CoA \rightarrow 2\,NADH + 1\,CO_2$
5	Oxid. phosphorylation	$1\,NADH + 0.5\,O_2 \rightarrow \delta\,ATP$
6	Anabolism	$1.267\,Ac\text{-}CoA + 0.2\,NH_3 + 2.16\,ATP \rightarrow CH_{1.8}O_{0.5}N_{0.2} + 0.267\,CO_2 + 0.434\,NADH$

Table 2.3. Stoichiometric yields derived from the metabolic reactions and balances for the conserved moieties, expressed as a function of the efficiency of oxidative phosphorylation δ.

Feast phase				
Growth	$Y_{CO_2/X}^{feast} = \frac{0.1\delta + 3.16}{2\delta - 1}$	$Y_{O_2/X}^{feast} = -\frac{3.21}{2\delta - 1}$	$Y_{X/Ac}^{feast} = -\frac{2\delta - 1}{2.1\delta + 2.16}$	$Y_{NH_3/X}^{feast} = -0.2$
PHB production	$Y_{CO_2/PHB}^{feast} = \frac{0.25\delta + 1}{2\delta - 1}$	$Y_{O_2/PHB}^{feast} = -\frac{1.125}{2\delta - 1}$	$Y_{PHB/Ac}^{feast} = -\frac{2\delta - 1}{2.25\delta}$	
Catabolism	$Y_{CO_2/Ac}^{feast} = -1$	$Y_{O_2/Ac}^{feast} = 1$	$Y_{ATP/Ac}^{feast} = 2\delta - 1$	
Famine phase				
Growth	$Y_{CO_2/X}^{fam} = \frac{2.41 - 0.15\delta}{2.25\delta - 0.25}$	$Y_{O_2/X}^{fam} = -\frac{2.693}{2.25\delta - 0.25}$	$Y_{X/PHB}^{fam} = -\frac{2.25\delta - 0.25}{2.1\delta + 2.16}$	$Y_{NH_3/X}^{fam} = -0.2$
Catabolism	$Y_{CO_2/PHB}^{fam} = -1$	$Y_{O_2/PHB}^{fam} = 1.125$	$Y_{ATP/PHB}^{fam} = 0.25 - 2.25\delta$	

Of the six reactions five need to be considered during the feast phase (no PHB degradation) and four during the famine phase (no acetate uptake, no PHB production). NADH, ATP and acetyl-CoA are treated as conserved moieties, i.e. there is no net production or consumption of these compounds. Balances of the conserved moieties produced and consumed can therefore be

used to calculate the stoichiometric maximum yields (van Aalst-van Leeuwen et al., 1997) as summarized in Table 2.3.

Kinetics

Feast phase

In the feast phase five reactions (reactions 1 - 2 and 4 - 6 in Table 2.2) occur. Due to the three conserved moieties NADH, ATP and acetyl-CoA, these five reactions are coupled to each other, reducing the degree of freedom from five rates to two rates, i.e. if two reaction rates are defined, the remaining reaction rates follow from these two. The biomass specific acetate uptake rate $\tilde{q}_{Ac}$ was modelled with saturation kinetics with a small half-saturation constant (see Table 2.4, equation (IV-1a)) (Dias et al., 2005). Likewise the biomass specific growth rate $\tilde{\mu}^{feast}$ can be modelled with a maximum growth rate and with saturation terms for acetate and ammonia (equation (IV-2)). As maintenance requirements were ignored in the reaction stoichiometry, they needed to be included at this stage. Maintenance was considered to be a constant biomass specific ATP consumption rate m_{ATP} . The rate of acetate consumption due to maintenance m_{Ac} can then be calculated with the stoichiometric yield of ATP on acetate (equation (IV-3)).

If the acetate uptake rate and growth rate are defined, all other rates can be calculated. The PHB production rate $\tilde{q}_{PHB}^{feast}$ follows as a kind of 'overflow' metabolism from the amount of acetate that is not used for growth and maintenance purposes (equation (IV-4a)).

If the culture reaches high PHB contents, it was observed that the PHB production rate was inhibited by the high PHB content (Dias et al., 2005). In our experiments under conditions of nitrogen limitation also the acetate uptake rate decreased at high PHB contents. Under these circumstances, PHB production needs to be defined with an independent rate description. Similar to Dias et al. (2005), the PHB production rate was defined as a maximum biomass specific rate multiplied with a saturation term for acetate and an inhibition term depending on the cellular concentration of PHB $\tilde{f}_{PHB}$ (equation (IV-4b)). At high PHB contents the rate of acetate uptake then follows from the acetate requirements for PHB production, growth (if any) and maintenance (equation (IV-1b)).

Whether the 'overflow' metabolism (equations (IV-1a) and (IV-4a)) was used to calculate the acetate uptake and PHB production rate or whether PHB inhibition needed to be considered (equation (IV-1b) and (IV-4b)) was set by the smallest PHB production rate (see Table 2.4).

Further rates that needed to be calculated were the biomass specific carbon dioxide evolution, oxygen uptake and ammonia uptake rate. These follow from the already known rates with the stoichiometric yields (equations (IV-5), (IV-6) and (IV-7)).

Table 2.4. Model kinetics.

Feast phase			
Acetate uptake	$\tilde{q}_{Ac,1}(t) = \tilde{q}_{Ac}^{\max} \cdot \frac{\tilde{c}_{Ac}(t)}{K_{Ac} + \tilde{c}_{Ac}(t)}$	If $\tilde{q}_{PHB,1}^{feast} \le \tilde{q}_{PHB,2}^{feast}$	(IV-1a)
- with PHB inhibition	$\tilde{q}_{Ac,2}(t) = \tilde{\mu}^{feast}(t) \cdot \frac{1}{Y_{X/Ac}^{feast}} + \tilde{q}_{PHB}^{feast} \cdot \frac{1}{Y_{PHB/Ac}^{feast}} + m_{Ac}$	If $\tilde{q}_{PHB,1}^{feast} > \tilde{q}_{PHB,2}^{feast}$	(IV-1b)
Growth	$\tilde{\mu}^{feast}(t) = \tilde{\mu}^{\max} \cdot \frac{\tilde{c}_{NH_3}(t)}{K_{NH_3} + \tilde{c}_{NH_3}(t)} \cdot \frac{\tilde{c}_{Ac}(t)}{K_{Ac} + \tilde{c}_{Ac}(t)}$		(IV-2)
Maintenance	$m_{Ac} = \frac{m_{ATP}}{Y_{ATP/Ac}^{feast}}$		(IV-3)
PHB production	$\tilde{q}_{PHB,1}^{feast}(t) = \left(\tilde{q}_{Ac}(t) - \mu^{feast}(t) \cdot \frac{1}{Y_{X/Ac}^{feast}} - m_{Ac} \right) \cdot Y_{PHB/Ac}^{feast}$	If $\tilde{q}_{PHB,1}^{feast} \le \tilde{q}_{PHB,2}^{feast}$	(IV-4a)
- with PHB inhibition	$\tilde{q}_{PHB,2}^{feast}(t) = \tilde{q}_{PHB}^{\max} \cdot \frac{\tilde{c}_{Ac}(t)}{K_{Ac} + \tilde{c}_{Ac}(t)} \cdot \left[1 - \left(\frac{\tilde{f}_{PHB}(t)}{\tilde{f}_{PHB}^{\max}(t)} \right)^{\alpha} \right]$	If $\tilde{q}_{PHB,1}^{feast} > \tilde{q}_{PHB,2}^{feast}$	(IV-4b)
CO_2 evolution	$\tilde{q}_{CO_2}^{feast}(t_i) = \tilde{\mu}^{feast}(t_i) \cdot Y_{CO_2/X}^{feast} + \tilde{q}_{PHB}^{feast}(t_i) \cdot Y_{CO_2/PHB}^{feast} + m_{Ac} \cdot Y_{CO_2/Ac}^{feast}$		(IV-5)
O_2 uptake	$\tilde{q}_{O_2}^{feast}(t_i) = \tilde{\mu}^{feast}(t_i) \cdot Y_{O_2/X}^{feast} + \tilde{q}_{PHB}^{feast}(t_i) \cdot Y_{O_2/PHB}^{feast} + m_{Ac} \cdot Y_{O_2/Ac}^{feast}$		(IV-6)
NH_3 uptake	$\tilde{q}_{NH_3}^{feast}(t_i) = \tilde{\mu}^{feast}(t_i) \cdot Y_{NH_3/X}^{feast}$		(IV-7)
Famine phase			
PHB degradation	$\tilde{q}_{PHB,1}^{fam}(t) = k \cdot \tilde{f}_{PHB}(t)^{2/3}$		(IV-8)
Maintenance	$m_{PHB} = \frac{m_{ATP}}{Y_{ATP/PHB}^{fam}}$		(IV-9)
Growth	$\tilde{\mu}^{fam}(t) = Y_{X/PHB}^{fam} \cdot (\tilde{q}_{PHB}^{fam}(t) - m_{PHB})$		(IV-10)
CO_2 evolution	$\tilde{q}_{CO_2}^{fam}(t) = \tilde{\mu}^{fam}(t) \cdot Y_{CO_2/X}^{fam} + m_{PHB} \cdot Y_{CO_2/PHB}^{fam}$		(IV-11)
O_2 uptake	$\tilde{q}_{O_2}^{fam}(t_i) = \tilde{\mu}^{fam}(t_i) \cdot Y_{O_2/X}^{fam} + m_{PHB} \cdot Y_{O_2/PHB}^{fam}$		(IV-12)
NH_3 uptake	$\tilde{q}_{NH_3}^{fam}(t_i) = \tilde{\mu}^{fam}(t_i) \cdot Y_{NH_3/X}^{fam}$		(IV-13)

Famine phase

Famine phase kinetics were applied when the modelled concentration of acetate was below 0.001 Cmmol/l. In the famine phase four of the six reactions (reactions 3 - 6 in Table 2.2) need to be considered. With the balances of the three conserved moieties NADH, ATP and acetyl-CoA, the degree of freedom can be reduced to one. We considered the PHB degradation rate ($\tilde{q}_{PHB}^{fam}$) as the rate limiting step in the famine phase and therefore chose to define this rate (Beun et al., 2002; van Loosdrecht et al., 2002). Murnleitner et al. (1997) suggested a reaction order of ⅔ for PHB degradation to reflect the specific surface area available to the PHA depolymerase on the shrinking PHB granule. This approach was adopted here (Table 2.4, equation (IV-8)).

The consumption of PHB due to maintenance m_{PHB} can be derived from m_{ATP} with the stoichiometric yield of ATP on PHB (equation (IV-9)). The biomass specific growth rate $\tilde{\mu}^{fam}$ follows from the PHB degradation rate and the maintenance requirements (equation (IV-10)).

The rates of carbon dioxide evolution, oxygen and ammonia uptake can be calculated from the known rates with the stoichiometric yields (equations (IV-11), (IV-12) and (IV-13)).

Material balance equations in the form of $\frac{d\tilde{c}(t)}{dt} = \tilde{q}(t) \cdot \tilde{c}_X(t)$ were applied for all relevant compounds (including PHB). With the concentrations of PHB and active biomass derived from the mass balances the molar fraction of PHB in the biomass $\widetilde{f}_{PHB}$ can be calculated (Appendix I).

Parameter estimation

The metabolic model was used to calculate all rates and concentrations of all relevant compounds (acetate, PHB, biomass, carbon dioxide, oxygen and ammonia) at time steps of 0.5 minutes. To be able to compute all rates and concentrations, several parameters needed to be defined. Some of these parameters were kept constant, whereas others were identified by fitting the model output to the measured concentrations and conversions. All parameters are summarized in Table 2.5.

The values of the half-saturation constants were chosen to avoid numerical problems during integration of the differential equations and do not reflect the true affinity constants. The exact value of these parameters does not influence the outcome of the SBR or fed batch simulations. The efficiency of oxidative phosphorylation (P/O ratio, δ) and the maintenance ATP requirement cannot be identified independently from a single measurement: a high P/O ratio and a high maintenance ATP consumption can give a similar result as a low P/O ratio and a low maintenance ATP consumption. We decided to use a P/O ratio for all experiments of 2 mmol ATP per mmol NADH as reported by Beun et al. (2000b) and to estimate m_{ATP} by fitting, as m_{ATP} seemed more likely to vary significantly due to differences in the conditions, e.g. stress caused by ammonia limitation.

The maximum specific acetate uptake rate ($\widetilde{q}_{Ac}^{\max}$) and specific growth rate in the feast phase ($\tilde{\mu}^{\max}$) were estimated in all experiments, while the parameters needed to describe the inhibitive effect of high PHB concentrations ($\tilde{q}_{PHB}^{\max}$, α, $\widetilde{f}_{PHB}^{\max}$) were only estimated for fed-batch experiments, as the PHB contents reached during SBR cycles were too low to inhibit acetate uptake. The rate constant for PHB degradation was only relevant for SBR cycle experiments since no PHB degradation occurred during fed-batch experiments. In all experiments the initial concentrations of acetate, PHB, biomass and ammonia were identified by optimization. The initial conditions for carbon dioxide evolution and oxygen uptake were set to 0 mmol.

Table 2.5. Model parameters.

Parameter / initial conditions	Value	Constant or estimated
Half-saturation constant for acetate	$K_{Ac} = 0.2 \frac{Cmmol}{l}$	Constant
Half-saturation constant for ammonia	$K_{NH_3} = 0.0001 \frac{mmol}{l}$	Constant
Efficiency of oxid. phosphorylation	$\delta = 2 \frac{mmol\ ATP}{mmol\ NADH}$	Constant
Maintenance ATP requirement	m_{ATP}	Estimated
Max. acetate uptake rate	$\tilde{q}_{Ac}^{\max}$	Estimated
Max. growth rate feast	$\tilde{\mu}^{\max}$	Estimated
Max. PHB production rate	$\tilde{q}_{PHB}^{\max} \left(= 2 \frac{Cmmol}{Cmmol \cdot h} \right)$	Estimated in fed-batch, constant in SBR experiments
Exponent of PHB inhibition term	$\alpha (= 1.24)$	Estimated in fed-batch, constant in SBR experiments
Max. fraction of PHB	$\tilde{f}_{PHB}^{\max}$	Estimated in fed-batch, constant in SBR experiments (value of fed-batch experiment)
Rate constant PHB degradation	k	Estimated for SBR experiments
Initial concentration of acetate	$\tilde{c}_{Ac}(t=0)$	Estimated
Initial concentration of PHB	$\tilde{c}_{PHB}(t=0)$	Estimated
Initial concentration of biomass	$\tilde{c}_{X}(t=0)$	Estimated
Initial concentration of ammonia	$\tilde{c}_{NH_3}(t=0)$	Estimated
Initial carbon dioxide evolution	$cumC\tilde{E}(t=0) = 0\ mmol$	Constant
Initial oxygen uptake	$cumO\tilde{U}(t=0) = 0\ mmol$	Constant

Both, the concentrations and rates computed with the model and measured, were converted into total amounts of acetate, ammonia and biomass, mol-fraction of PHB and cumulative carbon evolution and cumulative oxygen uptake (see Appendix I). In fed-batch experiments, the amounts of acetate were calculated as if all acetate had been provided right at the start of the experiment (considering the amount of acetate dosed and the concentration change in the liquid). The mol-fraction of PHB was used for fitting rather than the concentration of PHB per liquid volume as the fraction is directly derived from the PHB analysis and is independent of the biomass measurement. The weighted sum of squared relative errors between the measurements and the modelled values (E_M) was computed with the aim to minimize this error (see Appendix I).

For SBRs in steady state an additional error can be calculated for the deviation of the model from steady state (E_{SS}), using the measured SRT (see Appendix H and I). The model was fitted to the measurements by minimizing the sum of errors E_M and E_{SS} (for steady state systems) or the error E_M (for non-steady state systems) by adjusting the parameters described in Table 2.5.

Results & Discussion

Measurement corrections for sampling and pH control

The influence of the removal of reactor liquid by sampling and the addition of acid, base and substrate solutions on measured concentrations and conversions was considered by correcting all concentrations and conversions back to a standard reactor volume (see Appendix B). Required corrections naturally became more significant towards the end of an experiment.

In SBR cycles the amount of acid and base solution added for pH control was usually small and dilution effects on measured concentrations were found to be almost negligible in our experiments (up to 1 - 3% error). Gas conversions, however, were significantly influenced by sampling and had to be corrected by up to 7 - 19% to reflect conversions that would have been achieved in a non-sampled reactor.

Fed-batch reactors were supplied with either concentrated substrate pulses or with a continuous flow of a lower concentrated substrate solution. In both cases the diluting effect caused by the addition of substrate and acid and base solutions for pH control was significant, requiring the measured concentrations in the liquid phase to be corrected by up to 6 - 24%. Similarly the amount of sampling performed during fed-batch experiments made a correction of gas conversions and substrate dosage of up to 10 - 30% necessary.

It is therefore important to take the effects of sampling and dilutions into account if gaps in balances and misinterpretation of data are to be avoided.

Off-gas data treatment

The quality of the oxygen off-gas measurements was improved significantly with the off-gas recycling system (Figure 2.2). The depletion of oxygen in the off-gas increased by a factor of about 4 - 7, depending on the extent of off-gas recycling. The mass transfer of oxygen from the gas to the liquid phase was hardly affected as the off-gas concentration of oxygen never fell below 17% and the dissolved oxygen concentration in the reactor was kept at non-limiting conditions by adapting the stirrer speed and gas flow rate.

Carbon dioxide and oxygen transfer rates were calculated for all experiments as described in Table 2.1. Figure 2.3 shows an example of the two different oxygen transfer rates. The oxygen transfer rate calculated directly from the air entering the system and the off-gas leaving the system (OTR^S) clearly showed that it did not reflect the expected fast changes of the OTR at the beginning and end of the feast phase. These delays were due to the mixing and recycling of the gas within the system. Therefore the oxygen molar fractions in the gas entering and leaving the reactor liquid were calculated and used to determine a better estimate of the OTR (OTR^R). This method gave a more realistic picture of the actual OTR in the reactor (Figure 2.3). No significant delay (i.e. > 1 minute) was observed in this OTR^R profile compared to the oxygen concentration profile measured in the liquid phase (DO).

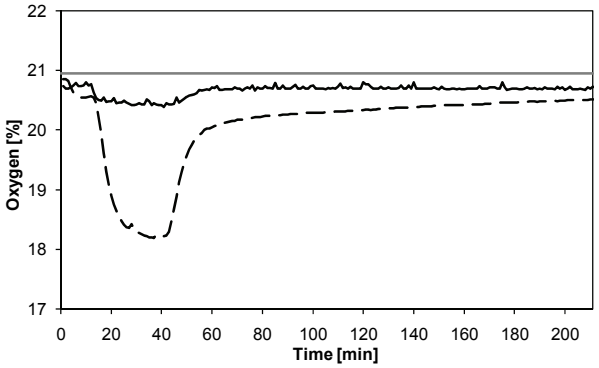

Figure 2.2. Off-gas oxygen molar fractions of similar experiments with (— —) and without (——) off-gas recycling. The grey line (——) indicates the oxygen molar fraction in air.

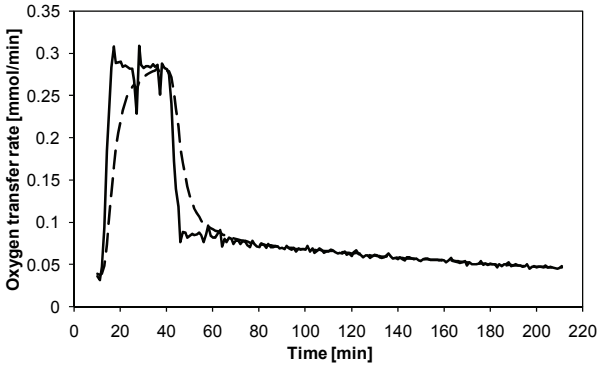

Figure 2.3. Oxygen transfer rates (OTR) during a SBR cycle with off-gas recycling (1 d SRT, 4 h cycles, 20°C, medium 8 Cmol/Nmol) calculated over the whole system - OTR^S (— —), or calculated over the liquid phase of the reactor - OTR^R (——).

Alternatively the OTR can also be calculated from the measured volumetric oxygen transfer coefficient ($k_{L,O_2}a$) and DO data, but this method was found to be less accurate than the use of the off-gas data for systems with off-gas recycling (see Appendix D).

The OUR deviated only slightly from the OTR as the concentration changes in the liquid (DO) were usually small apart from the time points when the feast phase started or ended or when the stirrer speed changed within one cycle.

In contrast, the changes of inorganic carbon in the liquid phase had a substantial influence on the CER. Figure 2.4 shows how the carbon dioxide produced by the microorganisms was distributed over the different inorganic carbon species in a typical SBR cycle. At the end of the feast phase (around 40 minutes) the majority of the carbon dioxide produced was present in the liquid in the form of bicarbonate and to a lesser extent as carbonic acid / aqueous carbon dioxide. Carbonate was included in all calculations but can be neglected at pH-values around 7 as the pK_a of the bicarbonate/carbonate system is greater than 10. In contrast, the pK_a of the reaction from aqueous carbon dioxide via carbonic acid to bicarbonate is around 6.4, making bicarbonate the dominant species in the liquid phase at pH 7. It is important to note that at the

end of the feast phase the amount of inorganic carbon dissolved in the liquid phase was far greater than the cumulative amount of carbon dioxide measured in the off-gas. The liquid phase acted as a buffer for the produced carbon dioxide, absorbing it in the feast phase and releasing it in the famine phase. This is also true in systems with higher SRTs and without off-gas recycling (Beun et al., 2000a). It is therefore vital to include the inorganic carbon dissolved in the reactor broth in the calculation of the CER and carbon balances.

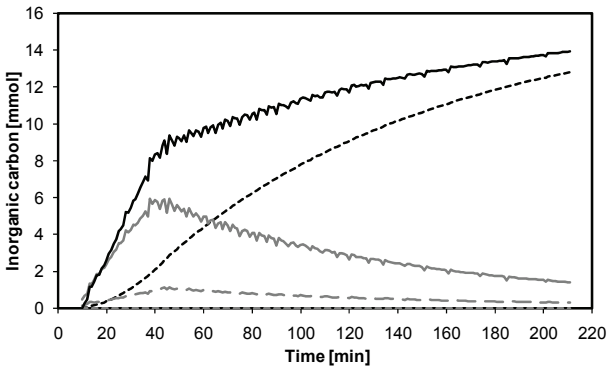

Figure 2.4. Distribution of the different inorganic carbon species in a SBR cycle with off-gas recycling (1 d SRT, 4 h cycles, 20°C, medium 8 Cmol/Nmol): cumulative CO_2 in the gas phase (**- - -**), sum of CO_2^{aq} and H_2CO_3 in the liquid phase (— —), HCO_3^- in the liquid phase (——), CO_3^{2-} in the liquid phase (▪▪▪), and total inorganic carbon evolution $cumCE$ (——).

Modelling

Comparison of model and experiments

A large number of experiments performed under different conditions has been evaluated with the procedure described above. It was possible to identify all model parameters from single SBR cycle measurements and fed-batch experiments.

Figure 2.5 shows examples for SBR and fed-batch experiments of model fits to measurements. The model described the measurements very well in all evaluated experiments irrespective of the conditions employed. The examples in Figure 2.5 were chosen to represent some of the different conditions and reactor behaviours that were modelled.

The greatest deviations between the model and the measurements can be found for off-gas data and in some cases in active biomass measurements. These measurements are considered the least reliable measurements and therefore received a low weighting in the fitting procedure. The measurements of OUR and CER are rate measurement which involve a number of individual measurements (off-gas analysis, gas flows, DO, pH, pressure) that each have their own inaccuracies. The individual measurement errors might add up and may thus explain the deviations between the measurements and model predictions for off-gas carbon dioxide and oxygen. Fed-batch experiments (Figure 2.5 D) can be modelled as well as SBR cycles (Figure 2.5 A-C). The model also handles the depletion or absence of ammonia in fed-batch experiments

very well (Figure 2.5 D). Equally the inhibiting effect of high PHB contents on the acetate uptake rate as observed from the measurements can be described with the model (Figure 2.5 D).

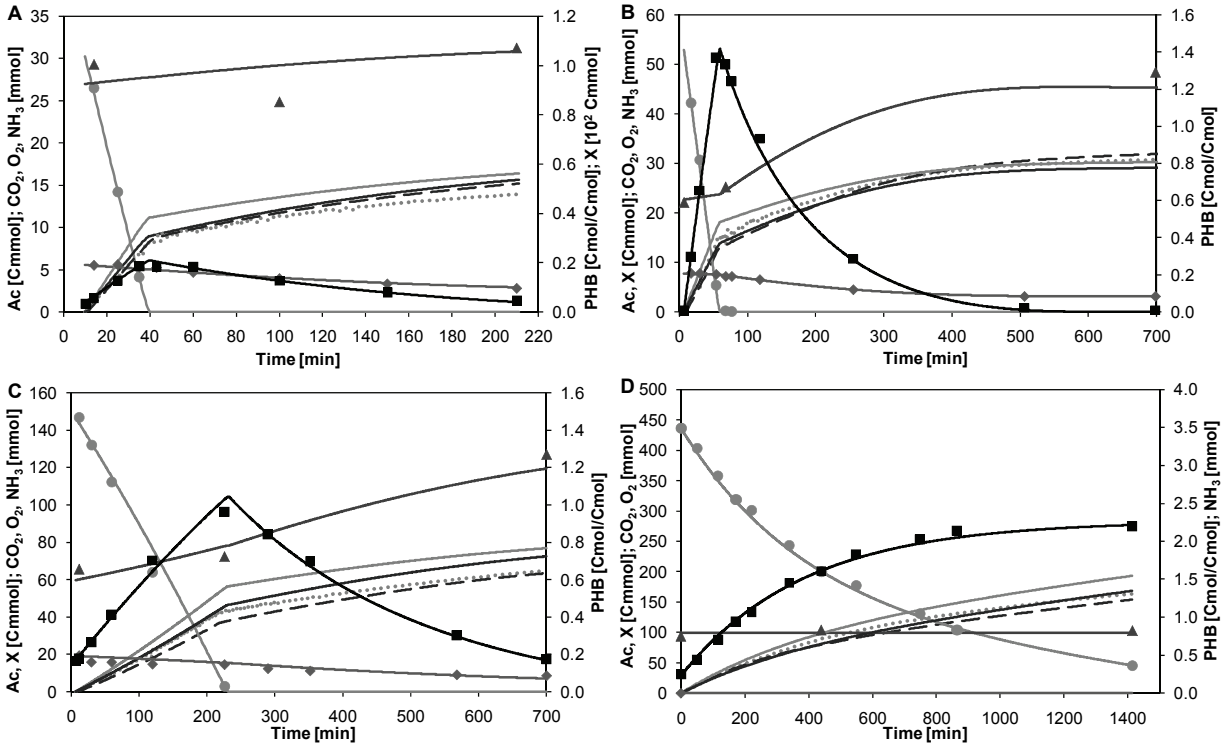

Figure 2.5. Examples of measurements and model estimates. Solid lines indicate modelled values corresponding to the respective symbols indicating measurements. (— —) cumulative oxygen uptake; (▪▪▪) cumulative carbon dioxide evolution; (●) acetate; (▲) active biomass; (◆) ammonia; (■) PHB. **A**: SBR cycle measurement 1 d SRT, 4 h cycles, 20°C, medium 8 Cmol/Nmol. Estimated parameters: $\tilde{q}_{Ac}^{\max}$ = -0.69 Cmol/Cmol/h, $\tilde{\mu}^{\max}$ = 0.06 Cmol/Cmol/h, k = -0.25 (Cmol/Cmol)$^{1/3}$/h, m_{ATP} = 0.000 mol/Cmol/h. **B**: SBR cycle measurement 1 d SRT, 12 h cycles, 30°C, medium 8 Cmol/Nmol. Estimated parameters: $\tilde{q}_{Ac}^{\max}$ = -2.71 Cmol/Cmol/h, $\tilde{\mu}^{\max}$ = 0.06 Cmol/Cmol/h, k = -0.30 (Cmol/Cmol)$^{1/3}$/h, m_{ATP} = 0.014 mol/Cmol/h. **C**: SBR cycle measurement 1 d SRT, 12 h cycles, 20°C, medium 8 Cmol/Nmol. Estimated parameters: $\tilde{q}_{Ac}^{\max}$ = -0.58 Cmol/Cmol/h, $\tilde{\mu}^{\max}$ = 0.07 Cmol/Cmol/h, k = -0.13 (Cmol/Cmol)$^{1/3}$/h, m_{ATP} = 0.000 mol/Cmol/h. **D**: Fed-batch experiment with biomass from a SBR with 0.5 d SRT, 4 h cycles, 20°C, medium 13.2 Cmol/Nmol. Estimated parameters: $\tilde{q}_{Ac}^{\max}$ = -0.52 Cmol/Cmol/h, $\tilde{q}_{PHB}^{\max}$ = 0.35 Cmol/Cmol/h, α= 1, $\tilde{f}_{PHB}^{\max}$ = 2.27 Cmol/Cmol, m_{ATP} = 0.12 mol/Cmol/h.

Ammonia measurements were generally in very good agreement with the model predictions as opposed to biomass measurements, indicating that ammonia measurements represent a more reliable measure for biomass growth. Nevertheless all indicators for biomass growth (i.e. increase in biomass concentration, ammonia uptake and for steady-state SBRs also measured SRT) were considered in the model, albeit with different weight, to include all available information and minimize errors.

Although the amount of growth can be estimated fairly well from ammonia measurements without the help of a metabolic model, one of the key advantages of the metabolic model is that the *concentration* of biomass in the reactor can be better estimated. For the experiment displayed in Figure 2.5 A for instance the best estimate for the actual biomass concentrations can hardly be derived from the TSS and ammonia measurements alone. However, since the biomass concentration has a significant influence on the calculated biomass specific rates, an over- or underestimation of biomass concentrations would lead to relatively large errors in the specific rates.

Figure 2.6 shows a comparison of feast phase rates calculated with and without the help of the metabolic model for experiments conducted at different temperatures. The reactor temperature was changed for one cycle to temperatures 5°C higher or lower than the steady state temperature. Considering that all rates in the feast phase basically follow zero order kinetics, and that the temperature range was small and the time frame too short for population changes, the rate changes were expected to comply with the Arrhenius equation for the temperature dependence of rate constants. The Arrhenius plot for the rates calculated without balancing them with the metabolic model (Figure 2.6 A) showed a low quality of fit (low R^2 values) especially for the growth rate, ammonia uptake rate and PHB production rate. The rates calculated with the metabolic model (Figure 2.6 B) on the other hand turned out to show an almost perfect fit with the Arrhenius equation. Our approach has therefore helped to identify a much clearer trend when comparing different conditions. The improvement achieved with the model is partly due to a better estimation of biomass concentrations and partly due a better estimation of total conversions of all involved compounds.

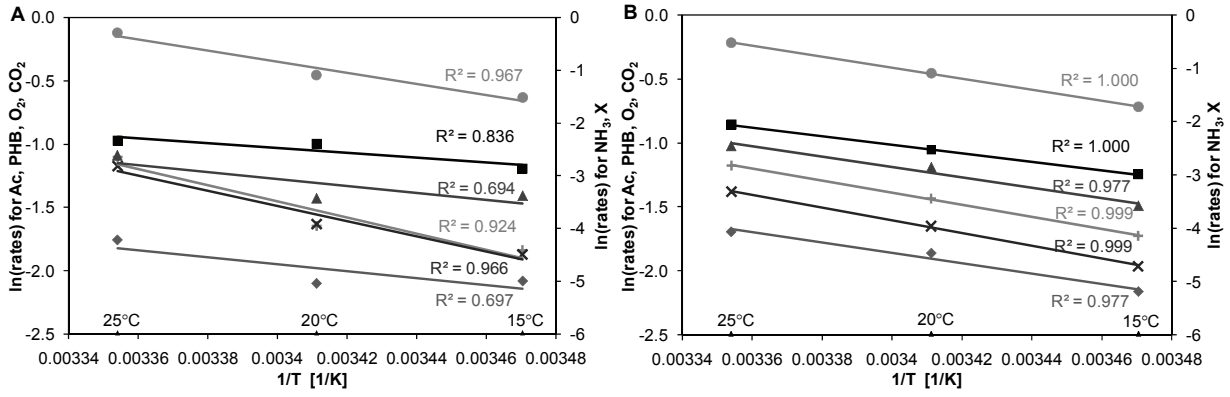

Figure 2.6. Arrhenius plot of average biomass specific reaction rates in the feast phase of a SBR at different temperatures. The SBR was in a steady state at 20°C (1 d SRT, 4 h cycles, medium 8 Cmol/Nmol) and the temperature was changed for one cycle to 15 or 25°C. Solid lines are linear trend lines corresponding to the respective symbols. (**x**) oxygen uptake rate; (+) carbon dioxide evolution rate; (●) acetate uptake rate; (▲) growth rate; (◆) ammonia uptake rate; (■) PHB production rate. **A**: Biomass specific reaction rates calculated directly from (corrected) measurements. Growth was estimated from ammonia samples. **B**: Biomass specific reaction rates calculated with the help of the metabolic model.

The application of the metabolic model helps thus to find good estimates for both, total conversions and concentrations, and consequently yields better estimates for biomass specific reaction rates. An additional advantage is that with the help of the kinetics implemented in the model, biomass specific reaction rates are not only calculated as averages over e.g. the feast or famine phase, but can be calculated and compared at any time point during an SBR cycle or fed-batch experiment (see also Figure 2.7). This would not be possible without a kinetic model, but can provide important information, particularly for the famine phase where reaction rates do not follow zero order kinetics.

Kinetics

Maintenance requirements of acetate in the feast phase or PHB in the famine phase were modelled with a constant substrate consumption rate independent of availability of the substrate, which is a different approach to Dias et al. (2005). If PHB is depleted in the famine phase, our model reverses the biomass formation reaction to 'produce' PHB for maintenance from biomass (see Figure 2.5 B). This was intentionally not prevented, as decay of biomass can be expected to occur if neither acetate nor PHB are available as a substrate.

Despite of this biomass 'decay' in the end of the famine phase in the SBR cycle shown in Figure 2.5 B, the average growth rate in the famine phase in this particular experiment still slightly exceeded that of the feast phase, which is different from previous observations (Beun et al., 2000a; van Loosdrecht et al., 2002). The growth rate at the beginning of the famine phase as calculated with the model was even about four times higher than the maximum growth rate in the feast phase (see Figure 2.7). This is due to the high PHB content of the culture (54 wt%) which according to the model results in high PHB degradation rates and consequently high growth rates. The ammonia measurements performed during this experiment match the modelled values very well and hence confirm that the culture indeed grew with a lower growth rate in the feast phase than at the beginning of the famine phase (Figure 2.7).

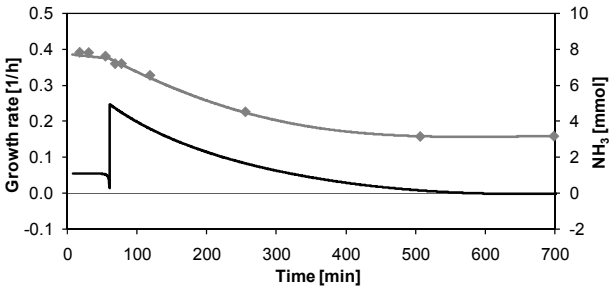

Figure 2.7. Growth during a SBR cycle of the experiment shown in Figure 2.5 B (1 d SRT, 12 h cycles, 30°C, medium 8 Cmol/Nmol). Modelled biomass specific growth rate (——), modelled amount of ammonia (——), measured amount of ammonia (◆).

The 'overflow' metabolism employed to model PHB formation in the feast phase (at low PHB contents) is based on the assumption that growth in the feast phase occurs at the maximum rate if nutrients and acetate are present in excess and that only the substrate that is not needed for growth and maintenance is available for PHB storage. However, the experiment shown in Figure 2.5 B and Figure 2.7 challenges this concept as the culture is evidently not growing at its maximum growth rate in the feast phase. Whether this is due to a limitation or due to a different type of regulation of the substrate split between storage and growth is not clear. The current model still describes the experimental results very well. Further research is required to investigate why the growth rate in the feast phase was lower than in the famine phase in order to improve the kinetic description of growth.

The reaction order of ⅔ for PHB degradation suggested by Murnleitner et al. (1997) described our PHB data very well, also at high PHB contents like in Figure 2.5 B. A reaction order of 1 overestimates the PHB degradation rate at the beginning of the famine phase and results in a worse description of the whole data set. Beun et al. (2000a) applied ⅔ order kinetics to a range of PHB data from SBRs at 20°C and found a rate constant k of -0.09, which is within the range of -0.09 to -0.14 that we identified for the rate constant k in carbon limited SBRs (12 h cycle length) at 20°C.

Dias et al. (2005) used a fairly different kinetic model, stoichiometry and experimental process parameters. A direct comparison of our estimated parameters with those reported by Dias et al. is therefore difficult. Nevertheless, parameters like the maximum acetate uptake and PHB production rate identified by us were in a good agreement with those reported be Dias et al.

Stoichiometry

The approach of fixing the P/O ratio δ while using the maintenance ATP requirement m_{ATP} as an estimation parameter worked well. Maintenance requirements in fed-batch experiments were generally calculated to be higher than for the same culture in a SBR cycle. This was expected due to the stress of ammonia limitation in fed-batch experiments. m_{ATP} values were in the range of 0.000 – 0.032 mol/Cmol/h for carbon limited SBR cultures at 4 or 1 d SRT and 20°C, which is in good agreement with values reported in literature, ranging from 0.01 - 0.02 mol/Cmol/h (Beun et al., 2000b). However, the chosen P/O ratio resulted in a stoichiometric PHB yield that was sometimes not efficient enough to provide the best fit of the measurements.

Dias et al. (2005) used a model that was based on measured stoichiometric yields rather than on the yields derived from the stoichiometric model suggested by van Aalst-van Leeuwen et al. (1997). The stoichiometric biomass yield on acetate used by Dias et al. (2005) was 0.42 Cmol/Cmol (0.47 Cmol/Cmol in our model) and the stoichiometric PHB yield on acetate was 0.75 Cmol/Cmol (0.67 Cmol/Cmol in our model). We found that many experiments could indeed be described better by the model if the PHB yield was chosen higher than calculated from stoichiometry.

A stoichiometric PHB yield of 0.75 Cmol/Cmol as estimated by Dias et al. (2005) could be derived from stoichiometry, if the efficiency of oxidative phosphorylation (P/O ratio, δ) slightly exceeded the theoretical maximum value of 3 mol ATP / mol NADH. Efficiency of oxidative phosphorylation is unlikely to even approach the theoretical maximum value of 3 mol ATP / mol NADH, as this value is derived from only using NADH as electron carrier. Other electron carriers such as FADH occur in metabolism, which are less efficient than NADH and will lower the overall value of δ to values below the theoretical maximum.

Another way of deriving a higher stoichiometric PHB yield is the use of less ATP for acetate uptake and activation than assumed in Table 2.2. This would also make biomass production more efficient, but Dias et al. (2005) report for biomass production from acetate a yield even lower than ours. It could however be that the ATP requirements for biomass formation are underestimated in the stoichiometry and that the lower stoichiometric yields for biomass as found by Dias et al. are indeed realistic.

The amounts of ATP involved in anabolism and acetate uptake as well as the P/O ratio obviously require re-evaluation to get a better insight into the true stoichiometry and yields.

Outlook

Another measurement that could provide useful information and could be included in the model in the future is the proton production rate derived from acid and base addition for pH control (Pratt et al., 2003; Pratt et al., 2004). The inclusion of this additional independent online measurement could help reducing the amount of offline sampling required for a good system description.

A limitation of the current model is that special cases like inhibition of acetate uptake by high PHB contents under ammonia limitation or ammonia limitation in the famine phase (not included here) are or would need to be incorporated into the model by using different kinetic equations for different situations. It would probably be more convenient and closer to reality, if a cell internal metabolite like intracellular acetate or acetyl-CoA would be included into the model. High levels of this metabolite would induce growth (if ammonia is present) and PHB production and decrease acetate uptake if above a certain threshold, while low levels would lead to PHB degradation. In this way one set of kinetic equations could be used to model all situations.

Conclusions

The aim of this research was to provide a tool to identify all biomass specific rates and observed yields at any time point of an experiment for a large amount of experiments as accurately as possible. The approach with the described data treatment and use of a metabolic model has proven to be a very useful tool to achieve this aim in fed-batch and SBR experiments. The metabolic model is employed to identify the best estimate of all state variables by using the

available knowledge of stoichiometry and kinetics of PHB production and degradation, while complying with material balances.

Our methodology is especially useful for experiments with high growth rates (low SRTs), where biomass specific rates can hardly be calculated otherwise. But also experiments conducted at lower growth rates or nutrient limitation benefit from this comprehensive data evaluation, particularly if balances do not close well.

In brief the advantages of using the metabolic model for data evaluation are: (i) material balances close, (ii) better estimates for biomass concentrations can be obtained, which is crucial for calculating biomass specific rates, (iii) better estimates for the conversion of all relevant compounds can be obtained, (iv) as a result of the latter two points better estimates for biomass specific reaction rates and observed yields can be obtained, leading to clearer trends when comparing different experiments, (v) concentrations and biomass specific rates can be computed and compared at any time point of an experiment, rather than just averaged rates between the measurement points, (vi) the model helps understanding the regulation of PHB production and degradation. A good understanding of the process is crucial for process optimization.

Appendices

Appendix A: Oxygen mass transfer measurements

Oxygen mass transfer between the liquid and the gas phase was measured in the same reactor as used for the experiments with a biomass-free medium. Different combinations of airflow rate, stirrer speed and temperature were used, reflecting conditions employed in all experiments. The reactor was sparged with nitrogen and subsequently with air. Dissolved oxygen concentrations were monitored with a DO electrode. The volumetric oxygen transfer coefficient $k_{L,O_2}a$ was determined from the mass balance over the liquid phase: $dc_{O_2}/dt = k_{L,O_2}a \cdot (c^*_{O_2} - c_{O_2})$.

Appendix B: Corrections for sampling and dilutions

The actual volume of liquid in the reactor $V^R_{L,true}$ at any time point t_i can be calculated from the standard reactor volume of liquid V^R_L by considering the volume of samples taken (V_S) and the volume of acid (V_{acid}) and base (V_{base}) added to the reactor up to that time point:

$$V^R_{L,true}(t_i) = V^R_L + \sum_{j=0}^{i} V_{acid}(t_j) + \sum_{j=0}^{i} V_{base}(t_j) - \sum_{j=0}^{i-1} V_S(t_j) \qquad (2.2)$$

The percentage of biomass remaining in the reactor after sampling can be calculated as follows:

$$\%biomass(t_i) = 100\% \cdot \prod_{j=0}^{i-1}\left(1 - \frac{V_S(t_j)}{V_{L,true}^R(t_j)}\right) \quad (2.3)$$

The measured amount of oxygen taken up, carbon dioxide produced and substrate fed (in fed-batch experiments with pH controlled substrate feeding) can then be multiplied with the correction factor f_G to yield the amounts that would have resulted without sampling.

$$f_G(t_i) = \frac{100\%}{\%biomass(t_i)} \quad (2.4)$$

The correction factor f_L for the measured concentrations in the liquid due to dilution with acid and base solutions can be calculated in a similar way:

$$f_L(t_i) = \prod_{j=0}^{i}\left(1 + \frac{V_{acid}(t_j) + V_{base}(t_j)}{V_{L,true}^R(t_{j-1}) - V_S(t_j)}\right) \quad (2.5)$$

All measured concentrations in the reactor liquid can be corrected for dilution effects by multiplying them with the respective time dependent f_L. Only the measured PHB contents do not need to be corrected as they are measured as a weight percentage of TSS rather than as a concentration in the reactor liquid.

In some fed-batch experiments without sufficient growth limitation it may be required to dilute the reactor content in order to avoid e.g. oxygen transfer limitations. The correction factors outlined above can also be used to consider these dilutions if any reactor liquid removed is treated as a sample and any liquid added is treated like acid or base.

Appendix C: Molar fractions of carbon dioxide and oxygen

The molar fractions of carbon dioxide and oxygen in the gas entering the liquid phase of the reactor ($x_{CO_2}^{R,in}$, $x_{O_2}^{R,in}$) can be derived from a mass balance over the "mixer" of fresh air and recycled gas (see also Figure 2.1):

$$x_{CO_2}^{R,in}(t_i) = \frac{\dot{Q}_G^{in} \cdot x_{CO_2}^{S,in} + \dot{Q}_G^{Rec} \cdot x_{CO_2}^{S,out}(t_i)}{\dot{Q}_G^R} \quad (2.6)$$

$$x_{O_2}^{R,in}(t_i) = \frac{\dot{Q}_G^{in} \cdot x_{O_2}^{S,in} + \dot{Q}_G^{Rec} \cdot x_{O_2}^{S,out}(t_i)}{\dot{Q}_G^R} \quad (2.7)$$

The molar fractions of carbon dioxide and oxygen in the gas leaving the liquid phase of the reactor and entering the headspace ($x_{CO_2}^{R,out}$, $x_{O_2}^{R,out}$) can be derived from the mass balance over the headspace gas volume V_G^{HS} (assuming that the headspace is ideally mixed):

$$x_{CO_2}^{R,out}(t_i) = \frac{V_G^{HS}}{\dot{Q}_G^R} \cdot \frac{dx_{CO_2}^{S,out}(t_i)}{dt_i} + x_{CO_2}^{S,out}(t_i) \quad (2.8)$$

$$x_{O_2}^{R,out}(t_i) = \frac{V_G^{HS}}{\dot{Q}_G^R} \cdot \frac{dx_{O_2}^{S,out}(t_i)}{dt_i} + x_{O_2}^{S,out}(t_i) \quad (2.9)$$

Appendix D: Alternatives for calculating OTR

Alternatively to calculating the OTR from the off-gas data alone, the oxygen transfer rate can also be calculated with the measured oxygen concentration in the liquid (DO) and the volumetric oxygen transfer coefficient $k_{L,O_2}a$ (Appendix A):

$$OTR^{R*}(t_i) = k_{L,O_2}a(t_i) \cdot V_L^R(t_i) \cdot P(t_i) \cdot H_{O_2} \cdot \left(\frac{DO(t_i)}{100\%} \cdot x_{O_2}^{S,in} - x_{O_2}^{R,out}(t_i) \right) \cdot f_G(t_i) \quad (2.10)$$

It is assumed that the gas in the reactor liquid is well mixed, i.e. it has the same oxygen molar fraction as the gas leaving the reactor liquid. V_L^R is the standard volume of liquid in the reactor, P the atmospheric pressure and H_{O_2} the Henry coefficient (Appendix F).

In many experiments OTR^{R*} deviated significantly from the OTR^R derived from off-gas data alone, but there was no clear pattern for these deviations. OTR^{R*} is very sensitive to the correct measurement of the oxygen mass transfer ($k_{L,O_2}a$) and correct calibration of the DO electrode, both of which could be sources of errors. In experiments with off-gas recycling OTR^R was therefore considered as the more realistic rate. It generally lead to better closing electron balances and was closer to the model predictions. In experiments without off-gas recycling, the errors in the off-gas measurement and thus also in OTR^R were generally so significant that OTR^{R*} was considered as the more realistic rate. Thus, in experiments with off-gas recycling the OUR was computed using OTR^R from the off-gas data, while in experiments without off-gas recycling OTR^{R*} calculated with the $k_{L,O_2}a$ and DO data (and off-gas data) was used.

If the depletion of oxygen in the off-gas is too small to be measured accurately enough (e.g. if no off-gas recycling is used) and if no off-gas data are available to calculate OTR^{R*}, the oxygen transfer rate can be approximated by assuming that the gas in the reactor liquid has the same molar fraction of oxygen as the unconsumed air entering the system (no depletion of oxygen in the off-gas), i.e. the equilibrium concentration of oxygen in the reactor liquid can be assumed to be $DO = 100\%$:

$$OTR^{R**}(t_i) = k_{L,O_2}a(t_i) \cdot V_L^R(t_i) \cdot P(t_i) \cdot H_{O_2} \cdot \frac{DO(t_i) - 100\%}{100\%} \cdot x_{O_2}^{S,in} \cdot f_G(t_i) \quad (2.11)$$

This is a similar approach to the traditional respiration measurements for OUR (Beun et al., 2000a). This OTR^{R**} overestimates the mass transfer if the depletion of oxygen is significant. If the depletion of oxygen in the off-gas is very small like in systems without off-gas recycling and with high gas flow rates, OTR^{R**} was found to be a good approximation for calculating the OUR.

Appendix E: Inorganic carbon in the liquid phase

The CTR from the liquid phase of the reactor to the gas phase (CTR^R) and the molar fraction of carbon dioxide in the gas phase can be calculated as described. The lumped concentration of carbon dioxide in the liquid and carbonic acid ($c_{H_2CO_3}$) can then be calculated from the mass transfer:

$$c_{H_2CO_3}(t_i) = P(t_i) \cdot H_{CO_2} \cdot x_{CO_2}^{R,out}(t_i) + \frac{CTR^R(t_i)}{V_L^R(t_i) \cdot k_{L,CO_2} a(t_i)} \quad (2.12)$$

The Henry coefficient H_{CO_2} also considers both species, carbon dioxide and carbonic acid, but not bicarbonate and carbonate (Appendix F). The volumetric carbon dioxide transfer coefficient $k_{L,CO_2}a$ can be calculated from the measured transfer coefficients for oxygen (Royce et al., 1991):

$$k_{L,CO_2} a(t_i) = k_{L,O_2} a(t_i) \cdot \left(\frac{D_{CO_2}}{D_{O_2}} \right)^{2/3} = k_{L,O_2} a(t_i) \cdot 0.89 \quad (2.13)$$

The concentrations of bicarbonate and carbonate can then be calculated from acid/base equilibria with the dissociation constants k_{a,H_2CO_3} and k_{a,HCO_3^-} (Appendix G):

$$c_{HCO_3^-}(t_i) = \frac{c_{H_2CO_3}(t_i)}{c_{H^+}(t_i)} \cdot k_{a,H_2CO_3} \quad (2.14)$$

$$c_{CO_3^{2-}}(t_i) = \frac{c_{HCO_3^-}(t_i)}{c_{H^+}(t_i)} \cdot k_{a,HCO_3^-} \quad (2.15)$$

The concentration of protons c_{H^+} is derived from the pH:

$$c_{H^+}(t_i) = 10^{-pH(t_i)} \quad (2.16)$$

The total amount of inorganic carbon dissolved in the liquid phase IC_{aq} is thus:

$$IC_{aq}(t_i) = \left[c_{H_2CO_3}(t_i) + c_{HCO_3^-}(t_i) + c_{CO_3^{2-}}(t_i) \right] \cdot V_L^R(t_i) \quad (2.17)$$

Appendix F: Temperature dependence of Henry coefficients

$$H_{O_2} = X_{O_2} \cdot \frac{1\ atm}{1.013\ bar} \cdot \frac{\rho_{H_2O}}{MW_{H_2O}} \quad \left[\frac{mol}{bar \cdot l} \right] \quad (2.18)$$

with $X_{O_2} = e^{-66.7354 + \frac{87.4755}{T^*} + 24.4526 \cdot \ln(T^*)}$ with $T^* = \frac{T}{100}$ (Gevantman, 2008) (2.19)

$$H_{CO_2} = 10^{-\left(\frac{-2622.38}{T+273.15} - 0.0178471\cdot(T+273.15)+15.5873\right)} \cdot \frac{1\ atm}{1.013\ bar} \quad \left[\frac{mol}{bar\cdot l}\right]$$ (Harned et al., 1943)

(2.20)

The Henry coefficient for carbon dioxide only considers carbon dioxide in solution and carbonic acid, not bicarbonate and carbonate.

Appendix G: Temperature dependence of dissociation constants

$$k_{a,H_2CO_3} = 10^{-\left(\frac{3404.71}{T+273.15} + 0.032786\cdot(T+273.15) - 14.8435\right)} \quad \left[\frac{mol}{l}\right]$$ (Harned et al., 1943)

(2.21)

$$k_{a,HCO_3^-} = 10^{-\left(\frac{2902.39}{T+273.15} + 0.02379\cdot(T+273.15) - 6.4980\right)} \quad \left[\frac{mol}{l}\right]$$ (Harned et al., 1941)

(2.22)

The dissociation constant for carbonic acid considers the reaction from aqueous carbon dioxide via carbonic acid to bicarbonate.

Appendix H: Sludge residence time (SRT) definition

The SRT is determined by the fraction of TSS removed from the reactor during the biomass (sludge) and effluent withdrawal phase. The SRT was defined and measured based on the amount of TSS present at the end of the cycle before sludge removal $TSS^R(t_{end})$:

$$SRT = \frac{TSS^R(t_{end})\cdot V_L^R}{TSS^{Sludge}\cdot V^{Sludge} + TSS^{Effl}\cdot V^{Effl}} \cdot t_{cycle}$$ (2.23)

With this definition the SRT will ideally equal the hydraulic residence time (HRT) if no settling step is used. The SRT is generally measured several times before a cycle measurement but not during a cycle measurement, as sampling will affect the amount of sludge and effluent withdrawn at the end of a cycle.

Appendix I: Parameter estimation

For comparison with the measured conversions, all concentrations computed with the model are converted into total or cumulative amounts:

$$\tilde{n}_i(t) = \tilde{c}_i(t)\cdot V_L^R$$ (2.24)

$$\tilde{f}_{PHB}(t) = \frac{\tilde{c}_{PHB}(t)}{\tilde{c}_X(t)}$$ (2.25)

$$cumC\tilde{E}(t) = \int \tilde{q}_{CO_2}(t) \cdot \tilde{c}_X(t) \cdot V_L^R dt \quad (2.26)$$

$$cumO\tilde{U}(t) = \int \tilde{q}_{O_2}(t) \cdot \tilde{c}_X(t) \cdot V_L^R dt \quad (2.27)$$

For comparison with the modelled values, all measured concentrations were corrected for dilution effects with the correction factor f_L (see Appendix B) and subsequently converted into total amounts n_{Ac}, n_{NH_3} and n_X:

$$n_{Ac}(t_i) = c_{Ac}(t_i) \cdot f_L(t_i) \cdot V_L^R \quad (2.28)$$

$$n_x(t_i) = TSS^R(t_i) \cdot f_L(t_i) \cdot \left(1 - \frac{w_{PHB}(t_i)}{100\%}\right) \cdot \frac{1}{MW_X} \cdot V_L^R \quad (2.29)$$

$$n_{NH_3}(t_i) = \frac{NH_4^+\text{-}N(t_i)}{MW_N} \cdot f_L(t_i) \cdot V_L^R \quad (2.30)$$

The measured fraction of PHB f_{PHB} was calculated from the measured weight percentage w_{PHB}:

$$f_{PHB}(t_i) = \frac{w_{PHB}(t_i)}{100\% - w_{PHB}(t_i)} \cdot \frac{MW_X}{MW_{PHB}} \quad (2.31)$$

The $cumCE$ and $cumOU$ were calculated as described in Table 2.1.

For each sampling time point t_i the modelled data for each compound were compared with the measured data and the relative errors were calculated, squared and summed up as shown for the example of acetate:

$$SSrelE_{Ac} = \sum_{i=1}^{N} \left(\frac{n_{Ac}(t_i) - \tilde{n}_{Ac}(t_i)}{\tilde{n}_{Ac}(t_i)} \right)^2 \quad (2.32)$$

Carbon dioxide evolution and oxygen uptake were only considered at time points where reactor samples were taken, therefore yielding a comparable number of data points.

Each sum of squared relative error for the different measurements is given a weight factor wf and summed up to the total error between measurements and model E_M:

$$E_M = \sum wf_i \cdot SSrelE_i \quad with\ i = Ac, NH_3, X, PHB, CO_2, O_2 \quad (2.33)$$

The weight factors were based on the accuracy of the respective measurement and the number of data points available. Typically values between 1 and 10 were chosen, with acetate receiving the highest weight (high accuracy, few data points), PHB and ammonia medium weight (high accuracy, many data points) and off-gas measurements (low accuracy, many data points) and biomass measurements the lowest weight (low accuracy, few data points).

In steady state the amount of solids (biomass and PHB) that is produced during one cycle is equal to the amount of solids removed at the end of the cycle, which in turn is set by the SRT.

The SRT definition (Appendix H) can thus be used to calculate the concentration change for biomass and PHB in one cycle:

$$c_X^{SRT}(t_{end}) = \tilde{c}_X(0) \cdot \frac{SRT}{SRT - t_{cycle}} \tag{2.34}$$

$$c_{PHB}^{SRT}(t_{end}) = \tilde{c}_{PHB}(0) \cdot \frac{SRT}{SRT - t_{cycle}} \tag{2.35}$$

The steady state error E_{SS}, which reflects the deviation of the model from steady state, was defined as follows:

$$E_{SS} = wf_{SRT,X}\left(c_X^{SRT}(t_{end}) - \tilde{c}_X(t_{end})\right)^2 + wf_{SRT,PHB}\left(c_{PHB}^{SRT}(t_{end}) - \tilde{c}_{PHB}(t_{end})\right)^2 \tag{2.36}$$

Here no relative errors were used as the PHB concentration will often be very close to 0. The weight factors $wf_{SRT,X}$ and $wf_{SRT,PHB}$ can instead be used to balance the weight of PHB and biomass in the error calculation, and to balance the weight of the steady state error relative to the error between model and measurements E_M. The total error is defined as the sum of E_{SS} and E_M:

$$E = E_{SS} + E_M \tag{2.37}$$

Nomenclature

$\%biomass$	Percentage of biomass remaining in reactor after sampling [%]
c_i	Measured concentration [(C)mol/l]
$\tilde{c}_i$	Modelled concentration [(C)mol/l]
c_i^*	Equilibrium concentration [mol/l]
c_{PHB}^{SRT}	Concentration of PHB calculated from SRT [Cmol/l]
c_X^{SRT}	Concentration of active biomass calculated from SRT [Cmol/l]
CER	Carbon dioxide evolution rate [mol/min]
CTR^R	Carbon dioxide transfer rate from balance over reactor liquid phase [mol/min]
CTR^S	Carbon dioxide transfer rate from balance over whole system [mol/min]
$cumC\tilde{E}$	Modelled cumulative carbon dioxide evolution [mol]
$cumCE$	Measured cumulative carbon dioxide evolution [mol]
$cumO\tilde{U}$	Modelled cumulative oxygen uptake [mol]
$cumOU$	Measured cumulative oxygen uptake [mol]
D_{CO_2}	Diffusion coefficient for carbon dioxide [m^2/s]
D_{O_2}	Diffusion coefficient for oxygen [m^2/s]
DO	Dissolved oxygen in the liquid phase [%]
E	Total error

E_M	Error between model and measurements
E_{SS}	Error of model deviating from steady state
f_G	Correction factor for sampling effects
f_L	Correction factor for dilution effects
f_{PHB}	Measured fraction of PHB on active biomass [Cmol/Cmol]
$\tilde{f}_{PHB}$	Modelled fraction of PHB on active biomass [Cmol/Cmol]
$\tilde{f}_{PHB}^{\max}$	Modelled maximum fraction of PHB on active biomass [Cmol/Cmol]
H_{CO_2}	Henry coefficient for carbon dioxide and carbonic acid [mol/l/bar]
H_{O_2}	Henry coefficient for oxygen [mol/l/bar]
IC_{aq}	Amount of inorganic carbon dissolved in the liquid phase [mol]
k	Rate constant of PHB degradation [(Cmol/Cmol)$^{1/3}$/h]
k_{a,H_2CO_3}	Dissociation constant of carbonic acid [mol/l]
k_{a,HCO_3^-}	Dissociation constant of bicarbonate [mol/l]
$k_{L,CO_2}a$	Volumetric carbon dioxide transfer coefficient [1/min]
$k_{L,O_2}a$	Volumetric oxygen transfer coefficient [1/min]
K_{Ac}	Half-saturation constant for acetate [Cmol/l]
K_{NH_3}	Half-saturation constant for ammonia [mol/l]
m_{Ac}	Biomass specific acetate requirement for maintenance [Cmol/Cmol/h]
m_{ATP}	Biomass specific ATP requirement for maintenance [mol/Cmol/h]
m_{PHB}	Biomass specific PHB requirement for maintenance [Cmol/Cmol/h]
MW_{H_2O}	Molecular weight of water = 18 g/mol
MW_N	Molecular weight of nitrogen = 14 g/mol
MW_{PHB}	Molecular weight of PHB = 21.5 g/mol
MW_X	Molecular weight of biomass (incl. ash) = 25.1 g/mol (Beun et al., 2002)
n_i	Measured amount [(C)mol]
$\tilde{n}_i$	Modelled amount [(C)mol]
NH_4^+-N	Ammonium-nitrogen [mg N / l]
OTR^R	Oxygen transfer rate from balance over reactor liquid phase [mol/min]
OTR^{R*}	Oxygen transfer rate over reactor liquid phase derived with $k_{L,O_2}a$ [mol/min]
OTR^{R**}	Oxygen transfer rate over reactor liquid phase derived from DO data [mol/min]
OTR^S	Oxygen transfer rate from balance over whole system [mol/min]
OUR	Oxygen uptake rate [mol/min]
P	Atmospheric pressure [bar]

$\tilde{q}_{Ac}^{\max}$	Maximum modelled biomass specific acetate uptake rate [Cmol/Cmol/h]
$\tilde{q}_i$	Modelled biomass specific rate [(C)mol/Cmol/h]
$\tilde{q}_{PHB}^{\max}$	Maximum modelled biomass specific PHB production rate [Cmol/Cmol/h]
$\dot{Q}_G^{in}$	Air flow into system [l_N/min]
$\dot{Q}_G^R$	Gas flow into reactor [l_N/min]
SRT	Sludge residence time [d]
$SSrelE_i$	Sum of squared relative errors between measurements and model for compound i
t	Model time [min]
t_i	Measurement time [min]
t_{cycle}	Cycle length [h]
T	Temperature [K]
T^*	Dimensionless temperature
TSS^{Effl}	Total suspended solids in the removed effluent [g/l]
TSS^R	Total suspended solids in the reactor liquid [g/l]
TSS^{Sludge}	Total suspended solids in the removed sludge [g/l]
V_{acid}	Volume of acid dosed [l]
V_{base}	Volume of base dosed [l]
V_G^{HS}	Headspace gas volume [l]
V_L^R	Standard volume of liquid in the reactor [l]
$V_{L,true}^R$	True volume of liquid in the reactor [l]
V_M	Molar gas volume = 22.414 l_N/mol
V_S	Sample volume [l]
V^{Sludge}	Volume of sludge removed [l]
V^{Effl}	Volume of effluent removed [l]
w_{PHB}	Weight percentage of PHB in TSS [wt%]
wf_i	Error weight factor for compound i
$wf_{SRT,i}$	Error weight factor for steady state error for compound i
$x_i^{R,in}$	Molar fraction of compound i in the gas entering the reactor liquid [mol/mol]
$x_i^{R,out}$	Molar fraction of compound i in the gas leaving the reactor liquid [mol/mol]
$x_i^{S,in}$	Molar fraction of compound i in the air entering the system [mol/mol]
$x_i^{S,out}$	Molar fraction of compound i in the gas leaving the system [mol/mol]
$Y_{i/j}^{fam}$	Stoichiometric yield of compound i on compound j in the famine phase [(C)mol/Cmol]

$Y_{i/j}^{feast}$	Stoichiometric yield of compound i on compound j in the feast phase [(C)mol/Cmol]
α	Exponent of PHB inhibition term
δ	Efficiency of oxidative phosphorylation [mol ATP / mol NADH]
$\tilde{\mu}^{fam}$	Modelled biomass specific growth rate in the famine phase [1/h]
$\tilde{\mu}^{feast}$	Modelled biomass specific growth rate in the feast phase [1/h]
$\tilde{\mu}^{\max}$	Modelled maximum biomass specific growth rate in the feast phase [1/h]
ρ_{H_2O}	Density of water = 1 kg/l

Subscripts

Ac	Acetate
CO_2	Carbon dioxide
CO_3^{2-}	Carbonate
H^+	Protons
H_2CO_3	Carbonic acid
HCO_3^-	Bicarbonate
NH_3	Ammonia
O_2	Oxygen
PHB	Polyhydroxybutyrate
X	Active biomass

Abbreviations

Ac-CoA	Acetyl-Coenzyme-A
ATP	Adenosine triphosphate
CER	Carbon dioxide evolution rate
CoA	Coenzyme A
COD	Chemical oxygen demand
CTR	Carbon dioxide transfer rate
DO	Dissolved oxygen
FADH	Reduced form of flavin adenine dinucleotide
HAc	Acetic acid
HRT	Hydraulic residence time
IC	Inorganic carbon
NADH	Reduced form of nicotinamide adenine dinucleotide
OTR	Oxygen transfer rate
OUR	Oxygen uptake rate
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
pKa	Acid dissociation constant
SBR	Sequencing batch reactor

SRT	Sludge residence time
TSS	Total suspended solids

CHAPTER 3

Influence of the C/N Ratio on the Performance of Polyhydroxybutyrate (PHB) Producing Sequencing Batch Reactors at Short SRTs

Katja Johnson, Robbert Kleerebezem, Mark C. M. van Loosdrecht

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ABSTRACT

Many waste streams that are suitable substrates for mixed culture bioplastic (polyhydroxyalkanoate, PHA) production are nutrient limited and may need to be supplemented to allow sufficient growth of PHA accumulating bacteria. The scope of this study was to investigate the necessity of nutrient supplementation for the enrichment of an efficient PHA producing mixed culture. We studied the influence of different degrees of carbon and nitrogen limitation on the performance of an acetate-fed feast-famine sequencing batch reactor (SBR) employed to enrich PHA storing bacteria. The microbial reaction rates in the SBR showed a shift with a change in the limiting substrate: high acetate uptake rates were found in carbon limited SBRs (medium C/N ratios 6 - 13.2 Cmol/Nmol), while nitrogen limited SBRs (medium C/N ratios 15 - 24 Cmol/Nmol) were characterized by high ammonia uptake rates. Biomass in strongly nitrogen limited SBRs had higher baseline PHA contents in the SBR, but carbon limited SBRs resulted usually in biomass with higher maximal PHA storage capacities. The PHA storage capacity in a nitrogen limited SBR operated at 0.5 d SRT decreased significantly over less than 5 months operation. For the microbial selection and biomass production stage of a PHA production process carbon limitation seems thus favourable and nutrient deficient wastewaters may consequently require supplementation with nutrients for the selection of a stable PHA storing biomass with a high storage capacity.

Introduction

Polyhydroxyalkanoates (PHA) are microbial storage polymers which increasingly attract interest as bioplastics. Industrial processes for the production of PHA are currently based on pure cultures of either genetically modified bacteria (e.g. Metabolix, U.S.A) or wild type PHA producing bacteria (e.g. Tianan Biological Materials, China). Process optimization, a growing competition on the PHA market, and larger scale production facilities have helped reducing the price of PHA to about 4.90 $/kg (Lunt, 2008), however, this price is still significantly higher than the price of conventional plastics such as polypropylene. As costs for raw materials play a crucial role for the economy of the PHA production process, alternative substrates derived from waste streams could help reduce the costs (Braunegg et al., 2004). The use of a PHA production process based on open mixed microbial cultures rather than on pure cultures would be favourable when using waste streams, since mixed microbial cultures can better adapt to changes in substrate supply and a sterilization of the substrate, which can have detrimental effects on the substrate quality, would not be required. Industrial wastewaters suitable for mixed culture PHA production such as paper mill wastewater or molasses are often nutrient deficient. If nutrient supplementation would be required for these streams, this could be an important cost factor. The aim of this study was to investigate the impact of nutrient limitation as opposed to carbon limitation on the enrichment of a stable and efficient PHA producing mixed culture, in order to judge whether nutrient supplementation would be necessary for deficient waste streams.

Typically, the upstream part of a mixed culture PHA production process from waste streams would consist of three steps: (i) feedstock production through anaerobic mixed culture fermentation of waste streams to volatile fatty acids, (ii) culture selection, and (iii) PHA production (Serafim et al., 2008a; van Loosdrecht, 2000). The second step makes use of the ecological role of PHA as a storage polymer in order to enrich a mixed culture capable of producing high amounts of PHA (van Loosdrecht et al., 1997). A successful strategy to enrich PHA producers in a mixed culture is the use of a dynamic feeding pattern consisting of alternating periods of presence (feast) and absence (famine) of the carbon source under fully aerobic conditions (also called aerobic dynamic feeding or feast-famine). Since this step is not only an enrichment step, but also the step in which the biomass is produced for the third step, nutrients and a carbon source are required for the biomass to proliferate. In the third step, the PHA production, biomass harvested from the second step is supplied with an excess of carbon source under conditions of nutrient limitation in order to minimize growth and maximize the cellular PHA content.

While the benefit of nutrient limitation on the maximum PHA content reached during the PHA production step has been documented (Bengtsson et al., 2008a; Serafim et al., 2004), the optimal composition of the feed in terms of nutrient versus carbon source during the culture selection step remains unclear as indicated by the following examples.

Using nutrient deficient waste streams such as fermented molasses (Albuquerque et al., 2007), fermented paper mill effluent (Bengtsson et al., 2008a) and fermented olive oil mill effluent (Beccari et al., 2009), PHA producing mixed cultures were successfully enriched, but in all studies the fermented substrate streams had to be supplemented with nutrients. Albuquerque et al. (2007) found that if the supplementation with a nitrogen source was not sufficient (nitrogen limitation), no stable PHA storing culture could be obtained. On the other hand other studies with cultures grown on acetate or propionate did show that stable PHA producing cultures with fairly high PHA storage capacities can be enriched with nutrient limited feeds (Serafim et al., 2004; Lemos et al., 2006). The influence of carbon versus nutrient limitation on the enrichment of a PHA producing culture requires thus further investigation.

We investigated the influence of the carbon to nitrogen ratio (C/N ratio) of the medium on the performance of a mixed culture in a selection reactor and on the culture's PHA production capacity. We performed culture selection experiments in sequencing batch reactors (SBR) at a range of C/N ratios from carbon to nitrogen limited with acetate as the substrate. When a stable reactor operation was obtained the steady state behaviour was documented by measurements of the relevant state variables. The selected cultures were subsequently transferred into nutrient limited fed-batch reactors in order to evaluate the capacity of the selected cultures to store PHA. The PHA produced from acetate was pure polyhydroxybutyrate (PHB).

As it can be expected that also the sludge residence time (SRT) in the SBR has an influence on whether a particular culture will be carbon or nitrogen limited at a certain C/N ratio, we also investigated cultures with different SRTs and the same medium C/N ratio.

Materials and Methods

Sequencing batch reactor (SBR) for culture selection

Two double-jacket glass bioreactors with a working volume of 2 l (Applikon, The Netherlands) were used for the cultivation of PHB producing bacterial cultures. The reactors (SBR I and II) were run in parallel under different conditions as specified in Table 3.1. Each reactor was equipped with a stirrer with two standard geometry six-blade turbines. Stirrer speeds were adjusted to establish the oxygen mass transfer rates required. The flow of air to the reactors was controlled with mass flow controllers (Brooks Instrument, USA) at fixed values in the range of 1.2 – 1.8 l_N/min, depending on experimental conditions. Each reactor was temperature controlled at 20°C by means of a water jacket and a thermostat bath (Lauda, Germany). The pH of the reactor liquid was maintained at 7.0 $\pm$ 0.1 using 1 M HCl and 1 M NaOH. The stirrers, the pH, the airflow and the pumps for feeding, biomass and effluent removal and pH control were controlled by a biocontroller (ADI 1030, Applikon, NL) that in turn was controlled by a PC using the software BIODACS (Applikon, NL). BIODACS was also used for data acquisition of the online measurements (DO, pH, acid dosage, base dosage, off-gas oxygen and carbon dioxide).

Table 3.1. Overview over SBR experimental conditions and carbon and nitrogen contents of the medium.

Experiment	1	2	3	4	5	6	7	8
SBR	I	I	I	I	II	II	I	I
Set SRT [d]	4	4	4	4	1	0.5	4	1
Set C/N [Cmol/Nmol]	6	24	11	13.2	13.2	13.2	15	8
Inoculum	Sludge	Exp. 1	Exp. 2	Exp. 3	Exp. 3	Exp. 4	Exp. 4	Exp. 7
Date started	16.09.04	28.01.05	01.04.05	10.06.05	13.06.05	25.07.05	05.08.05	09.02.06
Date measured	07.12.04	21.02.05	13.05.05	22.07.05	01.07.05	25.08.05	09.01.06	14.03.06
Date fed-batch	11.01.05	22.02.05	26.05.05		07.07.05	30.08.05	19.01.06	16.05.06
NaAc [mM]	30.0	60.0	30.0	30.0	56.1	165.0	30.0	90.0
NH_4Cl [mM]	10.00	5.00	5.45	4.55	8.50	25.00	4.00	22.5

The SRT of the SBRs was varied from 0.5 d to 1 d and 4 d, and the hydraulic residence time (HRT) was 8 h. Operation of the SBRs was based on 4 h batch cycles consisting of a start phase (10 min), an influent phase (3 min) in which 1 l fresh medium was supplied, a reaction phase, a biomass withdrawal phase (2 to 9 min depending on SRT) in which 83.3 ml (4 d SRT) or 333.3 ml (1 d SRT) or 666.7 ml (0.5 d SRT) mixed reactor liquor were withdrawn, a settling phase (15 min) and an effluent withdrawal phase (5 to 9 min depending on SRT) in which 916.7 ml (4 d SRT) or 666.7 ml (1 d SRT) or 333.3 ml (0.5 d SRT) of reactor supernatant were withdrawn.

The SBRs were considered to be running stable when for at least five days (i) the concentration of total suspended solids (TSS) at the end of the cycle, (ii) the measured SRT (considering also biomass removed with the effluent) and (iii) the length of the feast phase as indicated by the DO changes were constant. This definition of stable reactor operation was chosen in order to

be able to compare different SBR cultures at a similar level of development rather than to find a true long-term stable steady state, which may not exist in this kind of cultures. When a SBR was stable, a cycle was monitored additionally to the online measurements by offline samples (acetate, TSS, PHA, ammonia).

The reactors were cleaned about every 5 to 14 days (depending on SRT) to remove biofilms.

The initial inoculum of SBR I was aerobic activated sludge from the second aerobic stage of the Dokhaven wastewater treatment plant in Rotterdam, the Netherlands (September 2004). For each new experiment the sludge from a previous experiment was used as inoculum (see Table 3.1). Biomass withdrawn as mixed reactor liquor from the stable SBRs was collected and stored in the fridge for the cycles immediately before a fed-batch experiment. This biomass was used to restart the SBR after the fed-batch experiment.

The medium for the SBRs consisted of a carbon source, a nutrient solution and dilution water. The carbon source was sodium acetate with varying concentrations as described in Table 3.1. The nutrient solution was composed of NH_4Cl (concentrations as in Table 3.1), 8.3 mM KH_2PO_4, 1.85 mM $MgSO_4 \cdot 7H_2O$, 2.4 mM KCl, 5 ml/l trace elements solution according to Vishniac and Santer (1957) and 50 mg/l allylthiourea (to prevent nitrification). In the influent phase of each batch cycle 200 ml of carbon source, 200 ml of nutrient source and 600 ml of dilution water were mixed and simultaneously pumped into the reactor. Carbon and nutrient source were pumped with the same pump (two pump heads) to ensure that the ratio between these two solutions would be constant.

Fed-batch reactor for PHB production

Steady state biomass from the stable SBRs was used for PHB production in fed-batch experiments. For this purpose the same set-up as for the culture selection was used, but in a fed-batch mode. 1 l of culture from the end of a SBR cycle was mixed with 1 l of acetate- und ammonia-free medium (same composition as for the SBR, but no NaAc and NH_4Cl). To start the PHB production, a pulse of about 60 mmol concentrated sodium acetate solution was fed to the reactor. Further pulses of sodium acetate were supplied to the reactor whenever the DO signal increased (similar to Serafim et al. (2004)). The pulse feeding was continued for at least 13 h, in most experiments for over 20 h. Growth was limited in these experiments as no nitrogen source was supplied and only a small amount (if any) remaining nitrogen source from the previous SBR cycle was available. The progress of the experiments was monitored via online (DO, pH, acid and base dosage, off-gas CO_2 and O_2) and offline (acetate, TSS, PHA, ammonia) measurements.

Analytical Methods

The concentration of dissolved oxygen (DO) in the reactor was measured with a DO electrode (Mettler Toledo, USA) as percentage of air saturation and the pH was monitored with a pH

electrode (Mettler Toledo, USA). The amount of acid or base dosed for pH control was measured online. Carbon dioxide and oxygen partial pressures in the gas entering and leaving the reactor were analyzed in dried gas with a gas analyzer (NGA 2000, Rosemount, USA).

Concentrations of acetate, ammonia and total suspended solids (TSS) in reactor samples were analyzed as described previously (Johnson et al., 2009a). The amount of PHB was subtracted from the TSS to calculate the concentration of active biomass. The active biomass concentration was converted from g/l into carbon moles per litre (Cmol/l) assuming a composition of $CH_{1.8}O_{0.5}N_{0.2}$ with a molecular weight including ash of 25.1 g/Cmol (Beun et al., 2002).

Samples taken for PHB analysis were added to 15 ml tubes containing 5 drops of formaldehyde in order to stop all biological activity. Samples were subsequently washed with tap water and freeze-dried. The PHB content of the samples was determined as described previously (Johnson et al., 2009a). Results were expressed as weight percentage of PHB of the total solids.

Data evaluation

A comprehensive data analysis was carried out with the results obtained from SBR cycle measurements and fed-batch experiments. The data analysis is described in detail in Johnson et al. (2009b). In brief, measurements were corrected for the effects of sampling and addition of liquids to the reactor (acid and base solutions, feed in fed-batch experiments). Oxygen uptake and carbon dioxide evolution were determined from off-gas and DO measurements. Inorganic carbon dissolved in the liquid phase was considered when computing carbon dioxide evolution. Balances for carbon and electrons were calculated from the measured conversions at every sampling point. Finally, the available knowledge of the stoichiometry and kinetics of the PHB production and degradation metabolism was employed in the form of a metabolic model in order to compute the best estimates for all conversions and reaction rates from the measurements while complying with carbon and electron balances.

The metabolic model as described in Johnson et al. (2009b) already considered the effects of nitrogen limitation during the feast phase in order to be able to model fed-batch experiments, but it could not be used to model nitrogen limitation during the famine phase in the feast-famine SBR. The rate limiting step during the famine phase was assumed to be the rate of PHB degradation, which is dependent on the PHB content (Murnleitner et al., 1997):

$$\tilde{q}_{PHB}^{fam}(t) = k \cdot \tilde{f}_{PHB}(t)^{2/3} \qquad (3.1)$$

With PHB being degraded at a defined rate and a fixed rate of PHB consumption for maintenance processes, the growth rate in the famine phase resulted in our model from the rates of PHB degradation and maintenance:

$$m_{PHB} = \frac{m_{ATP}}{Y_{ATP/PHB}^{fam}} \qquad (3.2)$$

$$\tilde{\mu}^{fam}(t) = Y^{fam}_{X/PHB} \cdot (\tilde{q}^{fam}_{PHB}(t) - m_{PHB}) \quad (3.3a)$$

This model gave good results when growth was not limited by nutrients, but for the present study the model needed to be adjusted to account for ammonia limitation during the famine phase. A saturation term for ammonia was included in the growth equation to prevent growth in the absence of ammonia:

$$\tilde{\mu}^{fam}(t) = Y^{fam}_{X/PHB} \cdot (\tilde{q}^{fam}_{PHB}(t) - m_{PHB}) \cdot \frac{\tilde{c}_{NH_3}(t)}{\tilde{c}_{NH_3}(t) + K_{NH_3}} \quad (3.3b)$$

Now growth is no longer only a resultant from PHB degradation and maintenance, but is also dependent on the presence of ammonia. If growth does not occur anymore due to depletion of ammonia, the only PHB consuming reaction will be maintenance, and the rate of PHB degradation will therefore become equal to the rate of PHB consumption for maintenance purposes. There is no indication that the maintenance rate changes upon nitrogen depletion in our cultures. We defined therefore that if the ammonia concentration would become so small that the saturation term would be smaller than 0.5, the rate of PHB degradation was set equal to the rate of PHB consumption due to maintenance processes, i.e. maintenance became the rate limiting step:

$$\tilde{q}^{fam}_{PHB}(t) = m_{PHB} \quad \text{if} \quad \frac{\tilde{c}_{NH_3}(t)}{\tilde{c}_{NH_3}(t) + K_{NH_3}} < 0.5 \quad (3.4)$$

Else equation (3.1) was used to model PHB degradation.

The half-saturation constant K_{NH_3} in equation (3.4) was set to 10^{-7} mol/l since this value resulted in very good model fits to the measurements and did not cause numerical problems. This value has no physiological meaning. The purpose of using the metabolic model as a tool was to find better estimates for all reaction rates and yields, while complying with material balances. The aim was not to identify the actual half-saturation constant. The latter will require further research.

Results

SBR performance

Influence of the C/N ratio at constant SRT

We have evaluated the effect of the carbon to nitrogen ratio in the feed on the PHA accumulation and general reactor behaviour of a pulse-fed SBR operating at 4 d SRT. Data on the reactor behaviour after changing conditions are included in Appendix A. Figure 3.1 (A – E) shows the performances of the stable SBRs under the different employed conditions. Generally the metabolic model described the measurements very well in all experiments. Larger deviations were only observed for the conversions of carbon dioxide and oxygen which are

difficult to measure accurately due to the small differences in in- and outgoing gas concentrations (Johnson et al., 2009b). The experiments with medium C/N ratios of 6, 11 and 13.2 Cmol/Nmol (Figure 3.1 A - C) showed a similar behaviour: the acetate supplied at the beginning of the cycle was rapidly taken up and used simultaneously for growth and PHB production. Ammonia and PHB measurements during the famine phase indicated that the previously stored PHB was used for continued growth in this phase. Ammonia was not depleted at the end of the famine phase, suggesting that the cultures were not nitrogen limited, but rather carbon limited. The behaviour was very similar to previous reported experiments with acetate-fed SBRs under non-limiting nitrogen conditions (Beun et al., 2000a; Beun et al., 2002; Martins et al., 2003).

The feast phase of the experiment with a C/N ratio of 15 (Figure 3.1 D) resembled those of the experiments with a non-limiting amount of nitrogen in the feed; however, ammonia was depleted during the initial stage of the famine phase under these conditions. Although both, ammonia and acetate were completely taken up by the culture, this culture is considered as nitrogen limited due to the presence of PHB after ammonia depletion. PHB serves as an internal carbon source and therefore needs to be considered when judging whether a culture is carbon limited or not.

A C/N ratio of 24 (Figure 3.1 E) led clearly to a nitrogen limited culture with ammonia being depleted even before acetate was fully taken up by the culture. The feast phase was in this case split up in a growth phase (ammonia present) and a PHB production phase (ammonia absent). The decrease of the fraction of PHB during the growth phase of the feast phase was possibly not only caused by 'dilution' through biomass growth. The total amount of PHB present in the reactor as estimated with the metabolic model indicated that a small amount of PHB was actually used for growth simultaneously with the use of acetate. So effectively the bacteria may have grown on a mixed substrate in this case. Due to the absence of ammonia during the famine phase, PHB was probably consumed for maintenance processes only during this phase. The excess carbon supplied was effectively removed from the system in the form of PHB together with the produced biomass (elevated PHB levels at the end of the cycle, Figure 3.1 E).

Observed yields and biomass specific rates were estimated based on the metabolic model for all C/N ratio experiments at 4 d SRT (Table 3.2, left part). During the feast phase a clear trend can be observed in the ratio between acetate and ammonia uptake for the different C/N ratios: The more limiting ammonia was, the more important it was to take up ammonia fast rather than acetate and the ratio thus decreased. Although the experiment with a medium C/N ratio of 13.2 appears to have been carbon limited (ammonia was present throughout the cycle), the ratio between acetate and ammonia uptake was clearly decreased and the ammonia uptake rate was increased compared to the two other carbon limited cultures. This culture might therefore have been close to a dual limitation. The ratio between acetate and ammonia uptake for the SBR culture of a medium C/N ratio of 24 was even lower than the stoichiometric minimum value of 10.6 Cmol/Nmol for exclusive growth that can be calculated from the

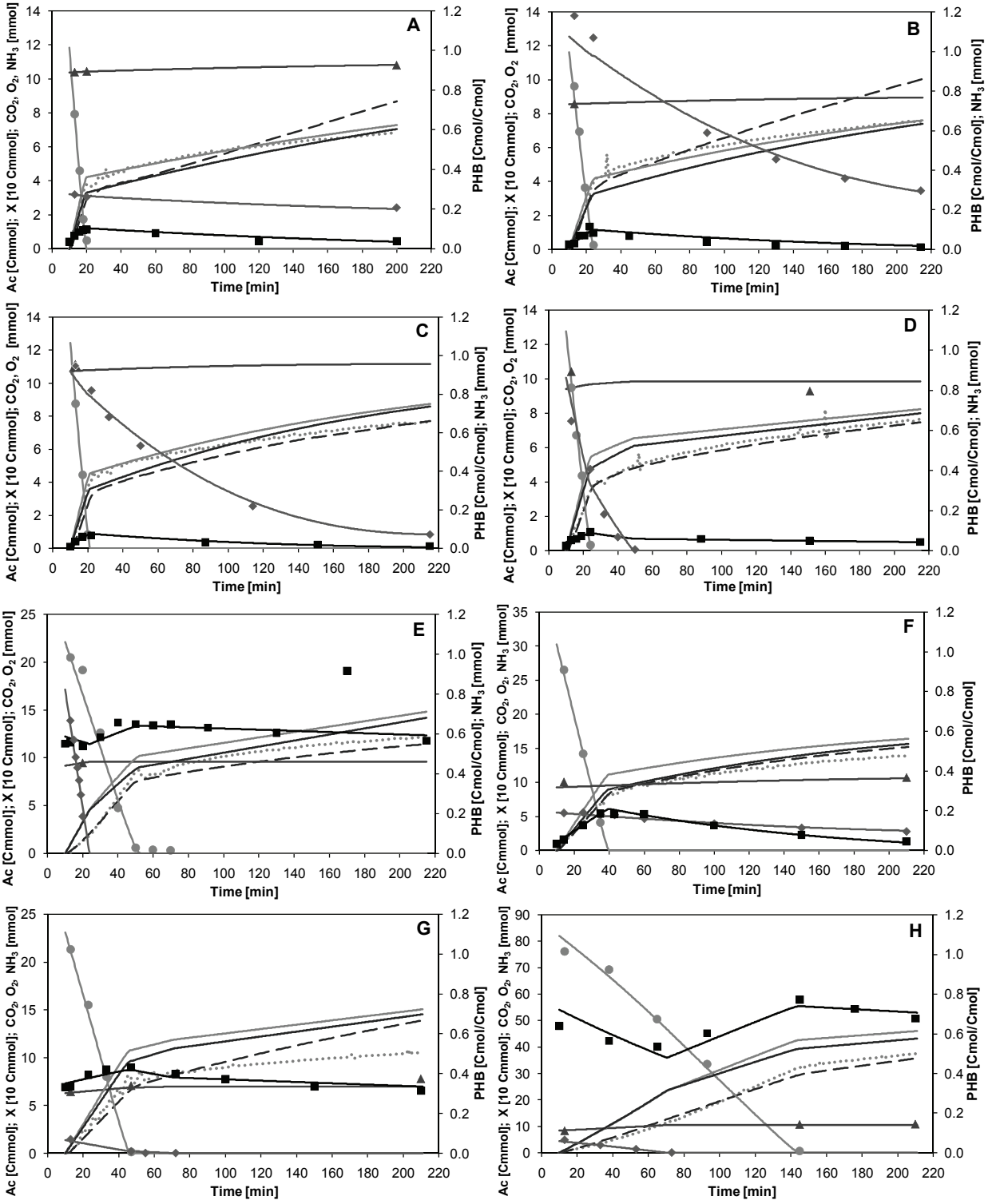

Figure 3.1. SBR performances with different C/N ratios of the medium and different SRTs. Solid lines indicate modelled values corresponding to the respective symbols indicating measurements. (▬ ▬) cumulative oxygen uptake; (▪▪▪) cumulative carbon dioxide evolution; (●) acetate; (▲) active biomass (X); (◆) ammonia; (■) fraction of PHB (PHB per active biomass). **A**: 4d SRT, C/N = 6 Cmol/Nmol. **B**: 4d SRT, C/N = 11 Cmol/Nmol. **C**: 4d SRT, C/N = 13.2 Cmol/Nmol. **D**: 4d SRT, C/N = 15 Cmol/Nmol. **E**: 4d SRT, C/N = 24 Cmol/Nmol. **F**: 1d SRT, C/N = 8 Cmol/Nmol. **G:** 1d SRT, C/N = 13.2 Cmol/Nmol. **H**: 0.5d SRT, C/N = 13.2 Cmol/Nmol.

Table 3.2. Influence of the C/N ratio of the medium and SRT on SBR performance. All rates are averages of biomass specific rates over the whole feast or famine phase apart from the specific ammonia uptake rate, which was averaged over the time that ammonia was present. Italic growth rates indicate values that include zero growth periods due to ammonia depletion. All yields are observed yields. All data were calculated with the help of a metabolic model (Johnson et al., 2009b).

Set SRT [d]		**4**					**1**		**0.5**
Set C/N [Cmol/Nmol]		**6**	**11**	**13.2**	**15**	**24**	**8**	**13.2**	**13.2**
Measured SRT [d]		4.1	3.8	4.2	3.7	3.8	1.2	1.4	0.7
Measured C/N [Cmol/Nmol]		5.8	11.3	13.7	14.8	26.9	7.4	17.0	18.9
Measured TSS [g/l][a]		1.4	1.1	1.4	1.4	1.7	1.3	1.0	1.6
Type of limitation		C	C	C	N	N	C	N	N
FEAST	Length feast [%][b]	5	7	6	7	21	15	19	68
	$Y_{PHB/Ac}^{obs}$ [Cmol/Cmol]	0.60	0.60	0.52	0.35	0.35	0.54	0.29	0.21
	$Y_{X/Ac}^{obs}$ [Cmol/Cmol]	0.04	0.04	0.10	0.21	0.19	0.09	0.24	0.27
	$Y_{CO_2/Ac}^{obs}$ [Cmol/Cmol]	0.36	0.36	0.38	0.43	0.46	0.37	0.47	0.52
	q_{Ac} [Cmol/Cmol/h]	-0.65	-0.52	-0.55	-0.52	-0.31	-0.63	-0.56	-0.36
	q_{PHB} [Cmol/Cmol/h]	0.41	0.32	0.30	0.19	0.11	0.35	0.16	0.07
	μ [Cmol/Cmol/h]	0.03	0.02	0.06	0.11	*0.06*[c]	0.06	0.14	*0.10*[d]
	q_{CO_2} [Cmol/Cmol/h]	0.24	0.20	0.21	0.23	0.15	0.24	0.26	0.19
	q_{O_2} [mol/Cmol/h]	-0.19	-0.15	-0.17	-0.20	-0.13	-0.19	-0.24	-0.18
	q_{NH_3} [mol/Cmol/h]	-0.006	-0.005	-0.011	-0.023	-0.038	-0.011	-0.028	-0.046
	q_{Ac} / q_{NH_3} [Cmol/Nmol]	107	113	48	23	8	55	20	8
	PHB max. [%]	7.7	9.1	5.3	7.4	36.0	13.7	27.0	39.8
FAMINE	$Y_{X/PHB}^{obs}$ [Cmol/Cmol]	0.56	0.50	0.61	0.37	0.00	0.67	0.21	0.00
	$Y_{CO_2/PHB}^{obs}$ [Cmol/Cmol]	0.44	0.50	0.39	0.63	1.00	0.33	0.79	1.00
	q_{PHB} [Cmol/Cmol/h]	-0.022	-0.025	-0.017	-0.014	-0.018	-0.05	-0.028	-0.029
	μ [Cmol/Cmol/h]	0.012	0.012	0.011	*0.005*[e]	*0.000*	0.04	*0.006*[f]	*0.000*
	q_{CO_2} [Cmol/Cmol/h]	0.010	0.012	0.007	0.009	0.018	0.02	0.023	0.029
	q_{O_2} [mol/Cmol/h]	-0.012	-0.015	-0.008	-0.010	-0.020	-0.02	-0.026	-0.033
	q_{NH_3} [mol/Cmol/h]	-0.002	-0.002	-0.002	-0.008	0.000	-0.007	-0.008	0.000
TOTAL	Time NH_3 present [%][c]	100	100	100	19	7	100	30	30
	$Y_{PHB/Ac}^{obs}$ [Cmol/Cmol]	0.01	0.01	0.00	0.01	0.14	0.02	0.06	0.17
	$Y_{X/Ac}^{obs}$ [Cmol/Cmol]	0.37	0.34	0.42	0.34	0.19	0.44	0.29	0.27
	$Y_{CO_2/Ac}^{obs}$ [Cmol/Cmol]	0.62	0.66	0.58	0.65	0.67	0.54	0.65	0.56
MODEL	$\tilde{q}_{Ac}^{\max}$ [Cmol/Cmol/h]	-0.78	-0.64	-0.65	-0.62	-0.36	-0.69	-0.63	-0.37
	$\tilde{\mu}^{\max}$ [Cmol/Cmol/h]	0.03	0.03	0.07	0.13	0.19	0.06	0.15	0.23
	k [(Cmol/Cmol)$^{1/3}$/h]	-0.13	-0.18	-0.17	-0.39	n.d.	-0.25	-0.15	n.d.
	m_{ATP} [mol/Cmol/h]	0.015	0.026	0.006	0.026	0.075	0.000	0.083	0.125
Fraction growth in feast [%]		12	13	24	63	100	20	85	100

a: at the end of the influent phase; *b*: percentage of time of the reaction phase (feast and famine phase); *c*: 0.19 during presence of ammonia; *d*: 0.23 during presence of ammonia; *e*: 0.041 during presence of ammonia; *f*: 0.041 during presence of ammonia

stoichiometric yields in the metabolic model (Johnson et al., 2009b). However, as PHB seems to have served as an additional carbon source during the feast phase, the value of 8 Cmol acetate taken up per Nmol seems realistic.

Observed yields and rates of PHB production were higher for carbon limited cultures, while observed biomass yields and growth rates in the feast phase were higher for nitrogen limited cultures. The growth rate in the feast phase for the culture with a medium C/N ratio of 24 only seems low, as it is the average growth rate over the whole feast phase, including thus the time of zero growth when ammonia was depleted (during presence of ammonia it was 0.19 Cmol/Cmol/h).

In the famine phase observed growth yields were higher for carbon limited cultures. In the nitrogen limited cultures a significantly larger fraction of PHB would be used for maintenance processes leading to a lower observed yield. The overall observed yields over the whole cycle (Table 3.2, 'TOTAL') indicate that the strongly nitrogen limited culture (C/N 24) 'wasted' some of the excess carbon by a net PHB production. The modelling parameters estimated during the fitting procedure (Johnson et al., 2009b) are also included in Table 3.2 ('MODEL'). The maximum specific acetate uptake rate decreased with higher C/N ratios, while the maximum growth rate increased. The rate constant of PHB degradation k could only be estimated in experiments where PHB was used for growth in the famine phase. The time frame for estimating k was very short in the experiment with C/N 15 (ammonia depleted); the accuracy of this particular value should therefore be doubted. An analysis of the impact of small deviations in the model parameters on the model outcome is included in Appendix B.

Additionally to the SBR experiments with 4 d SRT, also two experiments were performed at 1 d SRT with C/N ratios of 8 and 13.2 Cmol/Nmol (Figure 3.1 F and G). The C/N ratio of 8 resulted in a carbon limited culture with a similar behaviour to the carbon limited cultures of 4 d SRT, while the culture with a C/N ratio of 13.2 yielded a nitrogen limited culture. Also at 1 d SRT the differences between nitrogen and carbon limited cultures were reflected in the observed PHB yield and growth yield in the feast phase, the ratio between acetate and ammonia uptake, and the net production of PHB over the whole cycle (Table 3.2) in the same way as discussed above for 4 d SRT.

Influence of the SRT

The steady state of the SBR at 4 d SRT with a medium C/N ratio of 13.2 Cmol/Nmol was chosen as a base to study the influence of lowering the SRT while maintaining the same C/N ratio. Data on the reactor behaviour after changing conditions are included in Appendix A. The results for the stable cultures with 4, 1 and 0.5 d SRT and a medium C/N ratio of 13.2 are shown in Figure 3.1 C, G and H, respectively. While at 4 d SRT the culture had been carbon limited, albeit probably close to dual limitation, the cultures at 1 d and 0.5 d SRT were clearly nitrogen limited. Both cultures had elevated PHB levels and ammonia was depleted almost simultaneously with acetate (1 d SRT) or even well before acetate (0.5 d SRT). However, the measurements and

modelling of the SBR cycle at 1 d SRT and 0.5 d SRT indicated that the actual C/N ratios of the feed supplied in both cycles were significantly higher than 13.2 (17.0 and 18.9 respectively, Table 3.2), although the medium was prepared with C/N ratios of 13.2. Small deviations from the set C/N ratio can occur if not exactly the same volumes of carbon and nitrogen source are pumped into the reactor, however, these large deviations in these two experiments can not be explained. A direct comparison of the three experiments with C/N 13.2 is therefore not possible. But the nitrogen limited cultures at 1 and 0.5 d SRT can be compared with the two nitrogen limited cultures at 4 d SRT (C/N 15 and 24). Additionally the experiment at 1 d SRT under carbon limitation with a C/N ratio of 8 (Figure 3.1 F) can serve for comparison with the carbon limited cultures at 4 d SRT with comparable C/N ratios (C/N 6 and 11).

Generally, the length of the feast phase increased at lower SRTs (Table 3.2), as more substrate was supplied per unit of biomass per cycle and the cultures did not increase their acetate uptake rate. The nitrogen limited cultures at low SRTs (1 and 0.5 d) showed even lower observed PHB yields and higher observed biomass yields during the feast phase compared to the nitrogen limited cultures growing at a SRT of 4 days. Similarly also the carbon limited culture at 1 d SRT had a lower observed PHB and higher observed biomass yield in the feast phase than the carbon limited cultures of 4 d SRT and comparable C/N ratios (C/N 6 and 11).

The ratio between acetate and ammonia uptake rate (Table 3.2) proved again to be a good measure for the degree of nitrogen limitation: the ratio decreased with the intensity of nitrogen limitation. Similar to the experiment of 4 d SRT and C/N 24, the experiment with 0.5 d SRT and C/N 13.2 had a ratio of acetate uptake to nitrogen uptake lower than the theoretical minimum required for growth. Likewise, also in the 0.5 d SRT experiment PHB appears to have been consumed in the presence of acetate in the first part of the feast phase in order to provide the required amount of carbon for biomass growth.

During the famine phase reaction rates tended to be higher at lower SRTs than at higher SRTs. Growth was limited or completely prevented by the depletion of ammonia in the nitrogen limited SBRs with 1 and 0.5 d SRT, respectively, as reflected in the observed yields and rates in Table 3.2.

When considering the total cycle, it is apparent that also the nitrogen limited cultures at lower SRTs had increased observed net PHB yields corresponding to the extent of ammonia limitation. Net production of PHB was a way for the cultures to ‘waste’ excess carbon. The carbon limited culture of 1 d SRT had the highest observed biomass yield over the whole cycle. From the model-based parameters it is also apparent that nitrogen limitation was linked to a higher ATP requirement for maintenance (m_{ATP}, Table 3.2).

The reactor at 0.5 d SRT was not easy to operate. Rapid biofilm formation was a major problem and the reactor had to be cleaned at least once a week, indicating that it was operated close to the wash-out rate of the system (Tijhuis et al., 1994).

PHB production

The PHB production capacity of the SBR cultures enriched at different C/N ratios and SRTs was investigated in fed-batch experiments without nitrogen addition designed to maximize the cellular PHB content. No fed-batch experiment was performed for the SBR with 4 d SRT and C/N 13.2, as it was not considered to be different enough from the SBR with 4 d SRT and C/N 11 at the time. Figure 3.2 A displays all PHB results in one diagram for direct comparison. Cultures that had elevated PHB levels already during the SBR stage started the fed-batch experiments with these higher PHB levels. For a better comparison with the other cultures that started from near zero PHB content, the PHB results for the cultures with elevated PHB starting concentrations were shifted on the time axis (see figure legend).

The PHB accumulation kinetics were fairly similar in all cultures with the PHB production rate decreasing with increasing PHB contents. As an example, the average specific rate of PHB production over the whole fed-batch experiment of the culture of 4 d SRT and C/N 11 was 0.12 Cmol/Cmol/h while the highest rate reached was 0.39 Cmol/Cmol/h. A comparison of the modelled specific rates of acetate uptake, PHB production and ammonia uptake for the four fed-batch experiments of SBR cultures with 4 d SRT can be found in Appendix C. Maximum PHB contents reached in the different experiments differed significantly and ranged from about 1.8 Cmol/Cmol or just over 60 wt% (4 d SRT, C/N 6 and 15) to about 3.0 Cmol/Cmol or just over 70 wt% (4 d SRT, C/N 11 and 1 d SRT, C/N 8).

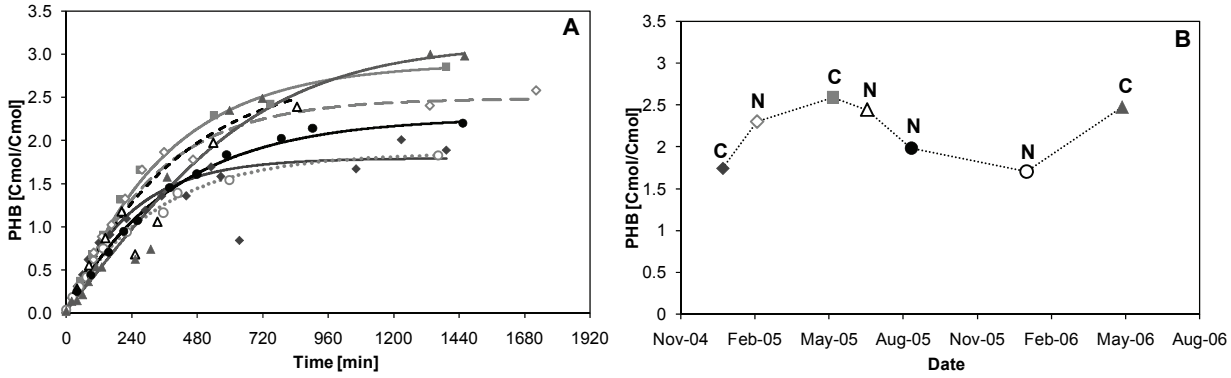

Figure 3.2. Fed-batch experiments. **A**: PHB production performances in fed-batch experiments for the cultures enriched in the different SBRs. Lines indicate modelled values corresponding to the respective symbols indicating measurements of the fraction of PHB (PHB per active biomass). (◆, ——) 4d SRT, C/N = 6 Cmol/Nmol; (■, ——) 4d SRT, C/N = 11 Cmol/Nmol; (○, ▪▪▪) 4d SRT, C/N = 15 Cmol/Nmol; (◇, — —) 4d SRT, C/N = 24 Cmol/Nmol, shifted by 100 min.; (▲, ——) 1d SRT, C/N = 8 Cmol/Nmol; (△, - - -) 1d SRT, C/N = 13.2 Cmol/Nmol, shifted by 40 min.; (●, ——) 0.5d SRT, C/N = 13.2 Cmol/Nmol, shifted by 40 min; no experiment was performed for 4d SRT, C/N = 13.2 Cmol/Nmol. **B**: Fraction of PHB after 800 min fed-batch at the respective dates of the fed-batch experiment. Same symbols as in Figure 3.2 A. C indicates carbon limited SBR cultures; N indicates nitrogen limited SBR cultures.

No clear correlation was found between PHB production capacity and the conditions employed in the SBR for culture enrichment. However, a pattern can be observed when considering the

time sequence in which the experiments were performed (Fig. 3.2 B). Initially the PHB contents increased with each experiment, likely due to continued enrichment of competitive PHB producers. After the experiment with 4 d SRT and a C/N ratio of 11 that led to a high PHB content, the three following SBR cultures were enriched under nitrogen limitation for a considerable number of generations (1 d SRT, C/N 13.5 - 24 SRTs; 0.5 d SRT, C/N 13.5 - 72 SRTs; 4 d SRT, C/N 15 - 42 SRTs) and all showed a lower PHB production performance. The reactor with 0.5 d SRT and C/N 13.2 was further maintained under the same conditions for a total of 272 SRTs at which point another fed-batch experiment was performed. At this stage the culture only reached 10 wt% PHB content (0.1 Cmol/Cmol) after more than 1500 minutes fed-batch and was dominated by filamentous bacteria (data not shown). In the previous fed-batch experiment filaments were a minor part of the culture. The behaviour of the culture in the SBR (e.g. length of feast phase) had, however, not noticeably changed. So while the SBR culture was stable from an operational point of view, it was apparently not stable in terms of microbial composition and PHB production capacity.

The experiment at 4 d SRT and C/N 24 was only maintained under these conditions for about 6 SRTs before the fed-batch experiment was performed. It could thus be that any potential detrimental effect of the nitrogen limitation on the PHB production performance might not have developed in this short time.

After the experiment with 4 d SRT and C/N 15 the conditions in this reactor were changed to 1 d SRT and a C/N ratio of 8. Under the new conditions with carbon limitation the PHB production capacity went back up to just over 70 wt% (3.0 Cmol/Cmol).

Discussion

Carbon versus nitrogen limitation

Accumulation of PHA is traditionally assumed to be related to limitation of a nutrient like nitrogen or phosphate and excess of a carbon compound for growth (Braunegg et al., 2004). During the enrichment of PHA accumulating bacteria in a mixed culture both, growth and PHA storage, need to be supported. The question arises thus whether carbon or nitrogen limitation are more beneficial for the enrichment of a culture with a high PHA storage capacity.

In a dynamically operated SBR with potential storage of carbon compounds in the cells it is not easy to determine the limiting compound from measurements of the reactor liquid. The results obtained in this study show that the state of the SBR in terms of carbon or nitrogen limitation can be characterized by measuring the ratio between acetate and ammonia uptake. Under carbon limiting conditions competition in the SBR is based on the carbon uptake rate leading to high specific acetate uptake rates, whereas under nitrogen limiting conditions competition is based on the specific ammonia uptake rate which was consequently found to be increased. As during nitrogen limitation the cultures depleted both, carbon and nitrogen source, the excess

carbon was 'wasted' by the cultures through a net production of PHB, which was removed from the system at the end of the cycle together with the harvested biomass. Only the nitrogen limited culture from the SBR at 4 d SRT and with a C/N ratio of 15 did not show a significant net PHB production. This culture seems to have been just slightly nitrogen limited.

Nitrogen limited cultures offer the advantage of a higher PHB content at the point of biomass harvesting, and would subsequently need less time in the PHA accumulation phase of the production process. However, nitrogen limited cultures had in most cases a decreased capacity to store PHB or even lost this capacity almost completely when maintained for a long time under nitrogen limiting conditions. It cannot be excluded that also other operational parameters may have played a role in obtaining the different PHB production capacities, but it seems likely that nitrogen limitation played a key role. Obviously there is no competitive advantage for carbon storage if nitrogen is the limiting compound for biomass production. It is thus unlikely that nitrogen limitation in the SBR will enrich efficient PHB producers. A carbon limited culture on the other hand was reported to increase its PHB storage capacity with time when maintained under the same conditions for a long time (Johnson et al., 2009a). This culture was shown to accumulate nearly 90 wt% PHB (9.6 Cmol/Cmol) (Johnson et al., 2009a).

The work of Albuquerque et al. (2007) confirms that also when real wastewater was used as a substrate, nitrogen limitation in the SBR led to a loss of the storage response, while carbon limitation yielded a stable PHA producing culture. On the other hand Serafim et al. (2004) reported a stable PHB producing culture in an acetate-fed SBR that was operated at a C/N ratio of 21.4 Cmol/Nmol for over a year. This SBR was clearly nitrogen limited: ammonia depletion occurred just after acetate depletion and PHB levels were around 37 wt% (0.7 Cmol/Cmol). In both works a SRT of 10 d was used, however, the feed C/N ratio applied by Albuquerque et al. (2007) was higher than the one used by Serafim et al. (2004), leading therefore to a strong nitrogen limitation in the first case (ammonia depleted long before carbon source) and a weak nitrogen limitation in the latter. The weak nitrogen limitation in the acetate-fed SBR is also reflected in the ratio between acetate and ammonia uptake which was about 33 Cmol/Nmol. This value is higher than for our nitrogen limited cultures (rate ratios ≤ 23 Cmol/Cmol), but lower than for our carbon limited cultures (rate ratios ≥ 48 Cmol/Cmol). It appears thus that a very weak nitrogen limitation can lead to a long-term stable PHB producing culture. But the maximum PHA storage capacity found by Serafim et al. for this nitrogen limited culture was with 65 wt% (2.2 Cmol/Cmol) (Serafim et al., 2008a) somewhat lower than for our carbon limited cultures.

Other authors have investigated nutrient limitation or starvation in PHA producing SBRs before, but they usually did not aim for a stable reactor operation (Punrattanasin et al., 2006; Chinwetkitvanich et al., 2004; Ma et al., 2000) or did not use a feast-famine regime or fully aerobic conditions (Chua et al., 1999; Chua et al., 2003; Rhu et al., 2003; Wang et al., 2007) and they generally did not report detailed measurements of the SBR cycle and reaction rates. A comparison of our result with those reported previously is therefore not possible.

A surprising finding in our study was that strongly nitrogen limited cultures seemed to degrade PHB during the beginning of the feast phase in order to facilitate the very fast ammonia uptake and associated growth. The enzymes for PHB degradation were apparently active independently of the presence of an external carbon source. It has been reported that the PHB synthase and a PHB depolymerase are expressed constitutively in *Ralstonia eutropha* (Lawrence et al., 2005), but the mechanisms by which the cells regulate PHA production and consumption are unclear (Uchino et al., 2007). The immediate switch that is observed in feast-famine SBR cultures from PHA production to PHA consumption and vice-versa supports the hypothesis that both types of enzymes are active throughout the cycle. From our experiments where PHB degradation seemed to occur during the feast phase it can be conjectured that the regulation of PHA production and degradation does not depend on the presence or absence of the external carbon source, but possibly rather on the concentration of cell-internal metabolites. If cells compete for ammonia and are thus forced to use carbon faster for growth than it can be taken up from the medium, cell internal concentrations of e.g. acetyl-coenzyme-A will be very low and those of free coenzyme-A high, which possibly triggers the degradation of PHB.

When applying nitrogen limitation to an open mixed culture, there is also a chance that nitrogen fixing bacteria may become part of the population (Patel et al., 2009). In that case the uptake of ammonia would no longer reflect biomass growth. However, we did not find any indication of significant presence of nitrogen fixation in the nitrogen limited reactors from the material balances.

Influence of SRTs

Unfortunately it was not possible to compare cultures growing at different SRTs with exactly the same medium C/N ratio, since the actual C/N ratios measured for the cultures at 1 and 0.5 d SRT (medium C/N ratio 13.2) were found to be significantly higher than 13.2 in the investigated cycles.

A comparison of the nitrogen limited cultures at 4 d SRT with those at 1 and 0.5 d SRT revealed that the lower the SRT was, the more the cultures used acetate for direct growth rather than storage (higher observed biomass and lower observed PHB yields, Table 3.2). This trend was similar for carbon limited cultures. The cultures at lower SRTs were supplied with more carbon and nitrogen per unit of biomass. Since these cultures apparently did not adjust their acetate uptake rate to the increased growth rate, the feast phases therefore became longer with decreasing SRT (Table 3.2). The cultures, however, did adjust their ammonia uptake rates in the feast phase (Table 3.2) and thus also their actual growth rates. As a result observed growth yields increased at lower SRTs while observed PHB yields decreased.

With increasing nitrogen limitation an increasingly larger fraction of the growth occurred in the feast phase directly on acetate rather than on PHB in the famine phase (Table 3.2, bottom row). Growth on acetate directly is more efficient than growth on acetate via PHB as the degradation of PHB requires ATP. Growth via PHB is about 7% less efficient than growth on acetate directly

(Beun et al., 2000a). However, since the cultures were nitrogen limited, they could not benefit from their more efficient use of carbon for biomass production. These cultures even 'wasted' the excess of carbon by higher maintenance requirements and net PHB production (Table 3.2). A culture that did benefit from growing more in the feast and less in the famine phase was the carbon limited culture of 4 d SRT and C/N 13.2, which was close to a dual limitation. This culture had already increased its growth rate in the feast phase as a response to the lower ammonia concentrations it experienced, but since it was not nitrogen limited, it did not 'waste' carbon in the form of PHB or large maintenance requirements. As a consequence this culture had a higher observed biomass yield over the whole cycle than the other SBR cultures at the same SRT (Table 3.2).

The highest observed growth yield over the whole cycle was found for the carbon limited culture at 1 d SRT. When comparing this culture with the cultures at 4 d SRT with comparable C/N ratios (6 and 11), it can be concluded that the culture at 1 d SRT reached its higher efficiency in biomass production partly through less maintenance requirements but mostly through growing relatively more in the feast phase. As discussed above, at lower SRTs the feast phase lasted longer, allowing thus for more growth in this phase. Beun et al. (2000a) compared SRTs from 3.8 to 19.8 d and also found that at lower SRTs cultures grew relatively more in the feast phase and had subsequently higher observed biomass yields over the whole cycle. If cultures grow more efficiently at lower SRTs, it can be expected that the same C/N ratio my lead to a carbon limitation at higher SRTs, while leading to increasing nitrogen limitation with decreasing SRTs.

A study that investigated two different SRTs with the same nutrient limited wastewater was conducted by Coats et al. (2007). Pulp and paper mill foul condensate was fed to aerobic feast-famine SBRs with an SRT of 4 or 6 d. At 4 d SRT the average PHA content of the enriched culture was higher than at 6 d SRT. This may indicate that the SBR at 4 d SRT was probably stronger nutrient limited than the SBR at 6 d SRT as a consequence of the different SRTs. The previously mentioned study conducted by Serafim et al. (2004) at 10 d SRT and a medium C/N ratio of 21.4 Cmol/Cmol resulted in a weakly ammonia limited culture with an uptake ratio of about 33 Cmol/Nmol. If we would use the same medium C/N ratio, but a SRT of 4 d, the results in Table 3.2 would suggest that the ratio between acetate and ammonia uptake would be between 8 and 23 Cmol/Nmol. A culture at 4 d SRT can therefore be expected to be stronger nitrogen limited than the culture at 10 d SRT with the same medium C/N ratio, which again confirms our hypothesis that nitrogen limitations get stronger if lower SRTs are used.

In order to reach high biomass productivities and yields in a potential commercial PHA production process, lower SRTs are favourable. An SRT of 1 d has been successfully applied in several studies before (e.g. Dionisi et al., 2006; Dionisi et al., 2001) and also we found this SRT feasible for enriching a PHB producing culture (Johnson et al., 2009a). The nitrogen limited culture at 0.5 d SRT caused problems due to substantial biofilm formation and lost most of its PHB storage capacity over time. When an attempt was made at a later stage to run an SBR at 0.5 d SRT under carbon limiting conditions, the enriched culture showed a very low PHB storage

capacity (27 wt%, 0.4 Cmol/Cmol). In experiments with 0.5 d SRT the feast phase was longer than the famine phase and the selective pressure for PHB producers was apparently not strong enough to obtain a stable PHB producing culture with a high storage capacity.

Although low SRTs are favourable for biomass production, we have also shown here that very low SRTs do not favour PHB storage. In order to increase the selective pressure for PHB production in cultures growing at low SRTs the SBR cycle length can be increased. In longer cycles cultures have to store a higher amount of PHB in order to grow throughout the longer famine phase.

Implications for PHA production from waste streams

One of the advantages of the use of open mixed cultures for PHA production is that waste streams can be used as a substrate. Suitable industrial wastewaters are for example streams form the food industry or paper industry (Bengtsson et al., 2008a; Albuquerque et al., 2007; Beccari et al., 2009), but many of these streams have a low nitrogen content and may require supplementation. Our results and those reported in literature indicate that a stable PHA producing culture with a high PHA accumulation capacity can best be enriched on carbon limited feeds. The long-term stability of the process is essential as mixed culture PHA production is a continuous process. Also a high PHA storage capacity is of major importance for keeping costs for downstream processing in an economically feasible range. Carbon limitation appears to support both – long-term process stability and a high and or even increasing PHA production capacity.

If severely nutrient deficient waste streams would not be supplemented with the limiting nutrient(s), the probability seems high that no stable PHA producing culture would be obtained (unless PHA producing nitrogen fixing bacteria could be enriched under nitrogen limitation). If weakly nutrient deficient waste streams were used, it might be possible to obtain a long-term stable PHA producing culture, but possibly high SRTs and thus low biomass productivities might be required. With a nutrient limitation during the enrichment step PHA contents could be expected to be higher already in this step, which would save time during the actual PHA production stage. However, the time saving is an insignificant advantage if maximum PHA contents reached during the PHA production step do not equal those obtained with a carbon limited strategy. And if such a nutrient limited strategy would indeed require higher SRTs, the productivity of the overall process would be lowered significantly.

Supplementation of nutrient deficient wastewaters with the limiting nutrient(s) to obtain a (just about) carbon limited feed stream seems thus advisable for the best performance of a mixed culture PHA production process. However, supplementation with nutrients is costly and the benefits will have to justify the costs involved. For wastewaters that require nutrient supplementation for the purpose of wastewater treatment anyway, the PHA production process might even offer savings in the addition of nutrients, because during the actual PHA

production step carbon would be removed from the wastewater without the need for nutrients, reducing the overall need for nutrient supplementation.

Conclusions

- Nitrogen limitation is a successful strategy for reaching high PHA contents during the PHA production step. However, a carbon limitation strategy was found to be favourable for the enrichment and long-term cultivation of a PHA producing community. Severely nutrient limited waste streams will need to be supplemented with sufficient nutrients for a successful enrichment of PHA producing cultures with high PHA storage capacities.
- Under carbon limitation microbial competition in the SBR was based on the specific acetate uptake rate, while under nitrogen limitation competition was based on the specific ammonia uptake rate. The ratio between acetate and ammonia uptake rate can thus give an indication for the kind and degree of limitation a culture is experiencing. Ratios below 33 Cmol/Nmol (Serafim et al., 2004) indicated nitrogen limitation, ratios above 48 Cmol/Nmol carbon limitation (at reactor temperatures around 20°C). Elevated cellular PHB contents at the end of the SBR cycle are another indication for the presence of a limitation other than carbon.

Appendices

Appendix A: Culture development between experiments

When the reactor operating conditions were changed to a new carbon to nitrogen ratio or a new SRT, online measurements were recorded to follow the development of the culture. From DO measurements the length of the feast phase can be evaluated. Only when the length of the feast phase became stable, also TSS and SRT were measured daily in order to judge if the reactor performance was stable. In most cases TSS and SRT values were stable when the length of the feast phase was constant. The average values of the measured TSS and SRT are reported in Table 3.2. Fig. 3.A shows the development of the length of the feast phase with operating time for all cultures apart from experiment 8 (1 d SRT, 8 Cmol/Nmol), for which the data are not available anymore.

In the first experiment (4 d SRT, 6 Cmol/Nmol; Fig. 3.A A) the reactor had been inoculated with activated sludge from a wastewater treatment plant. A clear feast-famine profile developed within a few cycles and the length of the feast phase continued to decrease during about the first 150 cycles. During this first experiment the set-up was slightly optimized at different time points, which disturbed the stable operation somewhat. The reactor was therefore operated for a long time before a cycle was measured. In most experiments the change of operating conditions caused a disturbance of the reactor for the first 50-100 cycles. The experiment of 4 d

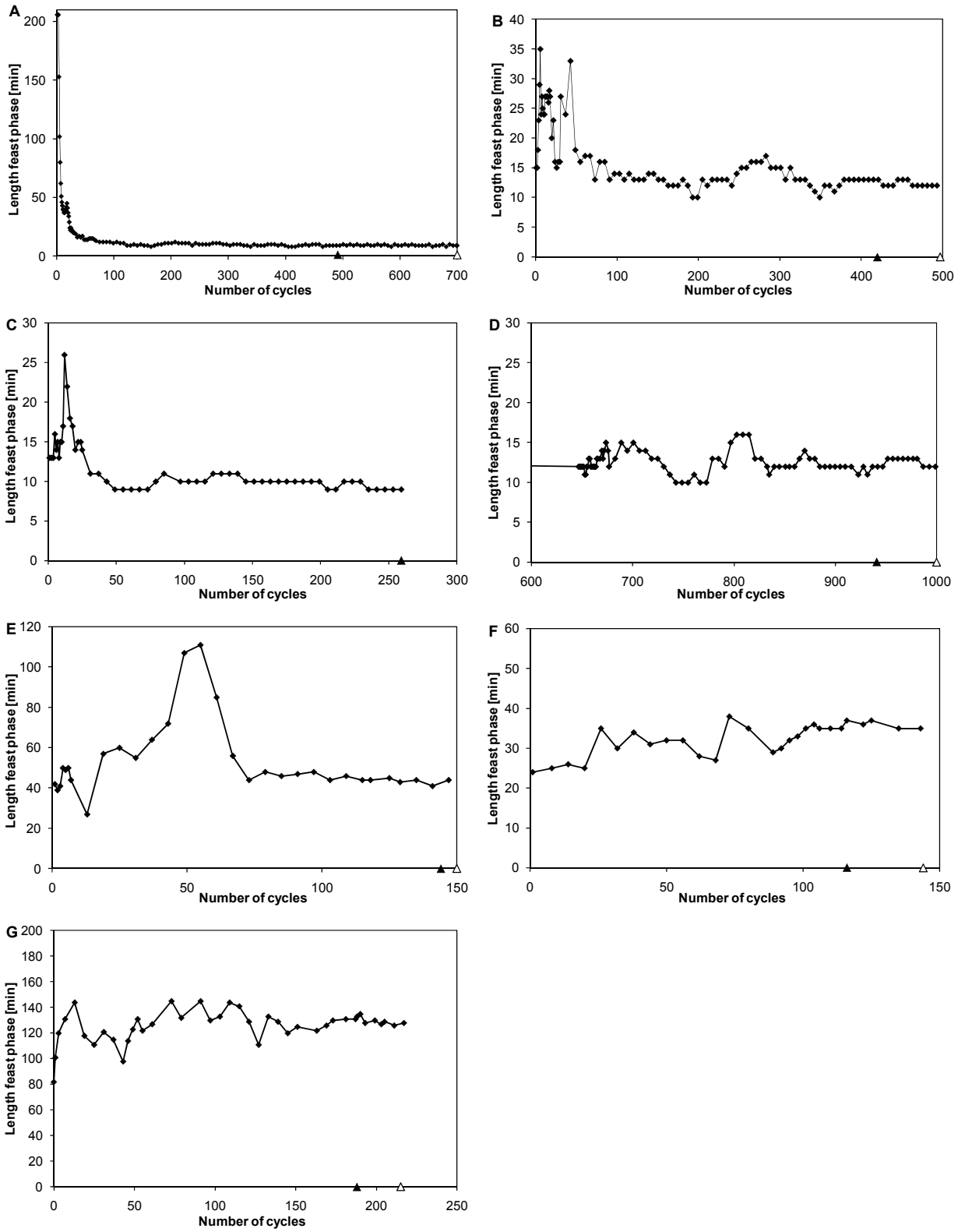

Figure 3.A. SBR feast phase lengths over operating time with different C/N ratios of the medium and different SRTs (◆). Solid triangles (▲) indicate the time of the cycle measurement, open triangles (△) the time of the fed-batch experiment. **A:** 4d SRT, C/N = 6 Cmol/Nmol. **B:** 4d SRT, C/N = 11 Cmol/Nmol. **C:** 4d SRT, C/N = 13.2 Cmol/Nmol. **D:** 4d SRT, C/N = 15 Cmol/Nmol. **E:** 4d SRT, C/N = 24 Cmol/Nmol. **F:** 1d SRT, C/N = 13.2 Cmol/Nmol. **G:** 0.5d SRT, C/N = 13.2 Cmol/Nmol.

SRT and 15 Cmol/Nmol (Fig. 3.A D) was not investigated for the first 650 cycles. This reactor run showed some changes in the length of the feast phase after about 700 and 800 cycles because of slight adjustments made to reach the SRT aimed for.

Small fluctuations in the length of the feast phase even after long reactor operation can always occur, because the amount of medium supplied to the reactor in the influent phase was not always constant. Deviations of ±10% (i.e. ± 1 min for a 10 min feast phase) were thus not considered as instabilities.

Appendix B: Impact of model parameters on estimated reaction rates

A sensitivity analysis was carried out to investigate the impact of the individual model parameters on the model outcome. The aim was to evaluate how sensitive the specific reaction rates estimated with the metabolic model were to small deviations in the model parameters. The experiment at 1 d SRT with a medium carbon to nitrogen ratio of 8 Cmol/Nmol was chosen for this purpose, since it reflects the conditions used in the majority of our experiments. The four key model parameters maximum specific acetate uptake rate, maximum specific growth rate in the feast phase, rate constant of PHB degradation and specific maintenance ATP requirement were individually varied by a small amount and fixed at the new value, while the remaining parameters were newly estimated with the model. The first three parameters were each changed up and down by 5% of the original value, while the maintenance ATP requirement, which had been estimated as 0 mol/Cmol/h for this experiment, was increased by 0.01 mol/Cmol/h. For each individual change (so seven times in total) the system was remodelled by taking the parameter under investigation out of the fitting procedure and only allowing changes for the remaining three parameters and the initial conditions (i.e. initial concentrations of biomass, acetate, ammonia and PHB). After a new solution with a minimal error between measurements and model predictions was found, all average specific reaction rates for the feast and famine phase were computed. The newly obtained model parameters, average specific reaction rates and total error between model and measurements were compared to the values previously obtained (Table 3.2) and the percentage of change was calculated.

Fig. 3.B displays the results of this analysis. Generally the error between model and measurements increased the least (0.1%, Fig. 3.B B) when the maximum specific growth rate in the feast phase was changed. Growth only accounted for 9% of the total carbon conversion in the feast phase. Due to the comparatively low rate and small contribution of the growth process on total conversions in the feast phase, a small deviation in the maximum growth rate will have a very small impact on the total model outcome. Consequently mainly the average specific growth rate in the feast phase and the specific ammonia uptake rate showed significant changes. The model outcome was thus not very sensitive to small changes in the maximum specific growth rate. The same can be expected for most carbon limited cultures, but in nitrogen limited cultures the growth rate in the feast phase will have a larger contribution to

the overall carbon conversion and the identifiability of the maximum specific growth rate would improve.

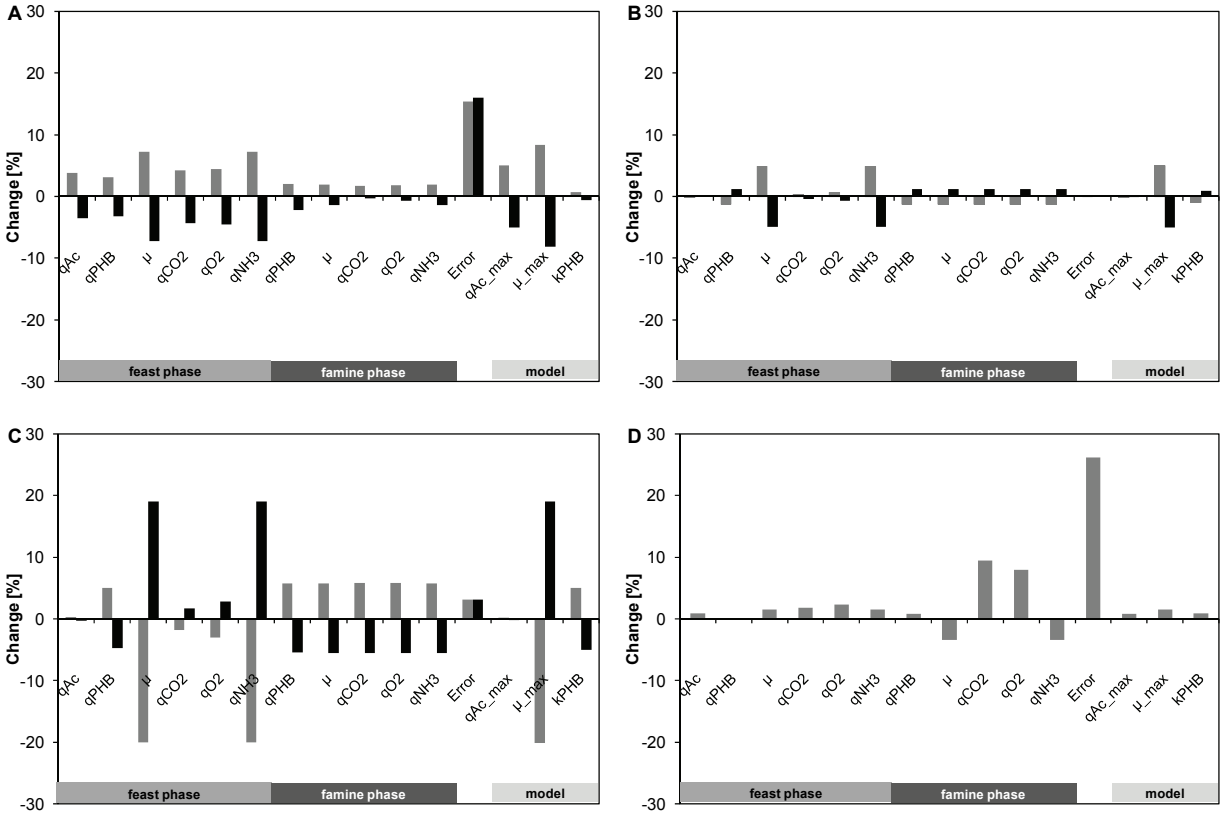

Figure 3.B. Change in estimated reaction rates if one model parameter is changed and the remaining parameters are re-estimated (experiment of 1 d SRT, 8 Cmol/Nmol). The error reflects the change in deviation between model and measurements. **A:** Increase (grey) or decrease (black) of the modelled maximum acetate uptake rate by 5%. **B:** Increase (grey) or decrease (black) of the modelled maximum growth rate by 5%. **C:** Increase (grey) or decrease (black) of the modelled rate constant of PHB degradation by 5%. **D:** Increase of maintenance ATP requirements from 0 to 0.01 mol/Cmol/h.

The change in the rate constant for PHB degradation led to a medium strong increase in the error (Fig. 3.B C). An increase in this rate constant obviously will result in an increase of all specific reaction rates in the famine phase (the rate of PHB degradation controls all other rates), but it also led to a higher specific PHB production rate and lower specific growth rate in the feast phase. If more growth takes place in the famine phase, less growth will occur in the feast phase, and overall steady state can be maintained.

The change in the maximum specific acetate uptake rate led to a large increase of the error (Fig. 3.B A). If this rate was increased, all other specific rates increased, too, and vice-versa. This is because the main change induced by changing the maximum specific acetate uptake was a change of the estimated biomass concentration. The volumetric rates and total conversions did thus hardly change, only the biomass specific rates. Adequate identification of the maximum

specific acetate uptake rate therefore relies strongly on the estimated concentration of active biomass and vice versa.

The greatest increase of error was found for an increase of the maintenance ATP requirement by 0.01 mol/Cmol/h (Fig. 3.B D). A higher maintenance coefficient results in higher oxygen uptake and higher production of carbon dioxide. This can particularly be seen for the famine phase, where the higher maintenance requirement caused a drop in the growth rate.

This analysis shows that relatively small changes of the model parameters generally led to considerably larger errors between measurements and model and thus to a significantly worse fit. The only exception was a change in the maximum specific growth rate, but this rate was so low compared to e.g. the maximum specific acetate uptake rate that a change by 5% would be insignificant for the whole process. One parameter that has a comparatively large impact on the estimated specific reaction rates is the biomass concentration. Being able to measure and estimate biomass concentrations accurately is thus of major importance. In the model we have therefore already incorporated three measurements related to biomass concentrations and growth in order to provide a higher certainty for these values: the measurement of biomass concentrations via TSS and PHB samples, the measurement of the actual SRT (and thus biomass production) and the measurement of growth via ammonia measurements.

Appendix C: Comparison of rates in SBR and fed-batch reactor

Reaction rates observed in the initial stage of a fed-batch experiment can be expected to be similar to those observed in the SBR feast phase for the same culture, if conditions applied (e.g. concentrations of carbon source and nutrients) are comparable. Acetate concentrations were higher in fed-batch experiments than in SBRs, while ammonia concentrations were lower. To which extent this influenced the observed reaction rates in the fed-batch experiments as compared to the SBRs is discussed below for the three key rates of acetate uptake, PHB production and ammonia uptake (which is coupled directly to growth rate).

Specific acetate uptake rate: The specific acetate uptake rate in SBRs was found to be lower in nitrogen limited cultures than in carbon limited cultures (Table 3.2). The same was observed in fed-batch experiments of SBR cultures operated at 4 d SRT (Fig. 3.C A): cultures from carbon limited SBRs had a higher initial specific acetate uptake rate in the fed-batch experiments than cultures from nitrogen limited SBRs. Initial acetate uptake rates estimated for the fed-batch experiments were somewhat higher than for the same culture in the SBR. Acetate concentrations were far higher in the fed-batch experiments than in SBRs, which may induce a somewhat higher uptake rate. On the other hand fed-batch experiments are more difficult to model than SBR cycles, since the gaps in carbon and electron balances are generally larger in fed-batch experiments and the additional constraints in the form of steady state assumptions cannot be applied for fed-batch experiments. Therefore the uncertainty in the modelled reaction rates for fed-batch experiments is likely larger than for SBR measurements, which may also explain deviations found between SBR and fed-batch acetate uptake rates.

Specific acetate uptake rates as well as specific PHB production rates (Fig. 3.C B) decreased with time, i.e. with increasing PHB contents.

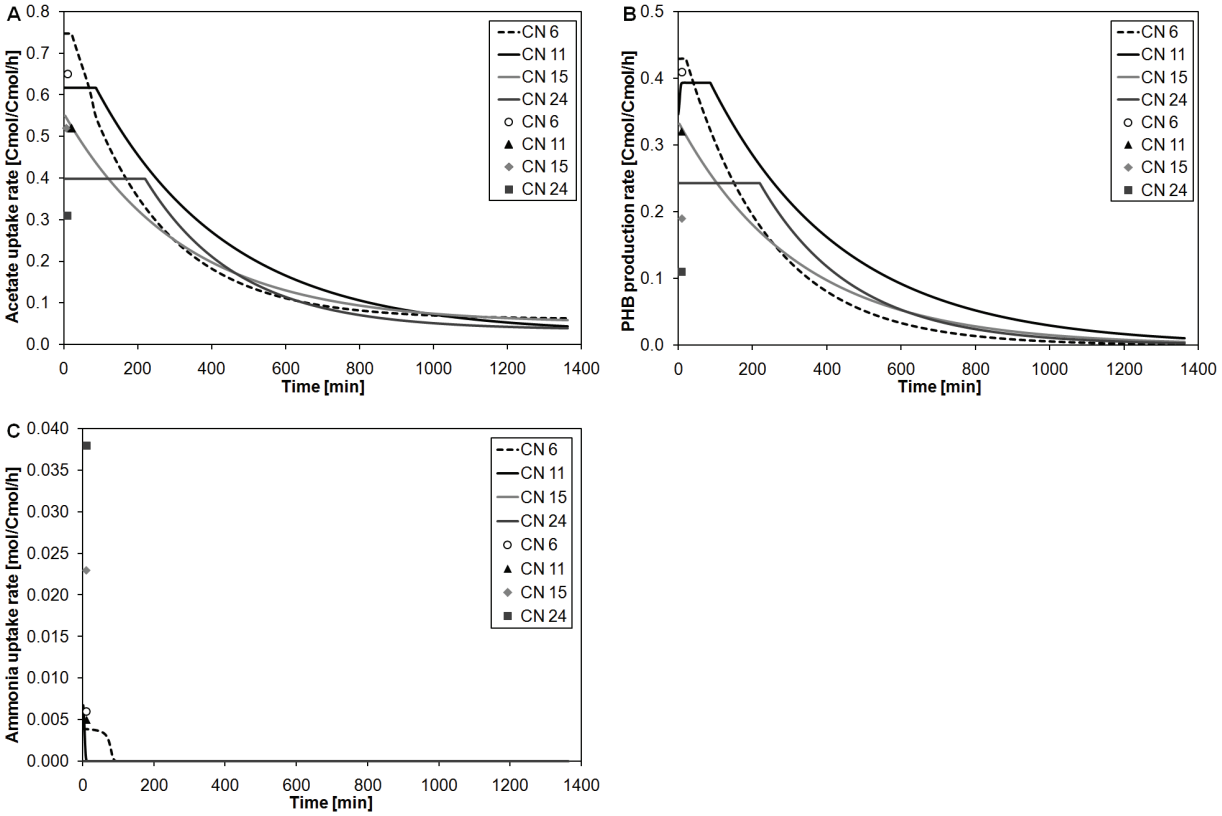

Figure 3.C. Modelled specific rates of acetate uptake (**A**), PHB production (**B**) and ammonia uptake (**C**) in fed-batch experiments of SBR cultures with 4 d SRT (lines) and comparison with average rates modelled for the feast phase of the SBRs (symbols).

Specific ammonia uptake rate: At the beginning of the fed-batch experiments ammonia was only present in a small amount in the carbon limited SBR cultures (0.2 mM CN 6, 0.02 mM CN 11) and absent in nitrogen limited SBR cultures. Initial specific ammonia uptake rates for the two carbon limited cultures were very similar for the fed-batch experiments compared to the SBR feast phase (Fig. 3.C C). Ammonia uptake rates decreased with decreasing ammonia concentration at these low concentrations. For the two nitrogen limited SBR cultures the rate of ammonia uptake in the fed-batch experiments was zero (Fig. 3.C C).

Specific PHB production rate: The modelled specific PHB production rates (Fig. 3.C B) showed the same trend as the acetate uptake rates, i.e. higher rates for cultures that had been carbon limited in the SBR and lower rates for cultures that had been nitrogen limited. The initial PHB production rates for the two carbon limited cultures (CN 6 and CN 11) were very similar for fed-batch and SBR experiments. The modelled fed-batch PHB production rate for the SBR culture, which had been supplied with medium with a C/N ratio of 11 Cmol/Nmol in the SBR, increased in the initial stage of the fed-batch experiment due to the constant acetate uptake (Fig. 3.C A)

combined with a decreasing growth rate (Fig. 3.C C; growth rate is equivalent to 5 times the ammonia uptake rate). Since less carbon was used for growth, more was available for PHB storage. This was not observed in the other carbon limited culture (CN 6), because ammonia only became limiting for growth at a later stage of the experiment (Fig. 3.C C), when PHB production rates were already limited by the increasing PHB content.

For the two nitrogen limited SBR cultures initial specific PHB production rates were much higher in the fed-batch experiment than in the SBR feast phase. This is because these cultures focused on growth in the SBR feast phase (high ammonia uptake rates, Fig. 3.C C), leaving less carbon for PHB production, while in the fed-batch experiments these cultures did not have any ammonia available for growth (ammonia uptake rate is zero, Fig. 3.C C), leaving thus much more carbon for PHB production.

Overall the specific reaction rates estimated for fed-batch experiments for the cultures enriched at different C/N ratios and 4 d SRT are thus in good agreement with those estimated for the SBR feast phase. Differences can be explained with the different concentrations of acetate and ammonia present in SBR and fed-batch experiments.

Nomenclature

ATP	Adenosine triphosphate
$\widetilde{c}_{NH_3}$	Modelled concentration of ammonia [mol/l]
DO	Dissolved oxygen [%]
$\widetilde{f}_{PHB}$	Modelled fraction of PHB in active biomass [Cmol PHB / Cmol active biomass]
HRT	Hydraulic residence time [h]
k	Rate constant of PHB degradation [(Cmol/Cmol)$^{1/3}$/h]
K_{NH_3}	Half-saturation constant for ammonia = 10^{-7} mol/l
m_{ATP}	Biomass specific ATP requirement for maintenance [mol/Cmol/h]
m_{PHB}	Biomass specific PHB requirement for maintenance [Cmol/Cmol/h]
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
q_{Ac}	Average biomass specific acetate uptake rate [Cmol/Cmol/h]
$\widetilde{q}_{Ac}^{\max}$	Modelled maximum biomass specific acetate uptake rate [Cmol/Cmol/h]
q_{CO_2}	Average biomass specific carbon dioxide evolution rate [Cmol/Cmol/h]
q_{NH_3}	Average biomass specific ammonia uptake rate [mol/Cmol/h]
q_{O_2}	Average biomass specific oxygen uptake rate [mol/Cmol/h]
q_{PHB}	Average biomass specific PHB production / degradation rate [Cmol/Cmol/h]
$\widetilde{q}_{PHB}^{fam}$	Modelled biomass specific PHB degradation rate [Cmol/Cmol/h]

SBR	Sequencing batch reactor
SRT	Sludge residence time [d]
t	Time [h]
TSS	Total suspended solids [g/l]
$Y_{ATP/PHB}^{fam}$	Stoichiometric yield of ATP on PHB in the famine phase [mol/Cmol]
$Y_{X/PHB}^{fam}$	Stoichiometric yield of biomass on PHB in the famine phase [Cmol/Cmol]
$Y_{CO_2/Ac}^{obs}$	Observed yield of carbon dioxide on acetate [Cmol/Cmol]
$Y_{CO_2/PHB}^{obs}$	Observed yield of carbon dioxide on PHB [Cmol/Cmol]
$Y_{PHB/Ac}^{obs}$	Observed yield of PHB on acetate [Cmol/Cmol]
$Y_{X/Ac}^{obs}$	Observed yield of biomass on acetate [Cmol/Cmol]
$Y_{X/PHB}^{obs}$	Observed yield of biomass on PHB [Cmol/Cmol]
μ	Average biomass specific growth rate [1/h]
$\tilde{\mu}^{fam}$	Modelled biomass specific growth rate in the famine phase [1/h]
$\tilde{\mu}^{\max}$	Modelled maximum biomass specific growth rate in the feast phase [1/h]

CHAPTER 4

Short- and Long-Term Temperature Effects on Aerobic Polyhydroxybutyrate Producing Mixed Cultures

Katja Johnson, Jorien van Geest, Robbert Kleerebezem,

Mark C. M. van Loosdrecht

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ABSTRACT

Short- and long-term temperature effects on polyhydroxybutyrate (PHB) producing mixed cultures enriched in feast-famine sequencing batch reactors (SBRs) were investigated in a temperature range of 15-35°C and 15-30°C, respectively. After short-term temperature changes (i.e. 1 cycle) from the steady state temperature of 20°C, reaction rate changes in the famine phase could be described over the whole temperature range with the Arrhenius equation with one temperature coefficient. For the feast phase different temperature coefficients were identified for acetate uptake, PHB production and growth. These were only valid for temperatures 5°C higher or lower than the steady state temperature.

Long-term temperature changes (i.e. new steady states) influenced not only the reaction rates but also the selective pressure in the SBR. At higher temperatures (30°C) the SBR feast phase was short and the rates of acetate uptake and PHB storage were very high. This culture was characterized by a storage strategy with high yields of PHB and low yields of biomass in the feast phase. The PHB storage capacity of this culture was 84 wt% as evaluated in fed-batch experiments. At lower temperatures (15°C) the feast phase was longer due to a lower rate of acetate uptake and the culture followed a strategy of direct growth on acetate rather than on PHB. This culture had a low maximal PHB storage capacity (about 35 wt%). The SBR culture enriched at 20°C was able to store up to about 70 wt% PHB. The temperature at which fed-batch experiments were conducted did not influence the maximal PHB storage capacity. The SBR temperature was found to be an important factor to consider when designing a mixed culture PHB production process.

Introduction

Mixed culture biotechnology is a promising alternative to pure culture biotechnology for the production of the bioplastic polyhydroxyalkanoate (PHA). Mixed culture biotechnology employs open undefined mixed cultures and ecological selection principles to produce a product such as bioplastics, ideally from a waste stream. It therefore combines the methodology of environmental biotechnology with the goals of industrial biotechnology. Optimization of the mixed culture PHA production process has led to cellular PHA contents and PHA production rates comparable or superior to those of pure cultures including genetically modified organisms (Johnson et al., 2009a). One of the important factors influencing PHA production is the process temperature, which was the subject of this study.

The critical step in the PHA production process with open mixed cultures is the enrichment of superior PHA producing bacteria in a mixed culture. This can be achieved by using a selective pressure for PHA production based on the ecological role of PHA as a microbial storage material (van Loosdrecht et al., 1997). Two different strategies can be applied: (i) alternating periods of presence and absence of the final electron acceptor (aerobic and anaerobic periods) with substrate being supplied during the absence of the final electron acceptor, or (ii) alternating

periods of presence and absence of the carbon source (feast and famine periods) (Reis et al., 2003). The first strategy selects for polyphosphate- and/or glycogen-accumulating organisms (PAOs, GAOs). The temperature effects on PAO and GAO cultures have been studied extensively (Brdjanovic et al., 1997; 1998; Lopez-Vazquez et al., 2007; 2008; 2009b; 2009a). In these studies the temperature was found to influence the metabolic reaction kinetics as well as the competition between these two different types of organisms.

In contrast to the PAO and GAO cultures, temperature effects on PHA storing cultures enriched with a feast-famine strategy have hardly been studied. Krishna and van Loosdrecht (1999) investigated the influence of temperature on acetate-fed feast-famine cultures and also found a strong influence of temperature on the kinetics of the process. However, these experiments were performed on cultures with an acetate uptake rate that was relatively low and limited by the acetate addition rate in the feast phase. Kinetics in dynamic processes with faster feeding and absence of substrate limitation will be more dominated by PHA storage and the impact of temperature on the kinetics will likely be different.

Neither the above mentioned PAO and GAO studies nor the study by Krishna and van Loosdrecht (1999) explored the influence of the temperature on the maximum PHA content that can be established depending on the temperature at which the mixed cultures were enriched.

When working with open mixed cultures a temperature change can have different effects depending on whether the temperature is changed for a short time or for a long time. Short-term changes are expected to mainly influence the kinetics of the metabolism of the organisms that are present in the reactor; long-term changes, however, would likely also lead to an adaptation of the present organisms and changes in the community structure (Brdjanovic et al., 1998; Lopez-Vazquez et al., 2009a). A change in the PHA storage capacity could therefore occur particularly after long-term temperature changes.

The impact of the temperature on PHA storage is particularly relevant when aiming at using industrial wastewaters, which are produced at a wider temperature range than communal wastewaters. For study purposes usually a synthetic wastewater with acetate as the sole carbon source is used. Once the mechanisms are understood for this simplified system, more complex media and wastewaters can be explored.

In this study we investigated both the effect of short- and long-term temperature changes on PHA accumulating cultures enriched with the feast-famine strategy. Additionally we examined the influence of temperature on the maximum PHA storage capacity of the enriched cultures. Sequencing batch reactors (SBRs) were used to enrich PHA storing bacteria with a feast-famine regime. Acetate was supplied as the sole carbon source and the polymer produced was consequently pure polyhydroxybutyrate (PHB). The sludge residence time (SRT) of the SBR was chosen as 1 day in order to aim for a high biomass productivity, which would be desirable for a commercial system. The SBRs were operated at temperatures of 15, 20 and 30°C until a steady state was reached (long-term temperature change experiments). The steady state obtained at

20°C was used as the base for the short-term temperature change experiments in which the reactor temperature was changed for one SBR cycle to 15, 25, 30 or 35°C. The maximum PHB storage capacity was determined for the three steady state cultures in fed-batch experiments with a continuous acetate supply under conditions of nitrogen limitation. The temperature applied in the fed-batch experiments was the same as in the respective SBR enrichment. For the steady state of 20°C fed-batch PHB accumulation experiments were additionally performed at 15 and 30°C.

Materials and Methods

Sequencing batch reactor (SBR) for culture enrichment

A double-jacket glass bioreactor with a working volume of 2 l (Applikon, the Netherlands) was used for the cultivation of PHB producing bacterial cultures. The reactor was equipped with a stirrer with two standard geometry six-blade turbines. Stirrer speeds were adjusted to establish the oxygen mass transfer rates required. The flow of air to the reactor was controlled with a mass flow controller (Brooks Instrument) at 0.23 l_N/min. The off-gas was recirculated through the reactor several times, resulting in gas flows of about 1.5 l_N/min through the reactor. Off-gas recirculation was used to improve the accuracy of oxygen conversion from off-gas measurements. The reactor was temperature controlled at either 15, 20, 25, 30 or 35°C by means of a water jacket and a thermostat bath (Lauda, Germany). The pH of the reactor liquid was maintained at 7.0 $\pm$ 0.1 using 1 M HCl and 1 M NaOH. The stirrer, the pH, the airflow and the pumps for feeding, biomass and effluent removal and pH control were controlled by a biocontroller (ADI 1030, Applikon) that in turn was controlled by a PC using the software BIODACS (Applikon). BIODACS was also used for data acquisition of the online measurements (dissolved oxygen (DO), pH, temperature, acid dosage, base dosage, off-gas oxygen and carbon dioxide).

The sludge residence time (SRT) of the SBR was 1 d, and the hydraulic residence time (HRT) was 8 h. Operation of the SBR was based on 4 h batch cycles consisting of a start phase (10 min), an influent phase (3 min) in which 1 l fresh medium was supplied, a reaction phase (198 min), a biomass withdrawal phase (5 min) in which 333.3 ml mixed reactor liquor were withdrawn, a settling phase (15 min) and an effluent withdrawal phase (9 min) in which 666.7 ml of reactor supernatant were withdrawn.

For the long-term temperature change experiments (15, 20, 30°C) the SBR was considered to be in steady state when for at least five times the SRT (i) the concentration of total suspended solids (TSS) at the end of the cycle, (ii) the measured SRT (considering also biomass removed with the effluent) and (iii) the length of the feast phase as indicated by the DO changes were constant. This steady state definition was chosen in order to be able to compare different SBR cultures at a similar level of development rather than to find a true long-term stable steady state. When the reactor was in steady state, a cycle of the SBR was monitored additionally to the online measurements by offline samples (acetate, TSS, PHA, ammonia). Biomass was

harvested from the SBRs in steady state in order to study the PHA production capacity of the biomass in fed-batch experiments.

For the short-term temperature change experiments the temperature of the steady state SBR of 20°C was changed for one cycle to either 15, 25, 30 or 35°C. Between each experiment the temperature was set back to 20°C until steady state was reached again.

For the long-term temperature change experiments the temperature was also changed immediately to the new temperature.

The inoculum of the 20°C steady state SBR came from a similar SBR operating at 4 d SRT and 20°C with 4 h cycles and acetate as the carbon source (medium C/N ratio 15 Cmol/Nmol) (see also Table 4.1). After the short-term temperature change experiments, all the biomass from the steady state SBR at 20°C was used to inoculate the SBR operating at 30°C, however, biomass washed out from this reactor and had to be re-inoculated from a similar SBR with a longer cycle length (12 h) but otherwise same conditions. Biomass from the same SBR with 12 h cycle length was also used to inoculate the SBR operating at 15°C. Table 4.1 summarizes the operating conditions for the three steady state temperatures.

The medium for the SBR consisted of a carbon source, a nutrient solution and dilution water. In the influent phase of each batch cycle 200 ml of carbon source, 200 ml of nutrient source and 600 ml of dilution water were mixed, heated or cooled to the desired temperature, and simultaneously pumped into the reactor. The carbon source was 90 mM sodium acetate and the nutrient solution was composed of 22.5 mM NH_4Cl, 8.3 mM KH_2PO_4, 1.85 mM $MgSO_4 \cdot 7H2O$, 2.4 mM KCl, 5 ml/l trace elements solution according to Vishniac and Santer (1957) and 50 mg/l allylthiourea (to prevent nitrification). Only in experiment IIb (Table 4.1) the influent concentrations were lowered to 90% of these concentrations after a first attempt to increase the cultivation temperature to 30°C had failed (experiment IIa in Table 4.1).

Table 4.1. Operational conditions for SBR steady-states and for the inoculum reactors.

Experiment	I	IIa[a]	IIb	III
Experimental reactor conditions				
Temperature [°C]	20	30	30	15
Set SRT [d] / set HRT [h]	1 / 8	1 / 8	1 / 8	1 / 8
Cycle length [h]	4	4	4	4
Organic load [Cmmol/l/d]	108	108	97	108
Operation time until cycle measurement [d]	34	-	33	20
Reactor conditions of inoculum reactor				
Temperature [°C]	20	20	20	20
Set SRT [d] / set HRT [h]	4 / 8	1 / 8	1 / 24	1 / 24
Cycle length [h]	4	4	12	12

a: biomass washed out, no stable reactor operation established

Fed-batch reactor for PHB production

Steady state biomass from the SBRs was used in fed-batch experiments with the aim to evaluate the PHB storage capacity. For this purpose the same set-up as for the culture selection was used, but in a fed-batch mode. The fed-batch reactor was operated at the following temperatures: 15°C for the 15°C steady state culture; 15, 20 and 30°C for the 20°C steady state culture; 30°C for the 30°C steady state culture. 1 l of culture from the end of a SBR cycle was mixed with 1 l of acetate- und ammonia-free medium (same composition as for the SBR, but without sodium acetate and NH_4Cl). To start the PHB production, a pulse of about 60 mmol sodium acetate was fed to the reactor. Further carbon source was supplied automatically with 1 M acetic acid via the pH control (set to pH 7). Growth was limited in these experiments as no nitrogen source was supplied and only a small amount remaining nitrogen source from the previous SBR cycle was available. The progress of the experiments was monitored via online (DO, pH, temperature, acid and base dosage, off-gas CO_2 and O_2) and offline (acetate, TSS, PHA, ammonia) measurements.

Analytical Methods

The concentration of dissolved oxygen (DO) in the reactor was measured with a DO electrode (Mettler Toledo) as percentage of air saturation and the pH was monitored with a pH electrode (Mettler Toledo). The temperature of the reactor broth was measured with a thermo element. The amount of acid or base dosed for pH control was measured online. Carbon dioxide and oxygen partial pressures in the gas entering and leaving the reactor were analyzed in dried gas with a gas analyzer (NGA 2000, Rosemount).

Concentrations of acetate, ammonia and total suspended solids (TSS) in reactor samples were analyzed as described previously (Johnson et al., 2009a). The amount of PHB was subtracted from the TSS to calculate the concentration of active biomass. The active biomass concentration was converted from g/l into carbon moles per litre (Cmol/l) assuming a composition of $CH_{1.8}O_{0.5}N_{0.2}$ with a molecular weight including ash of 25.1 g/Cmol (Beun et al., 2002).

Samples taken for PHB analysis were added to 15 ml tubes containing 5 drops of formaldehyde in order to stop all biological activity. Samples were subsequently washed with tap water and freeze-dried. The PHB content of the samples was determined as described previously (Johnson et al., 2009a). Results were expressed as weight percentage of PHB of the total solids.

Data evaluation

A comprehensive data analysis was carried out with the results obtained from SBR cycle measurements and fed-batch experiments. The data analysis is described in detail in Johnson et al. (2009b). In brief, measurements were corrected for the effects of sampling and addition of

liquids to the reactor (acid and base solutions, feed in fed-batch experiments). Oxygen uptake and carbon dioxide evolution were determined from off-gas and DO measurements. Inorganic carbon dissolved in the liquid phase was considered when computing carbon dioxide evolution. Balances for carbon and electrons were calculated from the measured conversions at every sampling point. Finally, the available knowledge of the stoichiometry and kinetics of the PHB production and consumption metabolism was employed in the form of a metabolic model in order to compute the best estimates for all conversions and reaction rates from the measurements while complying with carbon and electron balances.

The kinetics applied in the model are described in detail in Johnson et al. (2009b). Briefly, in the feast phase zero order kinetics were assumed for the biomass specific rates of acetate uptake, growth and maintenance. The kinetic equations for acetate uptake and growth were multiplied for modelling purposes with saturation terms with very small half saturation constants for acetate and for acetate and ammonia, respectively. The rates of PHB production, ammonia uptake, carbon dioxide production and oxygen consumption in the feast phase were computed from the other three rates with stoichiometric yields. For the famine phase, PHB consumption was considered as the rate limiting step. The rate of PHB consumption $\widetilde{q}_{PHB}^{fam}$ was modelled as a rate depending on the fraction of PHB $\widetilde{f}_{PHB}$ with a reaction order of ⅔:

$$\widetilde{q}_{PHB}^{fam}(t) = k_{PHB} \cdot \widetilde{f}_{PHB}(t)^{2/3} \qquad (4.1)$$

with k_{PHB} being the rate constant and t the time. Maintenance was also for the famine phase modelled with a constant rate, i.e. zero order kinetics. The rates of growth, ammonia uptake, carbon dioxide production and oxygen consumption in the famine phase followed from the other two rates.

The kinetic parameters such as the maximum biomass specific acetate uptake rate, the maximum biomass specific growth rate, the maintenance ATP requirement and the rate constant for PHB consumption were fitted for each experiment. An analysis of the impact of small deviations in the model parameters on the model outcome was reported previously (Johnson et al., under review, Water Research / Chapter 3 of this thesis). The half-saturation constants for ammonia and acetate have more numerical than physiological relevance and were assumed to be independent of the reactor temperature and treated as constants. Similarly the stoichiometric yields were considered as temperature independent constants. Temperature dependencies were, however, included for gas solubilities and dissociation constants (Johnson et al., 2009b).

Temperature dependence of reaction rates

The temperature dependence of a chemical reaction can usually be expressed with the Arrhenius equation:

$$k = A \cdot e^{\frac{E_a}{RT}} \quad (4.2)$$

with k being the rate constant of the reaction, A the pre-exponential factor, E_a the activation energy, R the gas constant and T the absolute temperature.

In previous studies for PAO and GAO cultures a simplified form of the Arrhenius equation was used to evaluate the effect of temperature on a rate constant relative to a reference temperature (Brdjanovic et al., 1997; 1998; Lopez-Vazquez et al., 2007; 2008; 2009b; 2009a):

$$k_T = k_{293K} \cdot \theta^{(T-293K)} \quad (4.3)$$

with k_T the rate constant at the temperature T, $k_{293\ K}$ the rate constant at the reference temperature of 20°C (293 K) and θ the temperature coefficient. For reactions that follow zero order kinetics the rate constants can be replaced by the reaction rates.

This simplified version of the Arrhenius equation was applied in this study in order to compare results with those obtained for PAO and GAO cultures. The simplified Arrhenius equation was fitted to the experimentally obtained rate constants with the least squares method by adjusting the temperature coefficient and the rate constant at the reference temperature.

Results

Short-term temperature change (SBR)

The SBR was maintained at 20°C until steady state was reached. The steady state was documented by measuring all relevant conversions in one cycle (Figure 4.1 B). The observed reactor behaviour was similar to those previously reported for similar SBRs (Beun et al., 2000a; 2002; Martins et al., 2003; Johnson et al., 2009a; 2009b): Acetate was taken up rapidly during the feast phase and partly converted into PHB and partly used for growth. In the famine phase the previously stored PHB was used as a carbon and energy source for continued growth.

In order to study the effect of the operating temperature in a range of 15 – 35°C on the observed yields and reaction rates of the PHB production and consumption process, the temperature of the steady state SBR was increased or lowered for one cycle and the new cycle behaviour was measured (non steady state). The reactor was always allowed to return to steady state at 20°C before a new experiment at a different temperature was performed. Reaction rates and observed yields as summarized in Table 4.2 ('short-term') were estimated for all experiments with the help of a metabolic model (Johnson et al., 2009b). The metabolic model helps to find better estimates for the reaction rates, leading to clearer trends in the temperature dependence (Johnson et al., 2009b).

The biomass specific reaction rates in the feast phase showed a clear trend only for the temperature range of 15 – 25°C, i.e. 5°C higher or lower than the steady state temperature (Table 4.2). The changes of the significant reaction rates of acetate uptake, growth and PHB production relative to those observed in steady state at 20°C are displayed in Figure 4.2 A. For

acetate uptake and growth the maximum rates were plotted as these are basically the rate constants for these reactions. PHB production is calculated as a linear combination from acetate uptake, growth and maintenance, all of which follow basically zero order kinetics. PHB production follows thus also zero order kinetics and the PHB production rate can be treated as a rate constant. The simplified Arrhenius equation was fitted to the data points in the temperature range of 15 – 25°C. The quality of the fits was generally very good (R^2 values of 0.98 – 1.00). Growth was found to be the most temperature sensitive process with a temperature coefficient of θ = 1.106, followed by acetate uptake with θ = 1.051, and PHB production with θ = 1.040. The growth rate almost tripled within the 10°C temperature increase from 15 – 25°C, while the rates of acetate uptake and PHB production did not even double. As a consequence of the different temperature coefficients of these three processes the observed biomass yield in the feast phase increased with increasing temperatures from 15 – 25°C significantly, while the observed PHB yield decreased (Table 4.2). At temperatures of 30 and 35°C reaction rates were lower than at 25°C (Table 4.2, Figure 4.2 A) and were therefore not included when fitting the simplified Arrhenius equation.

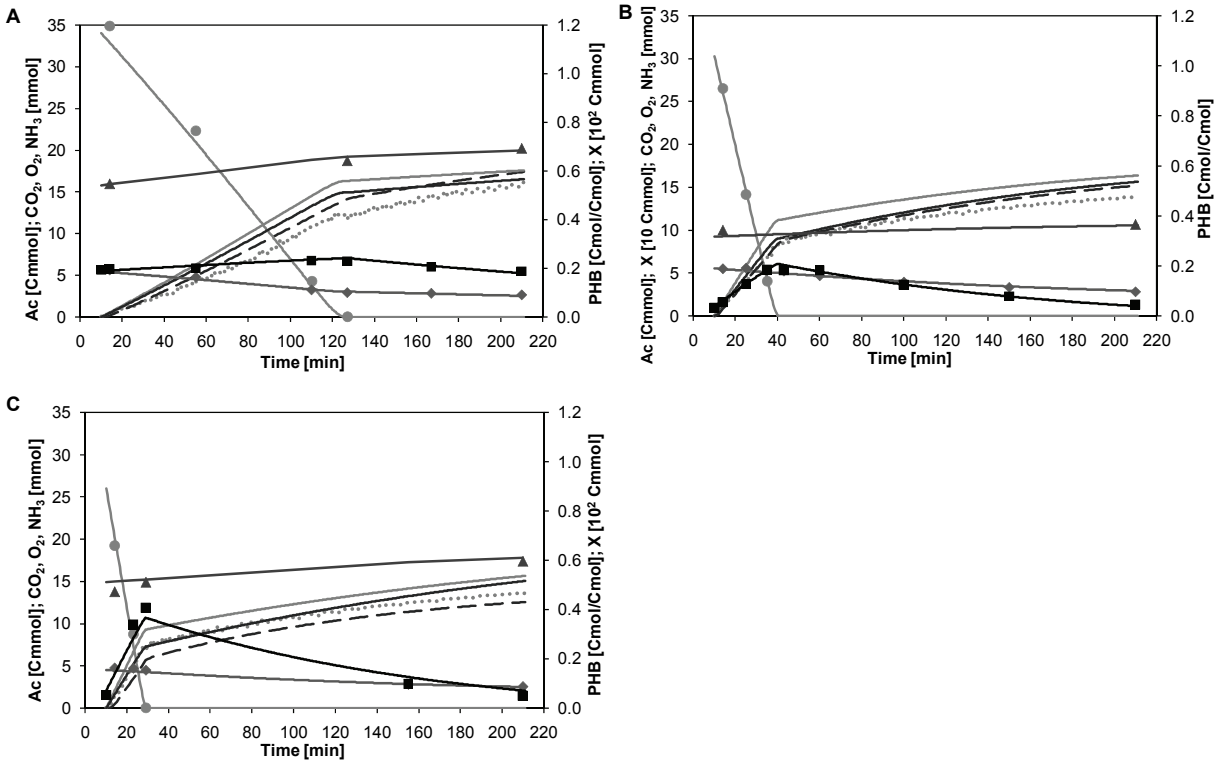

Figure 4.1. SBR steady state cycle behaviours at different temperatures (long-term). **A**: 15°C. **B**: 20°C. **C**: 30°C. Solid lines indicate modelled values corresponding to the respective symbols indicating measurements. (— —) cumulative oxygen uptake; (┈┈) cumulative carbon dioxide evolution; (●) acetate; (▲) active biomass; (◆) ammonia; (■) PHB.

Table 4.2. Influence of short-term (one cycle) and long-term (steady state) temperature changes on SBR performance. 20°C was the base temperature for the short-term experiments. All rates are averages of biomass specific rates over the whole feast or famine phase. All yields are observed yields. All data were calculated with the help of a metabolic model (Johnson et al., 2009b).

		Short-term					Long-term		
	Temperature [°C]	**15**	**20**	**25**	**30**	**35**	**15**	**20**	**30**
	Measured SRT [d]						0.9	1.2	1.0
	Measured TSS [g/l][a]						0.80	1.3	0.66
FEAST	Length feast [%][b]	29	15	13	12	12	59	15	9
	$Y^{obs}_{PHB/Ac}$ [Cmol/Cmol]	0.58	0.54	0.52	0.51	0.53	0.17	0.54	0.60
	$Y^{obs}_{X/Ac}$ [Cmol/Cmol]	0.06	0.09	0.10	0.11	0.09	0.35	0.09	0.04
	$Y^{obs}_{CO_2/Ac}$ [Cmol/Cmol]	0.36	0.37	0.38	0.38	0.38	0.48	0.37	0.36
	q_{Ac} [Cmol/Cmol/h]	-0.49	-0.63	-0.80	-0.73	-0.69	-0.29	-0.63	-1.55
	q_{PHB} [Cmol/Cmol/h]	0.29	0.35	0.42	0.38	0.38	0.05	0.35	0.95
	μ [Cmol/Cmol/h]	0.028	0.057	0.086	0.084	0.067	0.101	0.057	0.066
	q_{CO_2} [Cmol/Cmol/h]	0.18	0.24	0.31	0.28	0.27	0.14	0.24	0.57
	q_{O_2} [mol/Cmol/h]	-0.14	-0.19	-0.25	-0.23	-0.22	-0.13	-0.19	-0.44
	q_{NH_3} [mol/Cmol/h]	-0.006	-0.011	-0.017	-0.017	-0.013	-0.020	-0.011	-0.013
	PHB max. [%]	20	14	15	15	14	17	14	26
FAMINE	$Y^{obs}_{X/PHB}$ [Cmol/Cmol]	0.64	0.67	0.67	0.67	0.63	0.67	0.67	0.58
	$Y^{obs}_{CO_2/PHB}$ [Cmol/Cmol]	0.36	0.33	0.33	0.33	0.37	0.33	0.33	0.42
	q_{PHB} [Cmol/Cmol/h]	-0.063	-0.055	-0.062	-0.059	-0.058	-0.040	-0.055	-0.087
	μ [Cmol/Cmol/h]	0.040	0.037	0.042	0.040	0.036	0.027	0.037	0.050
	q_{CO_2} [Cmol/Cmol/h]	0.022	0.018	0.021	0.020	0.021	0.013	0.018	0.037
	q_{O_2} [mol/Cmol/h]	-0.028	-0.023	-0.026	-0.025	-0.027	-0.017	-0.023	-0.045
	q_{NH_3} [mol/Cmol/h]	-0.008	-0.007	-0.008	-0.008	-0.007	-0.005	-0.007	-0.010
TOTAL	$Y^{obs}_{PHB/Ac}$ [Cmol/Cmol]	0.25	0.02	-0.01	-0.11	-0.13	0.06	0.02	0.02
	$Y^{obs}_{X/Ac}$ [Cmol/Cmol]	0.27	0.44	0.46	0.53	0.51	0.42	0.44	0.37
	$Y^{obs}_{CO_2/Ac}$ [Cmol/Cmol]	0.48	0.54	0.55	0.59	0.62	0.51	0.54	0.60
MODEL	$\tilde{q}^{\max}_{Ac}$ [Cmol/Cmol/h]	-0.53	-0.69	-0.88	-0.80	-0.76	-0.32	-0.69	-1.70
	$\tilde{\mu}^{\max}$ [Cmol/Cmol/h]	0.030	0.061	0.092	0.090	0.072	0.111	0.061	0.071
	k_{PHB} (Cmol/Cmol)$^{1/3}$/h]	-0.17	-0.25	-0.28	-0.40	-0.54	-0.11	-0.25	-0.27
	m_{ATP} [mol/Cmol/h]	0.009	0.000	0.000	0.000	0.015	0.000	0.000	0.050

a: at the end of the influent phase

b: percentage of time of the reaction phase (feast and famine phase)

The reaction rates in the famine phase listed in Table 4.2 are all coupled to the rate of PHB consumption. Since PHB consumption is not following zero order kinetics, but is dependent on PHB contents as well as temperature, the average specific reaction rates computed for the famine phase do not show a clear temperature dependence (Table 4.2). However, the rate constant of PHB consumption k_{PHB} can be expected to have a temperature dependence that can be described with the simplified Arrhenius equation. Figure 4.3 A shows the temperature

dependence of the rate constant of PHB consumption as estimated when fitting the metabolic model to the measurements (Johnson et al., 2009b). For comparison the temperature dependence of the average growth rate in the famine phase is also included in Figure 4.3 A. The rate constant of PHB consumption showed a temperature dependence that can be described with a simplified Arrhenius equation with θ = 1.058 over the whole range of temperatures tested (15 – 35°C). The growth rate in the famine phase showed very little variation at the five temperatures tested (Figure 4.3 A), because this rate is calculated as an average over the whole famine phase and ultimately depends on PHB contents.

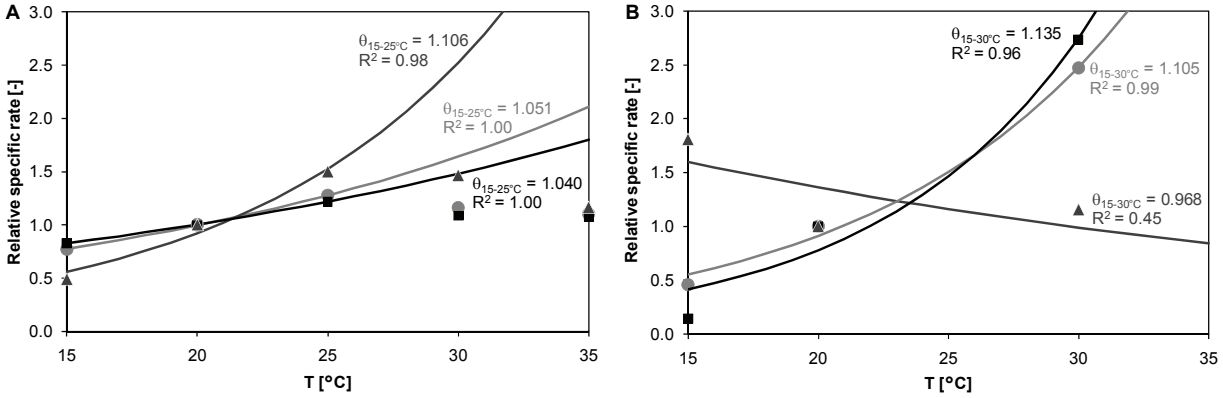

Figure 4.2. Relative biomass specific rates in the different SBR feast phases. **A**: Temperature changes for one cycle (short-term). **B**: Steady states at different temperatures (long-term). All rates are expressed relative to those at 20°C. (●) maximum biomass specific acetate uptake rate; (▲) maximum biomass specific growth rate; (■) average biomass specific PHB production rate. Solid lines indicate fits of the simplified Arrhenius equation to the respective rates in the range of 15 - 25°C (A) or 15 - 30°C (B).

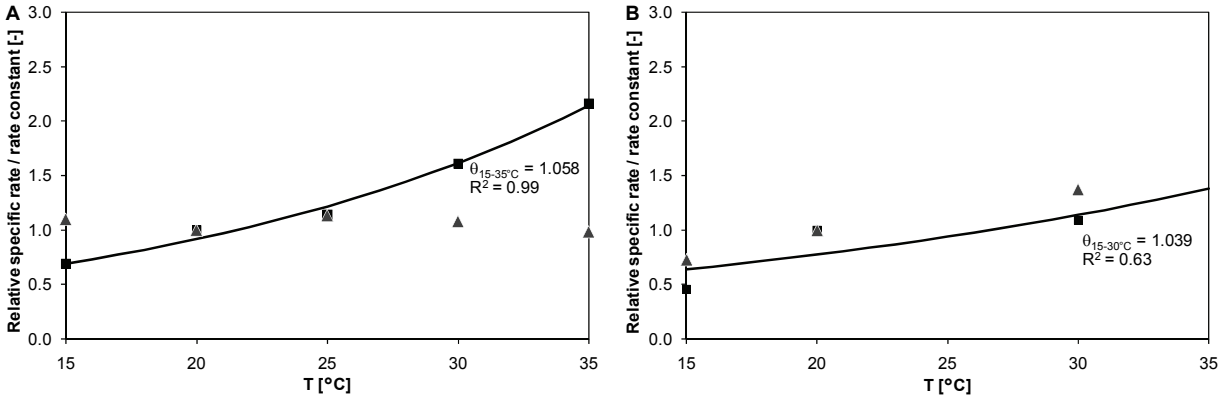

Figure 4.3. Relative biomass specific rates and rate constants in the SBRs' famine phases. **A**: Temperature changes for one cycle (short-term). **B**: Steady states at different temperatures (long-term). All rates are expressed relative to those at 20°C. (▲) average biomass specific growth rate; (■) rate constant for PHB consumption; (—) fit of simplified Arrhenius equation to modelled rate constant for PHB consumption at 15 - 35°C.

If the observed yields over the whole SBR cycle are considered (Table 4.2, 'TOTAL'), it becomes clear that at temperatures lower than the steady state temperature of 20°C not all of the PHB produced during the feast phase was degraded during the famine phase (positive observed PHB yield), while at higher temperatures more PHB was consumed than produced (negative observed PHB yield). The negative observed PHB yields were possible as cultures contained around 3 wt% PHB at the beginning of the cycle, which could be consumed additionally to the newly produced PHB from the feast phase. Since these cultures used more substrate than just the acetate supplied, observed carbon dioxide yields over the total cycle were increased. The culture at 15°C on the other hand did not use all of the supplied acetate for growth, but partly for a net PHB production resulting in a lower observed carbon dioxide yield (Table 4.2).

Long-term temperature change (SBR)

After the short-term temperature change experiments were completed the operating temperature of the SBR was increased to 30°C. The culture did not adapt well to the sudden temperature change, it did not settle well anymore and washed out (morphology remained unchanged). The reactor was restarted with biomass from a reactor running at 20°C at the same conditions as the previous steady state reactor, but with a longer cycle length of 12 h. Additionally the medium concentrations were lowered compared to the 20°C experiment (see also Table 4.1) in order to lower the biomass concentration and thereby avoiding oxygen limitation in the feast phase (caused by the very high reaction rates), and the biomass withdrawal was skipped until settling problems ceased (first week only). After this point the sludge settled very well. After about a month at 30°C the culture was considered to be in steady state and a cycle was measured in full (Figure 4.1 C). The SBR cycle at 30°C was similar to the one at 20°C (Figure 4.1 B), but the feast phase was much shorter and PHB contents reached at the end of the feast phase were higher.

The biomass from the SBR at 20°C with 12 h cycles was also used to inoculate the SBR operating at 15°C. After the temperature change also this reactor experienced briefly problems such as foaming and a feast phase stretching over the whole cycle, but it recovered within a few days. After 19 days at the new temperature the SBR was considered to be in steady state and a cycle was fully documented (Figure 4.1 A). Compared to the SBR at 20°C (Figure 4.1 B), the SBR at 15°C had a far longer famine phase and elevated PHB levels. The amount of PHB produced and consumed within a cycle were, however, very small.

The metabolic model described in Johnson et al. (2009b) was again used to estimate all reaction rates and yields for the three steady states at the different temperatures (Table 4.2, 'long-term'). Generally the metabolic model described the measurements very well in all experiments (Figure 4.1). Larger deviations were only observed for the conversions of carbon dioxide and oxygen which are difficult to measure accurately (Johnson et al., 2009b). In the feast phase the observed PHB yields increased with increasing temperature, while observed biomass yields decreased. This is the opposite trend to the short-term temperature change experiments. The

reason for the change of the observed yields with temperature lies also here in the different changes of the reaction rates with temperature. Figure 4.2 B shows how the maximum growth and acetate uptake rates and the PHB production rate changed relative to those at 20°C when steady states at 15 and 30°C were evaluated. Simplified Arrhenius equations were fitted to the three rates although the Arrhenius equation does obviously not fit the data very well. However, the calculated temperature coefficients are still useful for a comparison of the different rate changes and of short-term with long-term temperature effects.

Figure 4.2 B shows that in the steady state experiments the influence of temperature on the specific rate of PHB production was strongest (θ = 1.135), followed by the specific rate of acetate uptake (θ = 1.105). Interestingly, the specific growth rate decreased with increasing temperatures, leading to a temperature coefficient smaller than 1 (θ = 0.968). This is clearly an effect that was not caused by the temperature change directly since the short-term experiments showed a strong increase in growth rate with increasing temperatures (Figure 4.2 A). As the PHB production rate increased stronger with temperature in the long-term experiments than the acetate uptake rate, observed PHB yields increased with temperature. Observed biomass yields decreased, because the growth rate in the feast phase even decreased with increasing temperatures.

Figure 4.3 B displays the relevant rates and rate constants for the famine phase of the long-term temperature change experiments relative to the values of these parameters at 20°C. Only the rate constants for PHB consumption were fitted with the simplified Arrhenius equation like for the short-term experiments. Also in the famine phase the Arrhenius equation did not fit the data very well (R^2 = 0.63). The temperature coefficient for the rate constant of PHB consumption estimated for the long-term experiments (θ = 1.039) was somewhat lower than the one estimated for the short-term experiments (Figure 4.3 A, θ = 1.058). To get a better picture of the changes of this rate constant with temperature, we also looked at other steady state experiments we performed at similar conditions but with other SRTs or cycle lengths at temperatures of 20 and 30°C (Figure 4.4).

The values for k_{PHB} determined with the metabolic model varied considerably at each temperature for the different steady states. However, if the average value for k_{PHB} for each temperature was calculated, the simplified Arrhenius equation fitted these values very well (R^2 = 1.00). The temperature coefficient estimated with this approach of θ = 1.074 is equivalent to about a doubling of k_{PHB} every 10 degrees.

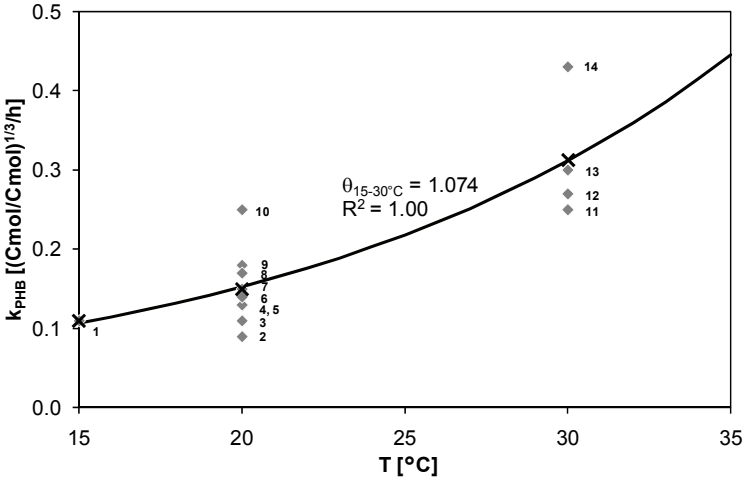

Figure 4.4. PHB consumption rate constant k_{PHB} obtained in various SBR steady states at different temperatures. (◆) individual steady states. 1: 4 h cycle, 1 d SRT, 8 Cmol/Nmol. 2-4 & 6: 12 h cycle, 1 d SRT, 8 Cmol/Nmol. 5: 4 h cycle, 4 d SRT, 6 Cmol/Nmol. 7: 4 h cycle, 0.5 d SRT, 8 Cmol/Nmol. 8: 4 h cycle, 4 d SRT, 13.2 Cmol/Nmol. 9: 4 h cycle, 4 d SRT, 11 Cmol/Nmol. 10: 4 h cycle, 1 d SRT, 8 Cmol/Nmol. 11 & 13: 12 h cycle, 1 d SRT, 8 Cmol/Nmol. 12 & 14: 4 h cycle, 1 d SRT, 8 Cmol/Nmol. (✕) average rate constants for each temperature. (—) fit of simplified Arrhenius equation to average rate constants for PHB consumption at 15 - 30°C.

Fed-batch experiments

Fed-batch experiments under nitrogen limitation were performed in order to investigate the PHB storage rate and capacity of the different cultures at different temperatures. The steady state culture from 20°C was used for fed-batch experiments at 15, 20 and 30°C. Fed-batch experiments for the steady state cultures from 15°C and 30°C were only performed at their respective steady state temperatures. The PHB accumulation over time in all five experiments is presented in Figure 4.5.

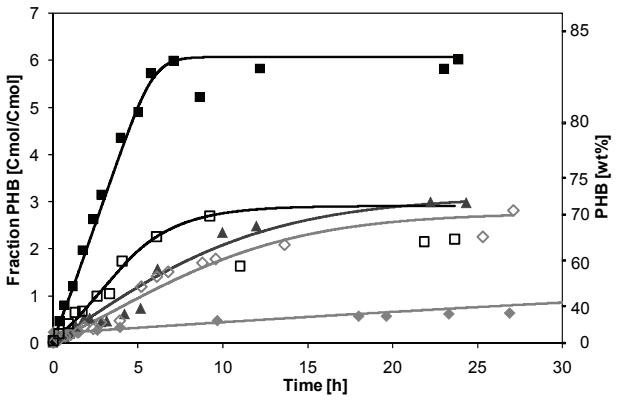

Figure 4.5. PHB production capacity of cultures from steady states at different temperatures as measured in fed-batch experiments conducted at different temperatures. Solid lines indicate modelled values corresponding to the respective symbols indicating measurements. (◆) steady state 15°C, fed-batch 15°C. (◇) steady state 20°C, fed-batch 15°C. (▲) steady state 20°C, fed-batch 20°C. (□) steady state 20°C, fed-batch 30°C. (■) steady state 30°C, fed-batch 30°C.

The culture from 20°C steady state reached about 70 wt% PHB irrespective of the temperature at which the fed-batch experiment was performed. The maximum PHB content was reached faster at 30°C and slower at 15°C compared to the fed-batch experiment at 20°C.

The culture enriched in the SBR at 15°C showed a very low PHB storage capacity and reached only about 35 wt% PHB content after more than 25 h fed-batch. The culture enriched at 30°C on the other hand had a much higher PHB storage capacity than the culture from 20°C and reached 84 wt% PHB after just over 7 h fed-batch.

Discussion

Temperature effects on kinetics (short-term experiments)

The temperature coefficients identified in short-term experiments in this study were generally comparable to those proposed for ordinary heterotrophic organisms, fermentation and nitrification processes (1.07-1.12 (Henze et al., 2000)). For many heterotrophic microorganisms one temperature coefficient is sufficient to describe the temperature dependence of all occurring reaction rates, since most rates are coupled to each other. In PHB producing cultures enriched with a feast-famine strategy, however, three independent processes occur during the feast phase that each may have their own temperature coefficient: growth, PHB production and maintenance. These three reactions are coupled by the acetate uptake rate. Maintenance was negligible in most of our experiments (Table 4.2) and a temperature coefficient could therefore not be determined. The other three processes were found to have very different temperature coefficients ranging from 1.040 to 1.106. But all of these rates only abode by the Arrhenius equation in a temperature range of 5°C higher or lower than the steady state temperature, with the optimum temperature with maximum rates probably being just over 25°C. It can be expected that the Arrhenius equation can only be applied for a limited range of temperatures for microbial systems. Microorganisms usually have an upper and a lower temperature limit for growth. With increasing temperatures also the rate of denaturation of key cellular components will increase, leading to lower observed conversion rates. It has been suggested that at lower temperatures the transport of substrates over the cell membrane limits growth (Nedwell, 1999). These two effects will lead to deviations from the Arrhenius equation when moving towards the temperature extremes for a certain culture.

In the famine phase only two independent reactions occur with growth and maintenance, both of which are coupled by the PHB consumption rate. Since maintenance was negligible in most experiments, in effect only one temperature coefficient was required to describe the temperature dependence of rates in the famine phase. In contrast to the feast phase, reactions in the famine phase do not follow zero order kinetics, but appear to be dependent on the PHB content. A reaction order for PHB consumption of ⅔ (surface area limited reaction rate) was found to describe the results very well (Johnson et al., 2009b). The rate constants for PHB consumption with this kinetic model do show a good fit with the Arrhenius equation over the

whole temperature range tested in short-term experiments (Figure 4.3 A), indicating that the kinetic model is indeed realistic.

While the feast phase reaction rates decreased at temperatures of 30°C and higher, the rate constant of PHB consumption in the famine phase was not negatively affected by higher temperatures and continued to increase with a temperature coefficient of 1.058 over the whole temperature range tested. Since higher temperature did not appear to have a negative influence on growth during the famine phase, but only during the feast phase, it seems likely that the acetate uptake system may be the rate limiting step during the feast phase, which is negatively affected by higher temperatures and consequently results also in lower reaction rates downstream.

Temperature effects on competitive behaviour (long-term experiments)

The long-term change of the reactor temperature induced a major change for the mixed bacterial culture. Both the increase of temperature by 10°C as well as the decrease by 5°C disturbed the reactor considerably and led to instabilities. While the changes observed in short-term temperature change experiments were caused by a direct temperature effect on the rate of the prevailing metabolic processes, long-term temperature changes may additionally lead to an acclimatization of present organisms to the new temperature, and to considerable changes in the composition of the microbial community. We studied the microbial diversity of different steady state cultures in a separate study (Jiang et al., submitted for publication) by means of denaturing gradient gel electrophoresis (DGGE). The DGGE band patterns found for the SBRs at 30 and 20°C differed considerably (15°C was not investigated). The cultures were dominated by two different organism: the culture from 20°C was dominated by a species most closely related to *Zoogloea* while the culture from 30°C was dominated by a novel bacterium within the *Gammaproteobacteria* (Jiang et al., submitted for publication) (Johnson et al., 2009a). The difference in the microbial community seems to be a result of a combined effect of the inoculum and the reactor temperature (Jiang et al., submitted for publication). Differences in the community structure may explain why the Arrhenius equation showed generally a relatively weak fit with the measurements in the long-term temperature change experiments.

Acetate uptake rates and PHB production rates increased and decreased much stronger with higher and lower temperatures in the long-term experiments than in the short-term experiments (Table 4.2). The steady state culture at 30°C had thus increased its rates for acetate uptake and PHB storage. The competition became more dominated by an efficient substrate storage mechanism. The steady state culture at 15°C, however, showed an even lower acetate uptake rate than the short-term temperature change culture at 15°C. In carbon limited feast-famine cultures competition is usually based on the uptake rate of the carbon source. Cultures that can take up the carbon source quickly by storing most of it as PHB and growing on it in the famine phase would be able to produce the most biomass since they secured the most substrate. But the steady state culture at 15°C followed a different strategy:

The steady state culture at 15°C produced more than 80% of the newly formed biomass per cycle in the feast phase whereas the culture at 20°C only produced about 20% of its biomass in the feast phase and the culture at 30°C about 10%. This change from a storage to a growth strategy at lower temperatures was likely triggered by the relatively longer feast phase that resulted from the lower acetate uptake rate at lower temperatures. We observed a similar shift from competing for acetate (storage strategy) towards growing directly on acetate (growth strategy) with decreasing SRTs as the feast phase also got longer with shorter SRTs (Johnson et al., under review, Water Research). Dionisi et al. (2006) reported a similar strategy change with longer feast phases when investigating the effect of the applied organic loading rate. The benefit of growing directly on acetate rather than on PHB, if the feast phase is long enough, lies in the higher efficiency of biomass production: Growth on acetate directly is about 7% more efficient than growth on PHB (Beun et al., 2000a). It seems that with a relatively larger fraction of the cycle being feast phase, the best strategy is to grow directly on the substrate. These two different strategies of direct growth versus PHB storage explain the different reactor behaviours at the different steady state temperatures and the differences observed with the short-term temperature change experiments.

While the differences between short- and long-term experiments are clearly visible for the feast phase rates (Figure 4.2), this is not the case for the famine phase. In the famine phase all rates are linked to the rate of PHB consumption. The temperature coefficient for PHB consumption was 1.058 (Figure 4.3 A) for the short-term temperature change experiments and between 1.039 (Figure 4.3 B) and 1.074 (Figure 4.4) for the long-term experiments. In the different steady states displayed in Figure 4.4 the values estimated for k_{PHB} vary considerably at each temperature. Cells could adjust the amount of PHB depolymerase that is present per PHB granule surface. Variations in k_{PHB} are therefore not unrealistic. Also differences in the microbial communities between different steady states could be responsible for some variation. A significant adjustment of the amount of PHB depolymerase or of the microbial composition is not very likely to occur in the short-term experiments. Still, the differences between long-term and short-term experiments for the temperature dependence of k_{PHB} are comparatively small. This may be, because the competitive pressure in the feast-famine SBR mainly affects the feast phase conversions, and the steady state feast phase rates show thus a stronger difference to the short-term experiments than those of the famine phase.

Temperature effects on maximum PHB storage capacity

Fed-batch experiments (Figure 4.5) were performed to investigate the maximum PHB storage capacity of all enriched cultures.

When the temperature in the fed-batch reactor was changed for the same SBR enrichment culture, the storage capacity remained unchanged, but the PHB production rate did adjust. In contrast, the PHB storage capacity did change considerably when the cultures were enriched in the SBR at different temperatures. As a consequence of the different competition strategies in

the SBR, the 15°C culture, which showed mostly a growth response and hardly any PHB storage in the SBR, also had a very low PHB storage capacity in the fed-batch experiments. The 30°C culture on the other hand, which had competed on acetate uptake rate and PHB storage, had a much higher PHB storage capacity than the culture from the 20°C steady state. Working at higher temperatures in the SBR seems thus a good strategy for improving PHA storage capacities. Whether this improvement was only caused by the shorter feast phase and support of the storage strategy in the SBR or whether we simply enriched a very high capacity culture just because it incidentally happened to have its optimum temperature around 30°C is unclear and remains to be investigated. Also the source of the inoculum is likely to have played a role.

It appears that the enrichment step in the SBR determines the maximum storage capacity of a certain cultures, while the conditions in the fed-batch step only influence how fast and if the maximum storage capacity will be reached. In terms of process optimization the key lies thus most likely with finding the optimal conditions for the enrichment step.

The PHB storage capacity of the culture enriched at 15°C could potentially be improved if the operating conditions were adjusted to the lower reaction rates. The SRT could be increased according to the lower conversion rates. Additionally a longer cycle length could be used to obtain a longer famine phase, supporting bacteria that store a higher amount of PHB. A better result could possibly also be obtained with time if a more diverse inoculum acclimatized to 15°C could be used, for example from a wastewater treatment plant in a cool climate.

Comparison with other PHA storing cultures

Krishna and van Loosdrecht (1999) evaluated the long-term temperature effect on acetate-fed feast-famine cultures similarly to our experiments. It was found that observed PHB yields in the feast phase decreased with higher steady state temperatures while we observed the opposite (Table 4.2, 'long-term'). This difference is caused by the different feeding strategy applied by Krishna and van Loosdrecht (1999). In the latter study the length of the feast phase and the acetate uptake rate were controlled by feeding the substrate at a constant low rate. Effectively the ratio of feast phase to famine phase was therefore the same for each temperature in their study. In contrast, in the here reported experiments the relative length of the feast phase decreased with increasing temperature. When the acetate supply rate is strongly rate limiting like in the experiments of Krishna and van Loosdrecht (1999), obviously the growth response becomes relatively more important. In this case, at lower temperatures the growth rate will be relatively slower than at higher temperatures, leaving more substrate for PHA production, while at higher temperatures almost all acetate supplied will be consumed immediately for growth. This likely led to the observed higher PHA storage yield at colder temperatures by Krishna and van Loosdrecht (1999). Effectively the real competition in their experiments was based on substrate affinity rather than on maximum rates.

Table 4.3. Temperature coefficients for selected metabolic processes in PHA storing bacteria.

	Feast/famine				PAOs				GAOs			
	Short-term		Long-term		Short-term		Long-term		Short-term		Long-term	
	θ	T [°C]	θ	T [°C]	θ	T [°C]	θ	T [°C]	θ	T [°C]	θ	T [°C]
Acetate uptake	1.051	15-25	1.104	15-30	1.080	5-20	1.095	5-20	1.054	10-35	1.211	10-20
PHA production	1.040	15-25	1.105	15-30	1.081	5-20	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
PHA consumption	1.058	15-35	1.074	15-30	1.035	5-30	1.129	5-20	1.082	10-30	1.109	10-30
Reference	This study		This study		Brdjanovic et al., 1997		Brdjanovic et al., 1998		Lopez-Vazquez et al., 2007 Lopez-Vazquez et al., 2008		Lopez-Vazquez et al., 2009a	

n.r. – not reported

Temperature studies similar to our study have also been conducted for PAOs by Brdjanovic et al. (1997; 1998) and for GAOs by Lopez-Vazquez et al. (2007; 2008; 2009a). Although the metabolism of PAOs and GAOs differs considerably from that of PHB producing feast-famine cultures, the mechanisms involved in reactions like acetate uptake, PHB production and PHB consumption are similar and temperature dependencies could thus be comparable. Table 4.3 shows a comparison of the temperature coefficients reported in literature for these three reactions with those indentified in this study.

For short-term temperature changes differences of the temperature coefficients between our feast-famine culture and those from PAOs and GAOs were not larger than between the latter two very similar groups of organisms (Table 4.3). But there is a significant difference in the metabolism of feast-famine cultures on the one hand and PAO/GAO cultures on the other hand. In feast-famine cultures the phase of PHB production (feast phase) is the phase where temperature can influence different reactions to different degrees, because they are independent of each other, and several temperature coefficients will be required to describe the process satisfactory, while only one temperature coefficient is required in the phase of PHB consumption (famine phase), where reactions are coupled. In PAO and GAO cultures the opposite occurs (Brdjanovic et al., 1997; Lopez-Vazquez et al., 2007; 2008): In the PHA production phase (anaerobic phase) reactions are to some degree coupled since no growth occurs (e.g. acetate uptake and PHA production have similar temperature coefficients for PAOs, Table 4.3), while in the PHA consumption phase several independent PHA consuming reactions (growth, glycogen production, maintenance, polyphosphate replenishment) take place which each may have a different temperature coefficient. Feast-famine cultures require thus several temperature coefficients for the feast phase kinetics (PHB production) and only one for the famine phase kinetics (PHB consumption), while the temperature dependence of the kinetics of PAOs and GAOs can probably be described sufficiently with one temperature coefficient for the anaerobic phase (PHB production), but several are required for the aerobic phase (PHB consumption) (assuming that maintenance is negligible).

After long-term temperature changes all three types of cultures were found to have greater temperature coefficients than after short-term changes (Table 4.3). The difference between short- and long-term experiments in our feast-famine cultures and in the PAO cultures is probably mainly due to population changes (Jiang et al., manuscript in preparation) (Brdjanovic et al., 1998), however, the GAO culture was an almost pure culture of *Competibacter* and the differences between short- and long-term experiments are probably due to an adaptation or acclimatization of the organisms (Lopez-Vazquez et al., 2009a). In mixed cultures a long-term temperature change may therefore also lead to an acclimatization of present organisms (different regulation of metabolic pathways) as well as a population change.

In the famine phase of feast-famine cultures and in the aerobic phase of PAO and GAO cultures PHA consumption is generally assumed to be the rate limiting step for all other reactions. Since the mechanism of PHA mobilization is probably similar in different organisms, temperature coefficient could be expected to be similar, too. The temperature coefficients for the three cultures varied just as much as they did between short and long-term experiments (Table 4.3). Differences in the temperature effect may for example be due to different polymer composition and characteristics (PAOs and GAOs produce copolymers containing hydroxybutyrate and hydroxyvalerate monomers) or possibly due to different amounts of depolymerase and different numbers of PHA granules present per cell. Genomic and enzymatic studies on the PHA converting enzymes in these three types of organisms might therefore be of interest for understanding the general regulation principles for growth on intracellularly stored PHA.

Conclusions

- For short-term temperature changes rate changes of feast-famine SBRs could be very well described with the simplified Arrhenius equation. Independent temperature coefficients were identified for the different metabolic processes: acetate uptake, θ = 1.051; PHB production from acetate, θ = 1.040; growth on acetate, θ = 1.106; PHB consumption, θ = 1.058. Short-term temperature changes only influenced the reaction rates but not the maximal PHB storage capacity of the mixed culture.
- Rate changes after long-term temperature changes were also influenced by an adaptation of the culture. Temperature coefficients related to PHA metabolism were higher than for short-term temperature changes. The maximal specific growth rate on acetate on the other hand showed a decreasing value with increasing temperature. At higher steady state temperatures (30°C) PHB storage was the dominant process in the feast phase. This culture also had a very high PHB storage capacity (84 wt%). At lower temperatures (15°C) rates were decreased and the feast phase was therefore longer. As a consequence growth occurred predominantly directly on acetate rather than on stored PHB. This culture had a very low PHB storage capacity.

Nomenclature

A	Pre-exponential factor in Arrhenius equation
E_a	Activation energy
$\tilde{f}_{PHB}$	Modelled fraction of PHB
k	Rate constant of a reaction
k_T	Rate constant at temperature T
$k_{293\ K}$	Rate constant at the reference temperature of 20°C (293 K)
k_{PHB}	Rate constant of PHB degradation
l_N	Gas volume in litres at standard (normal) conditions (273 K, 1013 mbar)
m_{ATP}	Maintenance ATP requirement
q_{Ac}	Average biomass specific acetate uptake rate
$\tilde{q}_{Ac}^{\max}$	Maximum biomass specific acetate uptake rate in the model
q_{CO_2}	Average biomass specific carbon dioxide evolution rate
q_{NH_3}	Average biomass specific ammonia uptake rate
q_{O_2}	Average biomass specific oxygen uptake rate
q_{PHB}	Average biomass specific PHB production or consumption rate
$\tilde{q}_{PHB}^{fam}$	Modelled biomass specific PHB consumption rate
R	Gas constant
R^2	Coefficient of determination
t	Time
T	Absolute temperature
$Y_{CO_2/Ac}^{obs}$	Observed yield of carbon dioxide on acetate
$Y_{CO_2/PHB}^{obs}$	Observed yield of carbon dioxide on PHB
$Y_{PHB/Ac}^{obs}$	Observed yield of PHB on acetate
$Y_{X/Ac}^{obs}$	Observed yield of active biomass on acetate
$Y_{X/PHB}^{obs}$	Observed yield of active biomass on PHB
θ	Temperature coefficient in simplified Arrhenius equation
μ	Average biomass specific growth rate
$\tilde{\mu}^{\max}$	Maximum biomass specific growth rate in the model

Abbreviations

Ac	Acetate
ATP	Adenosine triphosphate
DGGE	Denaturing gradient gel electrophoresis
DO	Dissolved oxygen
GAO	Glycogen accumulating organism
HRT	Hydraulic residence time

PAO	Polyphosphate accumulating organism
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
SBR	Sequencing batch reactor
SRT	Sludge residence time
TSS	Total suspended solids

CHAPTER 5

Enrichment of a Mixed Bacterial Culture with a High Polyhydroxyalkanoate Storage Capacity

Katja Johnson, Yang Jiang, Robbert Kleerebezem, Gerard Muyzer,

Mark C. M. van Loosdrecht

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ABSTRACT

Polyhydroxyalkanoates (PHAs) are microbial storage polymers that attract interest as bioplastics. PHAs can be produced with open mixed cultures if a suitable enrichment step based on the ecological role of PHA is used. An acetate-fed sequencing batch reactor operated with 1 day biomass residence time and with feast-famine cycles of 12 hours was used to enrich a mixed culture of PHA producers. In subsequent fed-batch experiments under growth limiting conditions, the enriched mixed culture produced PHA up to a cellular content of 89 wt% within 7.6 hours (average rate of 1.2 g/g/h). The PHA produced from acetate was the homopolymer polyhydroxybutyrate (PHB). The culture was dominated by a *Gammaproteobacterium* which showed little similarity on 16S rRNA level with known bacteria (<90% sequence similarity). The mixed culture process for PHA production does not require aseptic conditions. Waste streams rather than pure substrates could be used as raw materials.

Introduction

Polyhydroxyalkanoates (PHAs) are polymers of hydroxy fatty acids which are naturally produced by many different bacteria as an intracellular carbon and energy reserve material (Lenz et al., 2005; Wältermann et al., 2005). The most abundant PHA is the homopolymer poly(3-hydroxybutyrate) (PHB) (Anderson et al., 1990). PHAs create interest as bioplastics as they can exhibit thermoplastic and/or elastomeric properties, are enantiomerically pure chemicals (only *R*-stereoisomer), non-toxic, biocompatible, made from renewable resources and biodegradable (Steinbüchel, 2001). Moreover, the polymer can be hydrolyzed and the hydroxy fatty acid monomers could serve as chiral building block chemicals for the production of biochemicals (Ren et al., 2005).

Some bacteria can accumulate PHAs up to a content of 70 to almost 90 wt% of their dry weight (Steinbüchel et al., 1998). For commercial production of PHA, genetically engineered bacteria are considered superior to natural PHA producers, producing 100 g/l PHA in 40 h or less from pure substrates like sugars and reaching up to 90 wt% cellular PHA content (Anon., 2002; Slater et al., 1988). Usually biotechnological processes employing pure cultures of microorganisms require well-defined feedstocks and aseptic process conditions. This results in high substrate costs, expensive equipment and high energy consumption, making industrial biotechnology based on pure cultures unfavourable for the large scale production of bulk biochemicals and biomaterials.

An alternative approach for the sustainable production of biochemicals and biomaterials could be the application of eco-biotechnology. Eco-biotechnology aims to produce products with processes employing open mixed cultures and ecological selection principles, thus combining the methodology of environmental biotechnology with the goals of industrial biotechnology. Mixed cultures have so far only been applied for waste treatment (e.g. biological wastewater treatment plants, composting facilities, anaerobic digesters) and partly for the production of

bioenergy (biogas), metals (biohydrometallurgy) or traditional fermented foods, but not for the production of biochemicals or biomaterials.

The principle of eco-biotechnology is based on natural selection and competition rather than on genetic or metabolic engineering (Kleerebezem et al., 2007). Selective pressure for a desired metabolism is applied on a diverse inoculum by choosing the substrate and operating conditions of the bioreactor in an appropriate way, i.e. to engineer the ecosystem rather than the organisms. Mixed culture fermentation can for example be directed towards different products depending on the chosen pH (Temudo et al., 2007). As PHA is a storage material that is naturally produced by many bacteria under dynamic conditions, an effective selective pressure for a PHA producing population is the application of a cyclic feast-famine regime, i.e. the repeatedly alternating presence (feast phase) and absence of the substrate (famine phase), as is typically established in a repeated batch cultivation mode (van Loosdrecht et al., 1997). An example of suitable substrates for mixed culture PHA production are fatty acids (Dias et al., 2006), which in turn can be produced by mixed culture fermentation – a process well-studied in the context of anaerobic digestion.

PHA production in mixed cultures has been studied in the past mostly in relation to its relevance in wastewater treatment rather than as a potential production process (van Loosdrecht et al., 1997). Currently research is focusing on the use of waste streams like olive oil mill effluent (Dionisi et al., 2005b), molasses (Albuquerque et al., 2007) and paper mill wastewater (Bengtsson et al., 2008a) for the commercial production of PHAs with mixed cultures. Optimization of the mixed culture PHA production process has led to PHB contents of up to 65 wt% with acetate as the substrate (Dias et al., 2006). However, this is still low compared to the best axenic cultures of natural producers and recombinant bacteria and requires further optimization for efficient downstream processing of the product.

The aim of this study was to improve the maximum PHA content that can be reached with mixed cultures towards the values achieved by engineered bacteria. A two-step process was employed to (i) enrich and grow a PHA producing mixed culture and (ii) produce PHA by maximizing the cellular PHA content of the biomass harvested from the first step. In the first step, a sequencing batch reactor (SBR) was used to achieve the dynamic conditions of alternating feast and famine periods, while the second step was realized in a fed-batch reactor. Acetate was used as a model substrate for simplicity reasons and as it is a major fermentation product.

Materials and Methods

Sequencing batch reactor for culture enrichment

A double-jacket glass bioreactor with a working volume of 2 litre (Applikon, The Netherlands,) was used for the cultivation of a PHA producing bacterial culture. The reactor has been running continuously for a period of 4 years as an open (i.e. non-sterile) sequencing batch reactor (SBR).

Operation of the reactor was based on a 12 h batch cycle consisting of a start phase (0 - 7 min), an influent phase in which 1000 ml fresh medium was supplied (7 - 17 min), a reaction phase (17 – 700 min) and a biomass withdrawal phase in which 1000 ml of reactor liquid was removed (700 - 720 min). The end of one batch was immediately followed by the start phase of the next batch, thus the reactor was running continuously sequences of batch reactions, with the remaining biomass from one batch being the inoculum for the following batch.

The reactor was equipped with a stirrer with two standard geometry six-blade turbines. The flow of air to the reactor was controlled at 0.2 l_N/min with a mass flow controller (Brooks Instrument, USA). The actual gas flow through the reactor was increased to 1.3 - 1.4 l_N/min through off-gas recirculation in order to reach an accurately measurable depletion of oxygen and production of carbon dioxide without compromising the oxygen mass transfer. The reactor was temperature controlled at 30±1°C by means of a water jacket and a thermostat bath (Lauda, Germany). Prior to feeding the reactor, the medium was heated to 30°C with a thermostat bath (Lauda, Germany). The pH of the reactor liquid was maintained at 7.0 $\pm$ 0.1 using 1 M HCl and 1 M NaOH. The pumps, the stirrer, the airflow and the pH were controlled by a biocontroller (BIOSTAT B plus, Sartorius) which in turn was controlled by a PC using the software MFCS/win (Sartorius Stedim Systems, USA). MFCS/win was also used for data acquisition of the online measurements (dissolved oxygen (DO), pH, temperature, acid dosage, base dosage, off-gas oxygen and carbon dioxide).

The amount of biomass removed during the biomass withdrawal phase determined the solids retention time (SRT). In steady state, the amount of biomass withdrawn in each cycle equalled the amount of newly formed biomass. The reactor was considered to be in steady state when for at least five days (i) the concentration of total suspended solids (TSS) at the end of the cycle and (ii) the length of the feast phase as indicated by the DO changes were constant. In this set-up the SRT was 1 d and equalled the hydraulic retention time (HRT). When the reactor was in steady state, a cycle of the SBR was monitored additionally to the online measurements by offline samples (acetate, TSS, PHA, ammonia).

The initial inoculum of the SBR was aerobic activated sludge from the second aerobic stage of the Dokhaven wastewater treatment plant in Rotterdam, the Netherlands (September 2004). For each new experiment the sludge from the previous experiment was used as inoculum. Over the months the sludge was subjected to different conditions. The reactor was initially operated with 4 h cycles at 4 d SRT and 20°C with different carbon to nitrogen ratios in the medium (carbon limited to nitrogen limited). After 1½ years the SRT was changed to 1 d and after another 3 months the cycle length was changed to 12 h. After about half a year at these conditions, the temperature of the reactor was slowly increased from 20°C to 30°C over a period of about 1 month (December 2006), therefore reaching the conditions described above. The SBR was further maintained at these conditions up to today (December 2008). Biomass was harvested from the reactor at different time points in order to study the PHA production capacity of the biomass in fed-batch experiments.

The medium for the SBR consisted of a carbon source, a nutrient source and dilution water. The carbon source was 270 mM $NaAc \cdot 3H_2O$ and the nutrient source was composed of 67.5 mM NH_4Cl, 24.9 mM KH_2PO_4, 5.55 mM $MgSO_4 \cdot 7H_2O$, 7.2 mM KCl, 15 ml/l trace elements solution according to Vishniac and Santer (1957) and 100 mg/l allylthiourea (to prevent nitrification). In the influent phase of each batch cycle 100 ml of carbon source, 100 ml of nutrient source and 800 ml of dilution water were mixed and pumped into the reactor.

The reactor was cleaned about once per week in order to remove biofilms from the glass walls and the metal parts and electrodes.

Fed-batch reactor for PHA production

Steady state biomass from the SBR (end of cycle) was used for PHA production in three fed-batch experiments (February 2007, July 2007 and April 2008). For this purpose the same set-up as for the culture selection was used, but in a fed-batch mode. The same stirring speed, pH set point, temperature and aeration rate as in the SBR cycle's reaction phase were applied. 1 l of acetate- und ammonia-free medium (same composition as for SBR, but no NaAc and NH_4Cl) was fed to the reactor. To start the PHA production, a pulse of about 40 - 60 mmol sodium acetate was supplied to the reactor. 1.5 M acetic acid and 1 M NaOH were used for pH control. The acetic acid served simultaneously as carbon source (fed-batch) and for pH control. Using this approach, non-limiting acetate concentrations (10 - 30 mM) could be maintained throughout the experimental period. Growth was limited in these experiments as no nitrogen source was supplied and only a small amount (if any) remaining nitrogen source from the previous SBR cycle was available. In the first experiment (Feb. 2007) the reactor liquid contained still 0.3 mM ammonia from the previous SBR cycle, in the second experiment (July 2007) 1 mM ammonia was still present. In the third experiment (April 2008) no ammonia remained at the beginning of the fed-batch experiment.

The progress of the experiment was monitored via online (DO, pH, acid and base dosage, offgas CO_2 and O_2) and offline (acetate, TSS, PHA, ammonia) measurements. The experiments were stopped after about 12 h.

Analytical methods

The concentration of dissolved oxygen (DO) in the reactor was measured with a DO electrode (Mettler Toledo, USA) as percentage of air saturation and the pH was monitored with a pH electrode (Mettler Toledo, USA). The temperature of the reactor broth was measured with a thermo element. The amount of acid or base dosed for pH control was measured online. Carbon dioxide and oxygen partial pressures in the gas entering and leaving the reactor were analyzed in dried gas with a gas analyzer (NGA 2000, Rosemount, USA). Gas measurements and calibrations were corrected with the actual air pressure for standard conditions. Cumulative oxygen consumption and carbon dioxide evolution were calculated with mass balances from

the air flow rate supplied and the partial pressures measured on-line, considering the mixing effects and delays caused by the headspace volume and gas recirculation (also via mass balances). Inorganic carbon in the liquid phase (carbon dioxide, carbonic acid, bicarbonate, carbonate) was included in the carbon dioxide evolution with acid-base equilibria and liquid-gas mass transfer calculations.

Samples taken from the reactor for analysis of acetate and ammonia were immediately filtered with a 0.45 µm pore size filter (PVDF membrane, Millipore, Ireland). The acetate concentration in the supernatant was measured with a Chrompack CP 9001 gas chromatograph (Chrompack, The Netherlands) equipped with a FID, on a HP Innowax column. The ammonia concentration was determined spectrophotometrically with a commercial cuvette test kit (Hach Lange, Germany). The biomass concentration was measured as total suspended solids (TSS) by filtration according to standard methods (Taras et al., 1971). The amount of PHA was subtracted from the TSS to calculate the concentration of active biomass. The active biomass concentration was converted from g/l into carbon moles per litre (Cmol/l) assuming a composition of $CH_{1.8}O_{0.5}N_{0.2}$ and a molecular weight including ash of 25.1 g/Cmol (Beun et al., 2002). The same biomass composition was used to calculate newly formed biomass from ammonia measurements.

Samples taken for PHA analysis were added to 15 ml tubes containing 5 drops of formaldehyde in order to stop all biological activity. Samples were subsequently washed with tap water and freeze-dried. Pure PHB (Sigma) was used as a standard in the analysis and treated alongside with the samples. Biomass samples and 3 standards were weighed with an analytical balance and transferred into tubes with screw caps. 1 mg of benzoic acid in 1-propanol was added as the internal standard. 1.5 ml of a mixture of concentrated HCl and 1-propanol (1:4) and 1.5 ml of dichlorethane was added. The closed tubes were heated for 2 h at 100°C. After cooling, free acids were extracted from the organic phase with 3 ml water. 1 ml of the organic phase was filtered over water-free sodiumsulfate into GC vials. The propylesters in the organic phase were analyzed by gaschromatography (model 6890N, Agilent, USA) equipped with a FID, on a HP Innowax column. Results were expressed as weight percentage of PHB of the total biomass.

All measured concentrations and conversions were corrected for the effect of sampling and for the diluting effect of the addition of substrate (fed-batch only) and acid and base solutions for pH control.

Carbon and electron balances

Total conversions of acetate, biomass (either from TSS or ammonia measurements), PHB, carbon dioxide and oxygen were calculated form the measurements for the SBR cycle and the fed-batch experiments. In fed-batch experiments acetate uptake was calculated from the amount of acetate dosed for pH control and the concentration change in the liquid. For carbon balances the total amount of acetate consumed, PHB produced or consumed, biomass produced and carbon dioxide produced were considered (all in carbon-mole). Electron balances

were calculated relative to the reference oxidation states of carbon dioxide and water for organic carbon and oxygen. Using this approach the oxidation state of the remaining reactants can be calculated per (carbon) mole as 4 for acetate, 4.2 for biomass, 4.5 for PHB and -4 for oxygen (Heijnen, 1999).

Culture characterization

Extraction, purification and amplification of genomic DNA from the SBR reactor

Culture samples from the SBR reactor were washed with demineralized water to remove medium prior to DNA extraction. The genomic DNA was extracted using the UltraClean soil DNA extraction kit (MoBio Laboratories), following the manufacturer's instructions. The quality and the yield of the extracted DNA were determined by agarose gel electrophoresis and NanoDrop 1000 (NanoDrop Technologies), respectively. Subsequently, the extracted DNA was used as template DNA in the PCR. PCR amplification was performed as described by Muyzer et al. (1995). A touchdown PCR program was done by using primers 341F with a GC clamp and 907R for DGGE analysis.

Denaturing gradient gel electrophoresis (DGGE) analysis

16S rDNA amplicons were applied onto 8% polyacrylamide gels with denaturing gradient from 35% to 55% urea-formamide. DNA was visualized after ethidium bromide staining by UV illuminator and photographed with a digital camera. Individual bands were excised from the gel by sterile blade and incubated in 50 µl water at 4°C. Re-amplification was performed by using the same primer pair and purity of re-amplification was checked by second round DGGE with the same gradient. Subsequently, PCR products were purified with a QIA-Quick gel extraction kit (QIAGEN laboratories) and sequenced (Macrogen, South Korea).

Clone library

Primers amplifying the 16S rRNA genes of all Bacteria, i.e. 63f-Mod and 1387r-Mod, were used to amplify the 16S rDNA gene from the total community DNA. All PCR reactions were performed in a thermal cycler under the following conditions: Initial denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min and 30 s, with an additional extension step at 72°C for 15 min. PCR products were run on 1% (wt./vol.) agarose gel along with a standard molecular weight marker, stained with ethidium bromide solution and visualized using a UV illuminator. The amplified rDNA was ligated into a pCR2.1-TOPO vector and transformed into Escherichia coli competent cells (Invitrogen) in accordance with the manufacturer's instructions. Clones were screened for insertion of the correct size by PCR amplification with M13-F and M13-R primers and sequenced (Macrogen, South Korea).

Comparative sequence analysis

The obtained 16S rRNA gene sequence was imported into the ARB software program (Ludwig et al., 2004) and aligned using the automatic aligner function. The alignment was further corrected manually, and an optimized tree was calculated using the Neighbour-Joining algorithm with Felsenstein correction.

Fluorescent in situ hybridization (FISH)

Biomass samples from the SBR (end of the feast phase) were fixed in paraformaldehyde, immobilized on microscopic slides and hybridized as follows: the samples were hybridized with probes in a mixture of 1 µl of probe solution (5 ng/µl or 8.3 ng/µl) and 9 µl hybridization buffer containing 0.9 M NaCl, 0.01% (wt./vol.) sodium dodecyl sulphate (SDS), 20 mM Tris-HCl (pH = 8 and 20% (vol./vol.) formamide). Hybridizations were carried out for at least 90 min at 46°C in a sealed moisture chamber and stopped by rinsing the non-bound probes from the slides with washing buffer. The slides were immersed in the washing buffer for 20 min and washed shortly with distilled water and dried at room temperature. Published probes and one designed probe (Table 5.1) were used in FISH. Probes were commercially synthesized and 5' labelled with either the FLUOS or with one of the sulfoindocyanine dyes Cy3 and Cy5 (Thermo Hybaid Interactiva, Ulm, Germany). The Design Probe tool of the ARB software package (Ludwig et al., 2004) was used to design a specific probe from the 16S rRNA sequence.

Table 5.1. Oligonucleotide probes used for FISH.

Probe	Sequence 5'-3'	Binding position [a]	Specificity	Reference
EUB338	GCT GCC TCC CGT AGG AGT	338-355	Bacteria	Amann et al. (1990)
EUB338II	GCA GCC ACC CGT AGG TGT	338-355	Bacteria	Daims et al. (1999)
EUB338III	GCT GCC ACC CGT AGG TGT	338-355	Bacteria	Daims et al. (1999)
UCB-823	CCT CCC CAC CGT CCA GTT	823-841	Bacteria	This study

a: Numbering according to the 16S rRNA of *E. coli*

Post Nile Blue A staining

After FISH, Nile Blue A staining was performed. The cover slip was removed from the FISH slide. The slide was stained with 1% (wt./vol.) Nile Blue A solution at 55°C for 15 min in a staining jar. After being stained, the slide was washed with 8% (vol./vol.) aqueous acetic acid for 1 min. Finally, the slide was air dried and covered by a glass cover slip. The field photographed by FISH was relocated and re-photographed for the Nile Blue A staining.

Results

Culture enrichment

Figure 5.1 shows a typical sequencing batch reactor (SBR) cycle, obtained after over a year of continuous operation under the same conditions. In the beginning of the cycle fresh medium was supplied to the reactor (7 - 17 min), marking the beginning of the feast phase. The acetate point at 7 minutes represents the total amount of acetate supplied to the reactor, while the other points are derived from actual acetate measurements in the reactor broth. Acetate was taken up very fast with an active biomass specific acetate uptake rate of 3.2 Cmol/Cmol/h (equivalent to 3.8 g acetic acid per g active biomass per hour). Acetate was used by the bacteria for the formation of new biomass (indicated by ammonia and biomass measurements), but mostly for the storage of substrate in the form of PHB (up to 53 wt%). Mixed feast-famine cultures produced the pure homopolymer PHB in all our studies, when enriched on acetate. The average biomass specific rate of PHB production was 2.1 Cmol/Cmol/h (1.8 g/g/h).

When the external substrate acetate was depleted, the famine phase commenced (at 50 min) in which intracellularly stored PHB was used by the bacteria as a substrate for continued growth. At the end of the SBR cycle half of the reactor content was removed to harvest biomass (resulting in a solids retention time (SRT) of 1 day) and in order to make space for fresh medium in the next cycle. In steady state, the amount of biomass in the SBR hence doubled in every 12 hour cycle.

Carbon and electron balances calculated over the whole SBR cycle closed very well (Table 5.2) and confirmed thus a high accuracy of the measurements. The measurements confirm that under the employed conditions a PHB producing mixed culture can be enriched.

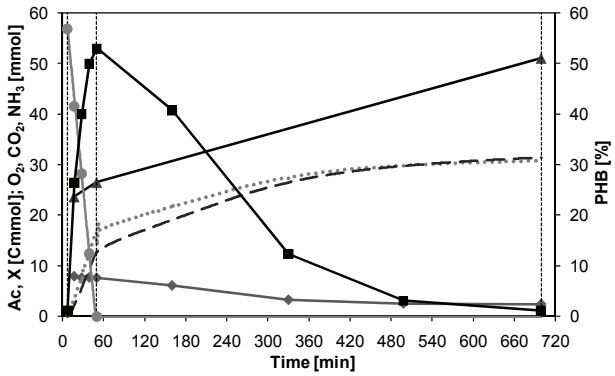

Figure 5.1. Sequencing batch reactor cycle (Feb. 2008). (●) amount of acetate present in the reactor, (▲) amount of active biomass (TSS excl. PHB) present in the reactor (X), (■) weight percentage of PHB in TSS, (···) cumulative carbon dioxide evolution, (— —) cumulative oxygen uptake, (◆) amount of ammonia present in the reactor. Vertical dashed lines indicate the beginning and end of the feast (7 - 50 min) and famine phase (50 - 700 min).

Table 5.2. Carbon and electron balances for the complete SBR cycle shown in Figure 5.1.

	Total conversions [(C)mmol]	C-balance [Cmmol]	Electron balance [e-mmol]
Acetate	-56.9	-56.9	-227.6
Biomass [a]	25.0	25.0	105.0
PHB	0.4	0.4	1.8
CO_2	30.8	30.8	
O_2	-31.5		126.0
Balance		-0.7	5.2
Relative error		1%	-2%

a: calculated from ammonia measurements

PHA production

The potential of the enriched microbial population to store large amounts of PHB was evaluated in a fed-batch reactor using a pH controlled feed of acetic acid and growth limiting conditions (nitrogen limitation). The PHB storage capacity and production rate of the selected culture were investigated at three different time points (Figure 5.2): After about two months of cultivation at the described conditions (February 2007) the culture was able to reach a PHB content of 79 wt% within 12 hours. About 5 months later 81 wt% PHB was produced within 8.1 hours. The third fed-batch experiment was performed 9 months after the second fed-batch experiment (April 2008). In this experiment a PHB content of 89 wt% was reached after about 7.6 h of continuous feeding. The PHA production capacity and rate both increased the longer the culture was cultivated in the feast-famine SBR. The average specific rate of PHB production over the first 7.6 h in the third experiment was 1.4 Cmol/Cmol/h (1.2 g/g/h) and the overall yield of PHB on acetate was 0.60 Cmol/Cmol (0.43 g/g), which is equivalent to an electron yield of 0.68 e-mol/e-mol.

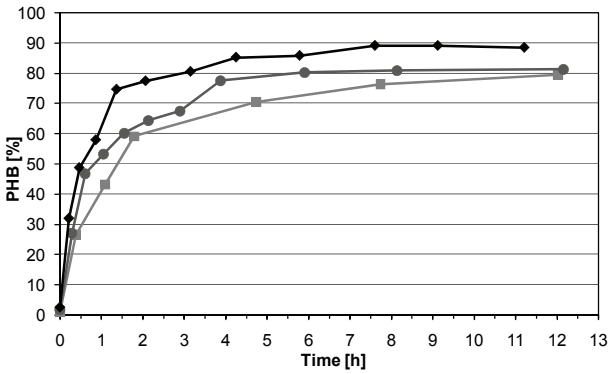

Figure 5.2. Weight percentages of PHB in total suspended solids in three fed-batch experiments under growth limiting conditions (see Materials and Methods) with biomass harvested from the SBR at three different time points: (■) February 2007, (●) July 2007, (◆) April 2008.

Figure 5.3 shows the complete data set of the third fed-batch experiment. The biomass specific PHB production rate of the culture was initially identical to the one measured in the SBR cycle (2.1 Cmol/Cmol/h), but decreased with increasing PHB content once PHB contents higher than during the SBR cycle were reached. The biomass specific acetate uptake rate was affected similarly by the increasing PHB contents, reaching 3.3 Cmol/Cmol/h only at the beginning of the experiment. The culture was starved of its nitrogen source ammonia throughout the whole experiment. Nevertheless, TSS samples indicated a slight increase in active biomass concentrations. A small amount of biomass growth could be possible through the conversion of the existing biomass into a biomass with a lower nitrogen content. This formation of new biomass was therefore considered as growth in the carbon and electron balances shown in Table 5.3.

Carbon and electron balances show errors of 12% and 10%, respectively, for this experiment, i.e. 10 - 12% of the measured acetate taken up was not found back in any of the measured products. Typically, balances for fed-batch experiments show larger errors than SBR cycle measurements, which might be partly due to an inaccuracy of the acetic acid dose monitor used in fed-batch experiments.

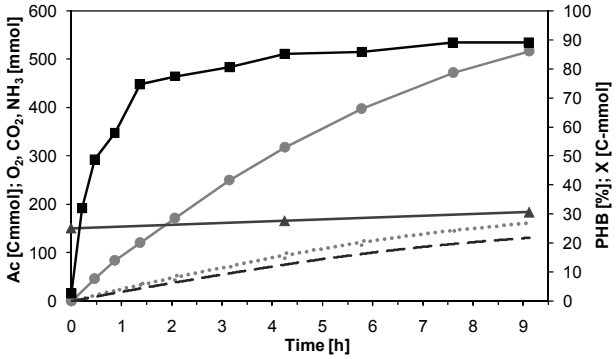

Figure 5.3. Fed-batch experiment from April 2008. (●) cumulative amount of acetate taken up, (▲) amount of active biomass (TSS excl. PHB) present in the reactor (X), (■) weight percentage of PHB in TSS, (▪▪▪) cumulative carbon dioxide evolution, (——) cumulative oxygen uptake.

Table 5.3. Carbon and electron balances for the fed-batch experiment shown in Figure 5.3, calculated over the first 9.1 hours

	Total conversions [(C)mmol]	**C-balance [Cmmol]**	**Electron balance [e-mmol]**
Acetate	-517	-517	-2068
Biomass [a]	6	6	25
PHB	290	290	1305
CO_2	159	159	
O_2	-131		524
Balance		-62	-214
Relative error		12%	10%

a: calculated from TSS measurements

Microbial diversity

We studied the microbial diversity of the culture enriched in the SBR using full cycle 16S rRNA and found one dominating organism. Comparative analysis of the nearly complete 16S rRNA sequence showed that this organism (MCB_clone37) was a member of the *Gammaproteobacteria* (Figure 5.4 A), only distantly related (<90% sequence similarity) to its closest known relatives. The dominance of MCB_clone37 was confirmed using fluorescence in situ hybridisation (FISH) with a specific probe designed for the 16S rRNA of the organism (Figure 5.4 B). The lipophilic stain Nile Blue A was used to verify the presence of PHA inside the cells of MCB_clone37 (Figure 5.4 C). The dominant organism was isolated from the mixed culture for further characterization. It was found to be able to grow aerobically on C2 - C9 fatty acids and C2 - C4 alcohols.

5

Discussion

Culture enrichment and PHA production

Carbon limited SBRs like the one described here are highly dynamic systems, which in contrast to chemostat type reactors do not have a continuous substrate addition and biomass removal. Competition in the SBR is based on substrate uptake rate rather than on growth rate: Simplified, bacteria with the highest substrate uptake rate can take up the most substrate in the short period of time it is available and can therefore produce the most new biomass and proliferate in the system. The PHA producers present in the system take up the substrate rapidly (using it for growth and PHA production simultaneously) and they continue to grow in the long period of absence of an external substrate, using stored PHA as an internal substrate. Bacteria that do not store PHA would have to take up the substrate as fast as the PHA producers and at least double their biomass in the 43 minutes of the feast phase in order to remain in the system. However, they would also have to survive the more than 11 hours of starvation and have virtually no lag-phase afterwards when the new feast phase starts. We believe that PHA storing bacteria outcompeted other bacteria in this reactor, as they store the majority of the substrate which results in a very high substrate uptake rate, and because they grow continuously throughout the cycle with a more or less continuous growth rate.

The PHA contents found in this study are considerably higher than the 65 wt% reported for mixed cultures before (Dias et al., 2006) and as high as reported for genetically engineered strains or the best pure cultures of natural PHA producers (Steinbüchel et al., 1998; Slater et al., 1988). The rate of PHA production was very high with our mixed culture, reaching up to 89 wt% of PHA within less than 8 h, whereas the commercial process with recombinant bacteria takes up to 40 h (Anon., 2002). The PHA production rate and storage capacity of the mixed culture improved over time due to the continuous selective pressure and competition (Figure 5.2). Apart from the time factor, a combination of factors is thought to have led to this extraordinary

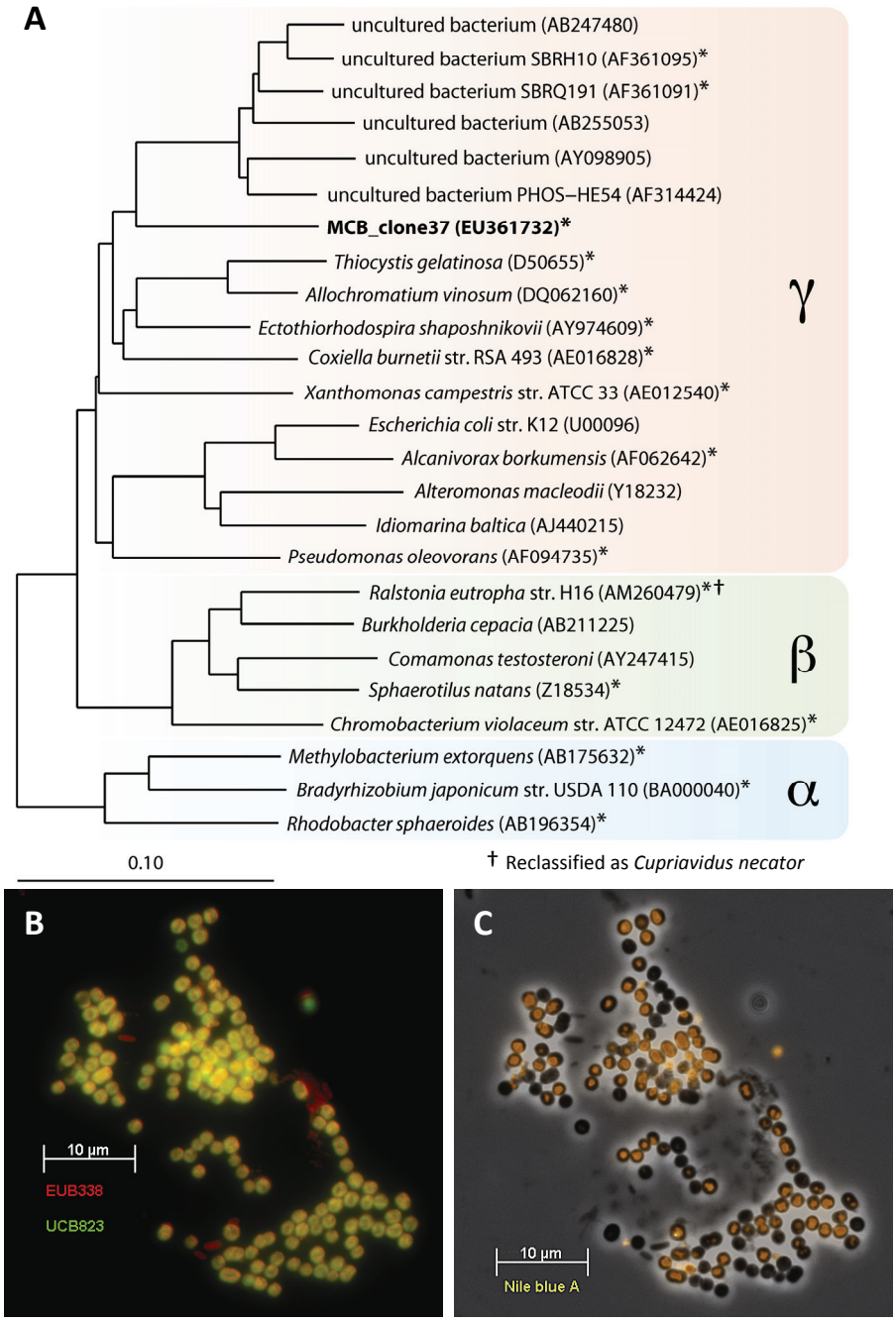

Figure 5.4. Phylogenetic tree and microscopic photographs. **A:** Neighbour joining tree based on nearly complete 16S rRNA sequences showing the phylogenetic affiliation of the dominant community member MCB_clone37. Names marked with an asterisk indicate bacteria that can produce PHA. Greek symbols indicate different subclasses of the *Proteobacteria*, i.e. *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*. Different members of the *Spirochaetae* were used as outgroup, but were pruned from the tree. The bar indicates 10% sequence difference. **B:** Fluorescence microscopy image of the mixed culture stained with fluorescein-labelled 16S rRNA probe for *Eubacteria* (EUB338, red) and Cy5-labelled 16S rRNA probe for the dominant community member MCB_clone37 (UCB823, green). The yellow colour indicates that both probes hybridized. **C:** Overlay of a phase contrast image and a fluorescence microscopy image of the same field as in Figure 5.4 B post-stained with the lipophilic stain Nile Blue A, indicating the presence of PHA.

culture enriched in the SBR: long SBR cycles, low SRT, carbon limiting conditions, and a high temperature compared to previous studies. Long SBR cycles combined with low SRTs select for bacteria with a high storage capacity, due to the long famine phase and the high rate of growth required. Carbon limitation ensures that the culture is not limited during the famine phase and can fully utilize its advantage of continued growth. The higher temperature leads to increased rates, but might have also played a role in the selection of this particular culture.

Microbial diversity

The approach of using a strong selective pressure for PHA producers that is based on the ecological role of PHAs led to a mixed culture that is dominated by an organism which is highly competitive under these conditions. Due to this competitiveness, the isolated dominant community member could be used as a virtually pure culture for the production of PHA without the need for aseptic conditions.

Potential of mixed culture PHA production

In this conceptual study we used acetate as the single carbon source, which yields pure PHB. Acetate is converted into acetyl-CoA, which is a direct precursor for the synthesis of PHB. If mixtures of volatile fatty acids such as acetate, propionate, butyrate and valerate are used as a substrate for PHA production, acetyl-CoA and propionyl-CoA are formed as precursors for PHA production, resulting in a copolymer containing hydroxybutyrate and hydroxyvalerate monomers (PHBV) (Dias et al., 2006; Albuquerque et al., 2007; Serafim et al., 2008b). Many waste streams from e.g. food and paper industries either contain these substrates or can be fermented to short-chain fatty acids by mixed culture fermentation and are therefore considered as suitable feedstocks for PHA production with mixed cultures (Dionisi et al., 2005b; Albuquerque et al., 2007; Bengtsson et al., 2008a). The dominant organism enriched in our culture was found to also use longer-chain fatty acids as a substrate. To our knowledge no study has evaluated the use of longer-chain fatty acids in mixed culture PHA production, yet. Longer-chain fatty acids could potentially lead to PHA monomers with longer side chains, but they could also be converted into acetyl-CoA and propionyl-CoA through fatty acid β-oxidation and therefore yield PHB and PHBV. Further research is required on this subject.

Conversion of fermentation products into PHA could also be an interesting option from a wastewater treatment point of view, as concentrated solid materials like PHA are generally easier to separate from the liquid than low concentrations of soluble fermentation products. Thus the PHA production process could generate clean water and an attractive product at the same time.

For commercial scale application, advantages of using eco-biotechnology rather than recombinant bacteria for PHA production would be: (i) lower substrate costs by using (fermented) waste streams rather than pure substrates, (ii) lower energy costs (no sterilization

required), (iii) lower equipment costs, (iv) a simpler process (strain selection and improvement by directed evolution within the system), (v) potential for continuous operation and (vi) a better acceptance in the public. The substrate costs are a major cost factor in the production of PHAs and the use of waste streams or agricultural residuals could contribute to a considerable reduction in these costs (Koller et al., 2007; Braunegg et al., 2004; Reis et al., 2003). Another advantage of using waste streams rather than e.g. sugars as a substrate for the production of PHAs is that competition with food production is avoided. On the other hand, the type of PHA produced in a waste based process depends primarily on the composition of the waste stream. Waste streams often vary in composition, which may result in a variable PHA composition and yield. Identification of suitable waste streams is therefore crucial for a successful commercial implementation of a waste based PHA production process. It should be noted that these considerations also apply to pure culture processes using waste streams.

A disadvantage of mixed culture PHA production might be the potentially higher costs for downstream processing, since lysis of cells might be more difficult than it is for recombinant bacteria. The stability of processes based on open mixed cultures could be another area of concern. However, our SBRs for enrichment of PHA producing cultures have been running continuously for over 4 years under various conditions without stability problems. Also Serafim and co-workers reported a stable reactor performance of a PHA producing SBR for over 2 years (Serafim et al., 2008b). Since the PHA production process is based on firm ecological principles, it can be argued that a mixed culture based process will be more stable compared to a pure culture based process. Upon changes in the process conditions, adaptation to the new process conditions will effectively select for a microbial community with a superior performance under the new conditions. While contaminations can be a problem for pure culture processes, the use of an open system for mixed culture PHA production actively supports the introduction of new organisms into the reactor that could potentially be more competitive PHA producers than the established community.

Conclusions

Here we show a considerable improvement of mixed culture PHA production, making it a viable option for the large scale production of bioplastics. Eco-biotechnology has been largely overlooked in the context of biorefineries and a lot of attention has focused instead on engineering microorganisms to overcome problems such as substrate specificity, product inhibition and low productivities and yields. We have shown that it is a promising alternative approach to explore the available diversity in nature by engineering the ecosystem rather than the organism and providing a niche for the particular metabolism required. Natural competition but also symbiosis between different microorganisms can be employed to optimize rates and yields and to overcome substrate specificity and product inhibition. Although the use of mixed cultures has a long history in wastewater treatment, we are only beginning to explore the full

potential of eco-biotechnology for the conversion of waste into products and we are hoping for more remarkable findings like in the case of PHA production.

CHAPTER 6

Influence of Ammonium on the Accumulation of Polyhydroxybutyrate (PHB) in Aerobic Open Mixed Cultures

Katja Johnson, Robbert Kleerebezem, Mark C. M. van Loosdrecht

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ABSTRACT

Mixed microbial cultures enriched in feast-famine sequencing batch reactors (SBRs) can accumulate large amounts of the bioplastic PHB under conditions of ammonium starvation. If waste streams are to be used as a substrate, nutrient starvation may not always be achievable. The aim of this study was to investigate the influence of ammonium on PHB production in the PHB production stage of the process. The biomass was enriched in an acetate-fed (carbon limited) feast-famine SBR operated at 30°C, 1 day sludge residence time and with a cycle length of 12 h. The biomass was used in three fed-batch experiments with medium C/N ratios of ∞ (ammonium starvation), 40 (ammonium limitation) and 8 Cmol/Nmol (ammonium excess) and acetate as the carbon source. Under ammonium starvation the biomass reached a maximum PHB content of 89 wt% after 7.6 h, under ammonium limitation 77 wt% after 9.3 h and under ammonium excess 69 wt% after 4.4 h. PHB contents decreased after these maxima were reached. PHB production slowed down more with time with larger ammonium availability. Growth led to a dilution of the PHB pool after the maximum PHB content was reached. Nutrient starvation seems thus to be the best strategy for maximal PHB production.

Introduction

The production of the microbial storage polymer polyhydroxyalkanoate (PHA) as a bioplastic with open mixed cultures is a promising alternative to processes based on pure cultures (Johnson et al., 2009a). Using acetate as a carbon source, mixed cultures have been shown to accumulate PHA up to 89 wt% of their cell dry weight in fed-batch experiments under conditions of ammonium starvation (Johnson et al., 2009a). Ammonium starvation was chosen to avoid biomass growth and obtain a higher PHA yield. Since mixed culture PHA production is ultimately aiming at using waste streams as a substrate, ammonium starvation (or starvation of any other nutrient essential for growth) during the PHA production step may be difficult to achieve. Previous studies indicated that the presence of an excess of nutrients may have detrimental effects on cellular PHA contents (Serafim et al., 2004; Bengtsson et al., 2008a; Dionisi et al., 2005a). Detailed studies evaluating the extent of this detrimental effect and its underlying causes are missing, but would be important for process design and optimization.

The aim of this study was to investigate in detail the influence of ammonium starvation, ammonium limitation and ammonium excess in fed-batch PHA accumulation experiments on (i) the maximal cellular PHA contents obtainable, (ii) all reaction rates, (iii) all observed yields, and (iv) the adaptation of the culture and changes in reaction rates over time.

The mixed culture used for this study had been enriched for PHA storing microorganisms in a sequencing batch reactor (SBR) by employing a feast-famine strategy under carbon limiting conditions as described previously (Johnson et al., 2009a). Biomass was harvested for each fed-batch experiment at the end of an SBR cycle. The ammonium starvation experiment was performed under absence of ammonium with a pH controlled feed of acetic acid. In the

ammonium limitation experiment a feed of acetic acid and nutrients with a carbon to nitrogen ratio of 40 Cmol/Nmol was supplied continuously via the pH control and in the ammonium excess experiment the feed solution had a carbon to nitrogen ratio of 8 Cmol/Nmol. As reaction rates and biomass concentrations in these fed-batch experiments changed continuously, a metabolic model (Johnson et al., 2009b) was applied to calculate the biomass specific reaction rates.

Materials and Methods

Sequencing batch reactor (SBR) for culture enrichment

The SBR was set up and operated like described in Johnson et al. (2009a). In brief the following conditions were employed: The reactor was fully aerated, the temperature was controlled at 30°C and the pH of the reactor liquid was maintained at 7.0 $\pm$ 0.1. The sludge residence time (SRT) and the hydraulic residence time (HRT) of the SBR were 1 d. Operation of the SBR was based on 12 h batch cycles consisting of a start phase (7 min), an influent phase (10 min) in which 1 l fresh medium was supplied, a reaction phase (683 min), and a biomass withdrawal phase (20 min) in which 1 l mixed reactor liquor were withdrawn. The reactor had been maintained under these conditions for about 1 year and 4 months at the time when the biomass was used for the fed-batch experiments.

The medium for the enrichment reactor was carbon limited and contained acetate as electron donor and carbon source and ammonium as a nitrogen source. Influent concentrations applied were 27 mM $NaAc{\cdot}3H_2O$, 6.75 mM NH_4Cl, 2.5 mM KH_2PO_4, 0.6 mM $MgSO_4{\cdot}7H_2O$, 0.7 mM KCl, 1.5 ml/l trace elements solution according to Vishniac and Santer (1957) and 10 mg/l allylthiourea (to prevent nitrification).

Fed-batch reactor for PHA production

Biomass for each fed-batch experiment was harvested as excess sludge from the SBR at the end of a cycle. The medium of the SBR was adjusted for the cycle of harvest with a lower ammonium content to ensure that all ammonium would be consumed by the time of biomass harvest. A similar set-up as for the culture selection was used, but in a fed-batch mode (Johnson et al., 2009a). The fed-batch reactor was fully aerated, operated at 30°C and pH controlled at 7 $\pm$ 0.1. 1 l of culture from the end of a SBR cycle was mixed with 1 l of acetate- and ammonium-free medium (same composition as for the SBR, but without NaAc and NH_4Cl). To start the PHA production, a pulse of about 60 mmol sodium acetate was fed to the reactor. Further carbon source was supplied automatically with the feed solution via the pH control. In the case of the experiment under ammonium starvation (C/N ratio ∞) the feed solution used for pH control was 1.5 M acetic acid. In the ammonium limitation experiment (C/N ratio 40 Cmol/Nmol) the feed solution consisted of 1.5 M acetic acid supplemented with 75.0 mM NH_4Cl, 27.6 mM KH_2PO_4, 6.2 mM $MgSO_4{\cdot}7H_2O$, 8.0 mM KCl, 16.7 ml/l trace elements solution

according to Vishniac and Santer (1957) and 75.0 mM NaOH. The feed solution for the ammonium excess experiment (C/N ratio 8 Cmol/Nmol) was composed of 1.5 M acetic acid supplemented with 375 mM NH_4Cl, 138 mM KH_2PO_4, 31 mM $MgSO_4 \cdot 7H_2O$, 40 mM KCl, 83 ml/l trace elements solution according to Vishniac and Santer (1957) and 150 mM NaOH. The NaOH addition was applied to compensate for the pH effect of ammonium uptake in the respective experiments.

The fed-batch cultures in the ammonium limitation and excess experiments produced so much biomass over time that it was necessary to dilute the cultures to be able to maintain non-limiting oxygen concentrations in the reactor liquid and for ease of sample treatment. The ammonium limited culture was diluted about 7.3 h after the beginning of the experiment, while the ammonium excess culture was diluted at 6 h, 11.6 h and 15.6 h. When the cultures were diluted about 1 l of reactor liquid was quickly withdrawn and replaced by 1 l of water. In the case of the ammonium excess experiments also a pulse of 30 mmol sodium acetate was added immediately after each dilution.

The progress of the experiments was monitored via online (dissolved oxygen (DO), pH, acid and base dosage, off-gas CO_2 and O_2) and offline (acetate, total suspended solids (TSS), PHA, ammonium) measurements.

All three fed-batch experiments were performed within a time frame of 2 weeks.

Analytical Methods and Data Treatment

Dissolved oxygen in the reactor liquid (DO), pH, acid and base dosage, carbon dioxide and oxygen partial pressures in the gas entering and leaving the reactor, and concentrations of acetate, ammonium, PHA and total suspended solids (TSS) in reactor samples were measured as described previously (Johnson et al., 2009a; Johnson et al., 2009b).

A comprehensive data analysis was carried out with the results obtained. The data analysis is described in detail in Johnson et al. (2009b). In brief, measurements were corrected for the effects of sampling, addition of liquids to the reactor (feed solutions) and the dilutions performed (i.e. results were expressed as if the reactors had not been diluted). Oxygen uptake and carbon dioxide evolution were determined from off-gas and DO measurements. Inorganic carbon dissolved in the liquid phase was considered when computing carbon dioxide evolution. Balances for carbon and electrons were calculated from the measured conversions at every sampling point. Finally, the available knowledge of the stoichiometry and kinetics of the PHA production and consumption metabolism was employed in the form of a metabolic model in order to compute the best estimates for all conversions and reaction rates from the measurements while complying with carbon and electron balances.

Results

General observations

The microbial culture enriched in a feast-famine SBR (Johnson et al., 2009a) was used for three fed-batch experiments under different conditions of nitrogen availability ranging from ammonium starvation to limitation and excess. The polymer produced from acetate was the homopolymer polyhydroxybutyrate (PHB). The PHB contents reached over time in these three experiments are displayed in Figure 6.1. During roughly the first hour of the experiments the biomass accumulated similar amounts of PHB in all three experiments, but subsequently the PHB contents differed. The biomass that had not been supplied with any ammonium reached a maximum PHB content of 89.2 wt% after 7.6 h, and PHB contents only declined very slightly after reaching the maximum. The biomass which was supplied with a limiting amount of ammonium throughout the experiment reached a maximum of 77.4 wt% PHB after 9.3 h, with PHB contents decreasing after this point. The maximum PHB content reached by the biomass that was supplied with an excessive amount of ammonium was 69.0 wt% after 4.4 h. The PHB contents declined rapidly in this biomass after the maximum PHB content was reached. The presence of ammonium had thus a significant influence on the maximum PHB contents reached by the mixed culture and also on the time point when this occurred.

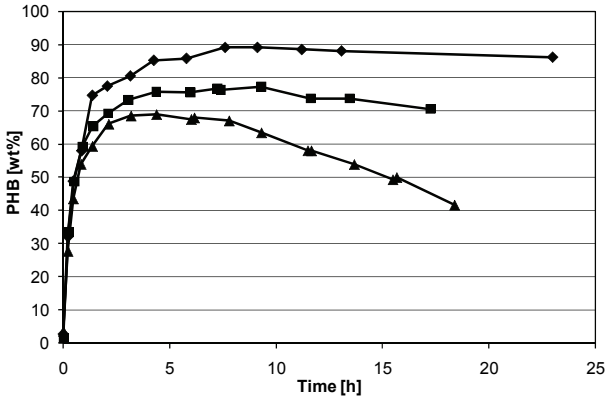

Figure 6.1. Cellular PHB contents over time in fed-batch PHB production experiments with ammonium starvation (◆), ammonium limitation (■), and ammonium excess (▲).

In order to understand the different behaviours of the mixed culture under these three different conditions, we applied the previously described metabolic model (Johnson et al., 2009b) to calculate all reaction rates at any time point by fitting the model to the measurements. The published metabolic model appeared to be only useful to model the experiments up to the time point of maximum PHB contents. The model had to be adjusted to be able to model the observed decline of PHB contents.

Presence of ammonium

From our observations it appeared that the PHB contents decreased after some time in experiments with presence of ammonium (ammonium limitation and excess). This was apparently not through PHB degradation but due to a slowdown of PHB production and continued growth. Effectively the PHB pool was diluted by newly produced biomass. The slowdown of PHB production appeared to depend on the length of time of presence of ammonium. We extended therefore the previously used kinetic description of PHB production (Johnson et al., 2009b) for the two experiments where ammonium was present by a time dependent inhibition factor (second set of brackets) similar to the inhibition term already used to model the inhibition of PHB production at high PHB contents (first set of brackets):

$$\tilde{q}_{PHB,2}^{feast}(t) = \tilde{q}_{PHB}^{\max} \cdot \frac{\tilde{c}_{Ac}(t)}{K_{Ac} + \tilde{c}_{Ac}(t)} \cdot \left[1 - \left(\frac{\tilde{f}_{PHB}(t)}{\tilde{f}_{PHB}^{\max}}\right)^{\alpha}\right] \cdot \left[1 - \left(\frac{t}{\tilde{t}^{*}}\right)^{\beta}\right] \quad (6.1)$$

With $\tilde{q}_{PHB,2}^{feast}$ the modelled biomass specific PHB production rate, t the time of simultaneous presence of ammonium and acetate, $\tilde{q}_{PHB}^{\max}$ the modelled maximum biomass specific PHB production rate, $\tilde{c}_{Ac}$ the modelled concentration of acetate, K_{Ac} the half saturation constant for acetate, $\tilde{f}_{PHB}$ the modelled fraction of PHB in biomass, $\tilde{f}_{PHB}^{\max}$ the modelled maximum fraction of PHB in biomass, α and β the exponents of the two inhibition terms, and $\tilde{t}^{*}$ the modelled time at which PHB production would not occur anymore. PHB fractions are expressed as carbon-moles PHB per carbon-mole 'active' biomass, with 'active' biomass being the total biomass without PHB. Growth and maintenance were modelled with their own rate descriptions basically as constant rates, and the acetate uptake rate followed with stoichiometry according to a Herbert-Pirt type of relation from these three reactions (Johnson et al., 2009b). Effectively this model assumes that the acetate uptake rate of the bacteria is a resultant of the three acetate consuming processes of growth, PHB production and maintenance.

This kinetic model yielded a good fit of the data sets for the two experiments where ammonium was present. Figures 6.2 B and C show that the model can describe the measured amounts and conversions of all compounds. Figures 6.2 E and F show the most important modelled reaction rates for these two experiments. In both experiments PHB production started with a high rate, but decreased as initially higher PHB contents inhibited PHB production and later on the length of time of simultaneous presence of ammonium and acetate inhibited PHB production. Acetate uptake and carbon dioxide evolution rates followed the trends of the PHB production rates, while the growth rates were constant. Figures 6.2 E and F also display the ratios of the carbon consuming reaction rates over the acetate uptake rate. These ratios are effectively the observed yield coefficients at each time point. Particularly under conditions of ammonium excess observed biomass yields increased with time while PHB yields decreased.

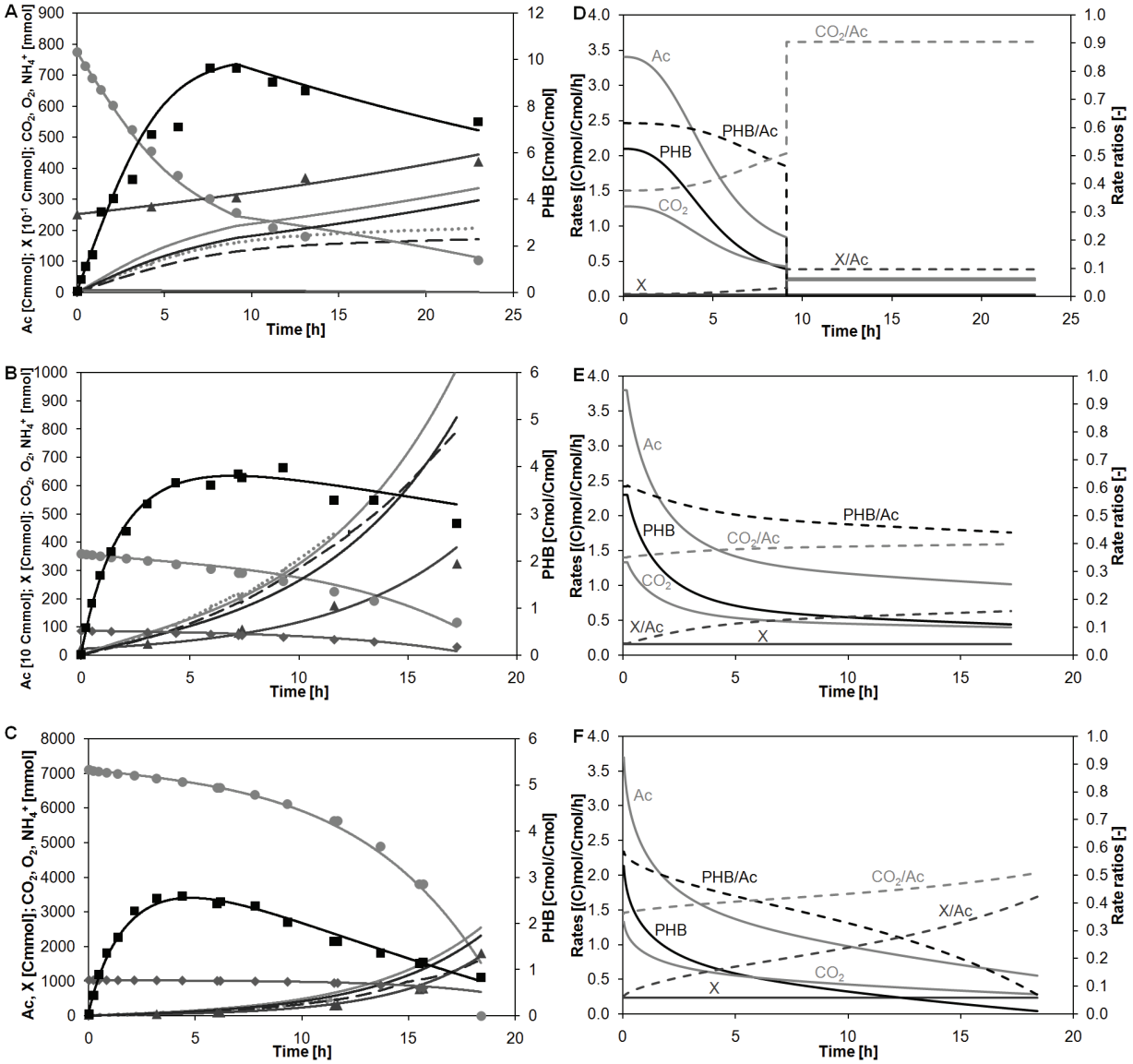

Figure 6.2. Modelled amounts (**A**-**C**) and biomass specific reaction rates (**D**-**F**) during fed-batch experiments with ammonium starvation (**A**, **D**), ammonium limitation (**B**, **E**), and ammonium excess (**C**, **F**). **A**-**C**: Solid lines represent modelled values corresponding to the respective symbols indicating measurements. (——) cumulative oxygen uptake; (┈┈) cumulative carbon dioxide evolution; (●) acetate; (▲) active biomass (X); (◆) ammonium; (■) fraction of PHB (PHB per active biomass). Acetate and ammonium were treated for modelling purposes as if the total amount had been fed at the beginning of the experiment. **D**-**F**: (—) biomass specific carbon dioxide evolution rate; (—) biomass specific acetate uptake rate; (—) biomass specific growth rate; (**—**) biomass specific PHB production rate; (– –) ratio of carbon dioxide over acetate uptake rate; (– –) ratio of growth over acetate uptake rate; (**– –**) ratio of PHB production over acetate uptake rate.

The kinetic parameters required to fit the model to the measurements are summarized in Table 6.1. These parameters should not be seen as absolute values but only be interpreted in the context of the used model. The PHB rate equation (equation 6.1) contains so many parameters that there is not necessarily just one good set of parameters. Parameters like maximum fraction of PHB and maximum PHB production rate are not representing the actual measured maximum

values in the two experiments with ammonium, but have to be chosen higher for a good fit, because the time inhibition term will lower the overall rate considerably. The maximum growth rate was lower in the ammonium limited experiment than in the ammonium excess experiment, since growth was limited by the ammonium feeding rate for most of the ammonium limited experiment. The time at which PHB production was estimated to cease completely was with almost 20 h much earlier under conditions of ammonium excess than under ammonium limitation (about 50 h, $\tilde{t}^*$ in Table 6.1).

Table 6.1. Estimated model parameters of the three fed-batch PHB production experiments and the steady state SBR for biomass production.

Parameters	NH_4^+ starvation	NH_4^+ limitation	NH_4^+ excess	SBR
$\tilde{q}_{Ac}^{\max}$ [Cmol/Cmol/h]	-3.41	-3.80	-3.70	-3.41
$\tilde{\mu}^{\max}$ [Cmol/Cmol/h]	0.025	0.163	0.234	0.051[a]
$\tilde{q}_{PHB}^{\max}$ [Cmol/Cmol/h]	2.10	3.00	3.30	-
$\tilde{f}_{PHB}^{\max}$ [Cmol/Cmol]	10.6	7.2	10.0	-
α [-]	2.6	1.0	1.4	-
$\tilde{t}^*$ [h]	-	49.7	19.8	-
β [-]	-	0.30	0.17	-
m_{ATP} [mol/Cmol/h]	0.615	0.000	0.000	-

a: in feast phase; maximum growth rate in famine phase was 0.210 Cmol/Cmol/h

Absence of ammonium

In the ammonium starvation experiment two observations were made that could not be modelled with the previously described metabolic model (Johnson et al., 2009b): Firstly, the PHB content decreased after a maximum PHB content was reached. This is more visible when considering the fraction of PHB (Figure 6.2 A) than when considering weight percentages (Figure 6.1). Secondly, the biomass concentration increased in this experiment indicating growth, although no ammonium was available (ammonium concentration was 0.003 mmol/l at the beginning of the experiment and no ammonium was supplied with the medium). It would have been possible that the nitrogen content of the biomass was not constant and actually decreased. Growth might explain why PHB contents decreased at the end of the experiment.

A decreasing nitrogen content of the biomass with time was difficult to model since we did not measure the change of the actual nitrogen content of the biomass with time. The small amount of growth observed was therefore modelled by allowing some growth in the model. This enabled us to fit the change in biomass concentration.

The decrease in PHB contents could not be satisfactorily modelled with a time inhibition factor as above (equation 6.1). A good fit of the measurement was only obtained when setting the PHB production rate to zero once the maximum measured PHB content was reached:

$$\tilde{q}_{PHB,2}^{feast}(t) = \tilde{q}_{PHB}^{\max} \cdot \frac{\tilde{c}_{Ac}(t)}{K_{Ac} + \tilde{c}_{Ac}(t)} \cdot \left[1 - \left(\frac{\tilde{f}_{PHB}(t)}{\tilde{f}_{PHB}^{\max}} \right)^{\alpha} \right] \quad \text{if} \quad \tilde{f}_{PHB}(t) \le f_{PHB}^{\max} \qquad (6.2a)$$

$$\tilde{q}_{PHB,2}^{feast}(t) = 0 \quad \text{if} \quad \tilde{f}_{PHB}(t) > f_{PHB}^{\max} \qquad (6.2b)$$

With $f_{PHB}^{\max}$ the maximum measured fraction of PHB. This approach did result in a good fit of all measurements (Figure 6.2 A), although the transition from equation 6.2a to 6.2b is not smooth as can be seen from the modelled reaction rates (Figure 6.2 D). After the maximum PHB content was reached maintenance was the dominating acetate consuming process, as growth was only contributing at a very low rate (see also Table 6.1). While maintenance was estimated to be negligible in the two experiments with presence of ammonium, this process does appear to require a significant amount of energy under conditions of ammonium starvation (Table 6.1).

Discussion

Influence of ammonium on PHB production

Our experiments show that the presence of ammonium had a negative effect on the maximum PHB content reached in fed-batch experiments for PHB accumulation. The storage capacity of the culture can be expected to be the same in all three fed-batch experiments; the culture was harvested from the same steady state enrichment reactor within a short time frame. The reason for the lower PHB contents in the experiments with presence of ammonium lies in the dilution of PHB through biomass growth. Biomass specific PHB production rates were lower the more ammonium was present, while growth rates were higher, leading to a dilution of the cellular PHB content.

The relation between biomass PHB content and rates of PHB production, acetate uptake and respiration for different levels of ammonium availability are shown in Figure 6.3. The rates are plotted over the PHB content since the PHB content influences the PHB production rate, and biomass specific rates can therefore best be compared at the same PHB content (Johnson et al., 2009b). The rates of PHB production, acetate uptake and respiration showed a similar behaviour: they started at high rates at the beginning of the three experiments and decreased much faster in experiments with ammonium than in the experiment without ammonium. Under conditions of ammonium starvation the PHB production rate decreased with time presumably due to an inhibition through increasing PHB contents. With less PHB being produced also less acetate and oxygen needed to be taken up, leading to decreasing rates. The rate of PHB production seems to be the rate controlling step.

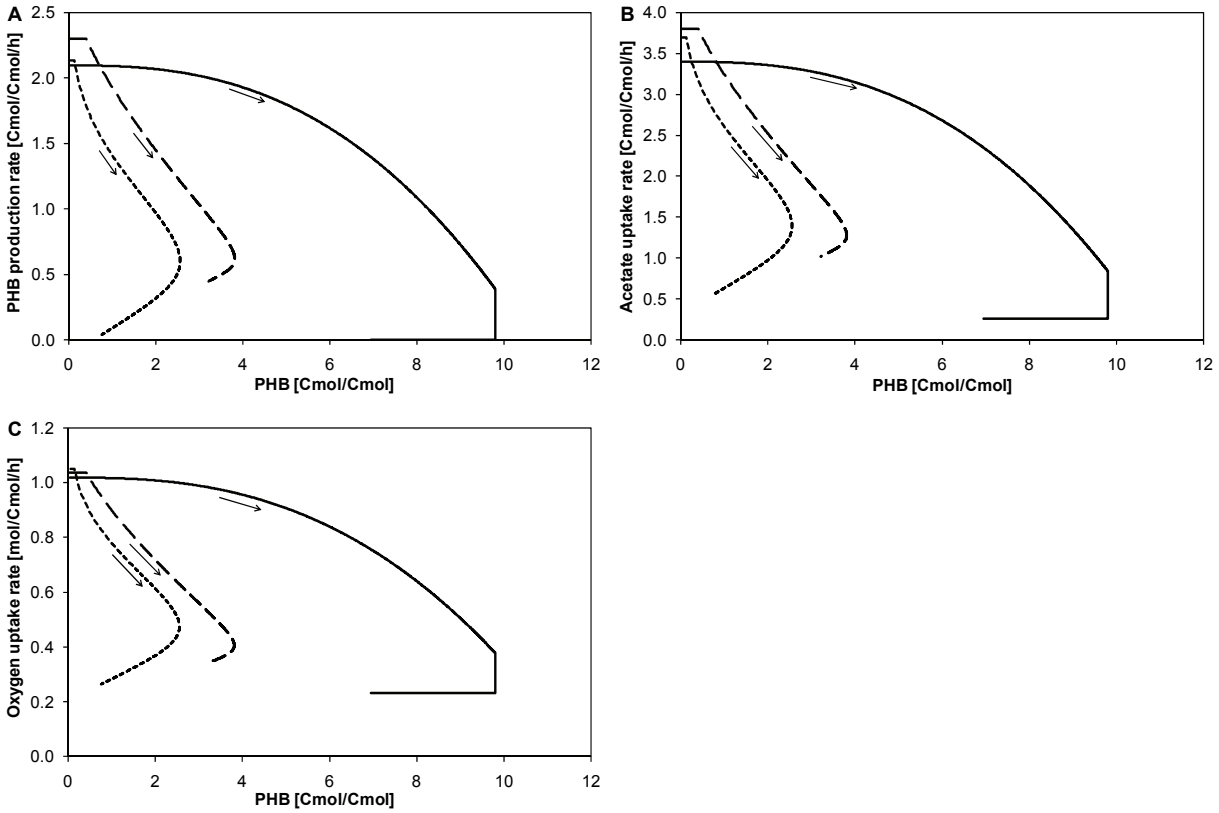

Figure 6.3. Selected biomass specific rates over fraction of PHB in fed-batch experiments with ammonium starvation (—), ammonium limitation (– –), and ammonium excess (- - -). Arrows indicate the direction of time. **A**: PHB production rate. **B**: Acetate uptake rate. **C**: Oxygen uptake rate.

In the two experiments with ammonium, the rate of PHB production decreased faster than in the ammonium starvation experiment. PHB production was thus inhibited or limited by another biochemical regulation instead of or additionally to the effect of increasing PHB contents. This limitation was, however, not caused by growth requiring a large amount of oxygen and acetate. The rate of acetate uptake and respiration could have proceeded faster in these experiments like in the ammonium starvation experiment. It was thus not the rate of acetate or oxygen uptake that limited PHB production. In the presence of sufficient carbon source and nutrients for growth the cells appeared to actively downregulate or inhibit PHB production more and more with time.

Interestingly the fraction of acetate used for PHB production and thus the observed PHB yield throughout the ammonium limitation experiment was comparable to the first part of the ammonium starvation experiment (ratio of PHB production over acetate uptake in Figures 6.2 D and E). The ammonium starved biomass seemed to 'waste' similar amounts of acetate for maintenance as the ammonium limited culture 'wasted' for growth. However, since biomass production dilutes PHB while maintenance does not, ammonium starvation leads to higher PHB contents and is thus the best strategy for efficient PHB production.

PHA accumulation experiments reported in literature for similar cultures with different amounts of ammonium are usually batch experiments rather than fed-batch experiments (e.g.

Serafim et al., 2004; Albuquerque et al., 2007; Bengtsson et al., 2008a; Dionisi et al., 2005a). If ammonium was not supplied in ample excess at the beginning of the experiment, biomass experienced a phase of ammonium excess in the beginning of the experiment followed by a brief period of ammonium limitation and finally ammonium starvation. Also the carbon source was in some experiments only present for a short time. It is therefore difficult to compare these experiments with our fed-batch experiments, particularly with the ammonium limitation experiment. Generally the trend from these experiments seems to be that ammonium starvation leads to higher biomass specific PHB production rates and PHB contents than an excess of ammonium (Serafim et al., 2004; Bengtsson et al., 2008a), which is in agreement with our observations. Dionisi et al. (2005a) performed batch experiments without and with an excess of ammonium and reported no increased storage rate under ammonium starvation, but also higher PHA contents. However, the compared storage rates appear to be volumetric rates rather than biomass specific rates. Considering that more biomass will have been present in the ammonium excess experiment due to growth, the biomass specific rates were probably also here higher for the ammonium starved biomass.

Long-term batch experiments with presence of ammonium and carbon source were reported by Dionisi et al. (2001; 2004; 2005a), which are comparable to our ammonium excess experiment. Dionisi et al. reported PHA storage to be initially the main process, with storage rates decreasing with time and finally growth becoming the dominating process and PHA contents decreasing. Figure 6.2 F shows that also in our experiment PHB storage initially consumed the majority of acetate, but since PHB production rates decreased, the fraction of acetate used for PHB production decreased with time while the fractions used for growth increased. As discussed above, this change seems to be only due to the change in PHB production rate, while the growth rate appears to be constant.

Maximum growth rate

While biomass subjected to an excess of acetate and nutrients seemed to downregulate PHB production with time, there was no indication from our experiments that the maximum growth rate was noticeably adjusted within the time frame of our experiment (18 h) – ammonium and biomass measurements could be described well with a constant growth rate in the ammonium excess experiment.

When comparing the three fed-batch experiments with the feast phase of the (carbon limited) steady state SBR (results reported in Johnson et al. (2009a)) it is striking that the maximum growth rates in the two fed-batch experiments with ammonium were significantly higher than in the SBR feast phase (Table 6.1). However, this SBR culture was rather extraordinary, as it grew with a much higher growth rate at the beginning of the famine phase than during the feast phase, i.e. the modelled maximum growth rate of the feast phase is not actually the maximum rate at which the culture can grow. This phenomenon has been discussed in Johnson et al. (2009b). The maximum growth rate estimated for the famine phase of the SBR was with

0.210 Cmol/Cmol/h similar to the growth rate observed in the ammonium excess fed-batch experiment (0.234 Cmol/Cmol/h, Table 6.1), which appears to be the actual maximum growth rate of the culture.

The feast phase in the SBR lasted just over 40 minutes. Conditions in the ammonium excess fed-batch experiment were similar to a SBR feast phase and the behaviour of the culture could thus be expected to be similar in the first 40 minutes. However, when calculating growth rates in the fed-batch experiment from the ammonium measurements, no evidence could be found for a lowered growth rate at the beginning of the experiment in contrast to the feast phase in the SBR cycle. It should be noted that the history of the biomass was slightly different in the fed-batch experiment compared to the normal SBR feast phase: the SBR feast phase biomass came out of a famine phase with an excess of ammonium (1.2 mmol/l), while the biomass used for fed-batch experiments was fed with a medium with reduced ammonium content in the SBR cycle previously to biomass harvest in order to start all three fed-batch experiments without any ammonium present. In the ammonium excess experiment the amount of ammonium left from the SBR cycle at the beginning of the fed-batch experiment was indeed near zero (0.003 mmol/l). The biomass had thus experienced ammonium starvation just before being harvested for the fed-batch experiment. It could thus be speculated that the experience of ammonium starvation might have triggered a fast ammonium uptake rate in the subsequent fed-batch experiment with ammonium in excess. We observed also in SBRs that were continuously supplied with an ammonium limited medium that the biomass could take up ammonium (and presumably grow) immediately with a high rate in the feast phase after experiencing a phase of ammonium starvation in the famine phase (Johnson et al., under review, Water Research / Chapter 3). Whether the high ammonium uptake rate in the fed-batch experiment can indeed be interpreted as a high growth rate after such a significant change in ammonium supply remains to be investigated.

Also the reason for the lower observed growth rate in the SBR feast phase requires further investigation. Curiously, a lower growth rate in the SBR feast phase might provide a competitive advantage for this culture in the SBR. PHB production was a far faster process in this culture than growth. In a feast-famine SBR where cells compete for acetate, it is the best strategy to store PHB rather than to grow in the feast phase, since PHB storage enables the cells to take up acetate faster. On the other hand, in a fed-batch reactor where cells are continuously under unlimited growth conditions, competition will ultimately be based on growth and not on the acetate uptake rate. It makes therefore sense for the cells to stop PHB production and concentrate on growth.

Implications for mixed culture PHA production from waste streams

Our results and also those reported in literature lead to the conclusion that PHA production with feast-famine mixed cultures is best performed under conditions of nitrogen starvation (or possibly starvation of another nutrient such as phosphorus). Nutrient starvation leads to the

highest PHA contents, which is important for efficient downstream processing. Moreover, PHA production rates and yields are high.

We found in a separate study that for the first step of the process, the culture enrichment for PHA producing organisms, carbon limitation is the best strategy (Johnson et al., under review, Water Research / Chapter 3). If a PHA production process was to be designed based on using (fermented) waste streams as a substrate for PHA production, two different kinds of waste streams would need to be used for optimal results: a carbon limited stream in the culture enrichment step and a nutrient limited stream in the PHA production step. Or one nutrient limited stream could be used for both, but would need to be supplemented with nutrients for the culture enrichment step, which could be costly. Lab studies using molasses, paper mill waste water and olive oil mill effluents are promising, but nutrient addition was required for efficient culture enrichment (Albuquerque et al., 2007; Bengtsson et al., 2008a; Beccari et al., 2009). The choice and availability of appropriate waste streams and nutrients will therefore be a challenge for large scale mixed culture PHA production from waste streams.

Conclusions

A mixed culture was enriched that is able to store up to 89 wt% of PHB under ammonium starvation. Starvation of a nutrient such as ammonium appears to be the best strategy in a fed-batch process for PHA production in terms of the highest PHA content, production rate and a high yield.

When sufficient nutrients and carbon source for growth were available during the fed-batch stage for PHA production, the biomass appeared to downregulate or inhibit PHB formation with time. In this case growth became the dominant process with time. The maximum growth rate did not appear to be adjusted under unlimiting growth conditions in the time frame of 18 h.

For efficient mixed culture PHA production based on waste streams as substrates, the stream used for culture enrichment should preferably be carbon limited, while the stream for PHA production should be nutrient limited.

Nomenclature

$\widetilde{c}_{Ac}$	Modelled concentration of acetate
$f_{PHB}^{\max}$	Maximum measured fraction of PHB
$\widetilde{f}_{PHB}$	Modelled fraction of PHB in biomass
$\widetilde{f}_{PHB}^{\max}$	Modelled maximum fraction of PHB in biomass
K_{Ac}	Half saturation constant for acetate
m_{ATP}	Maintenance ATP requirement
$\widetilde{q}_{Ac}^{\max}$	Modelled maximum biomass specific acetate uptake rate

$\widetilde{q}_{PHB}^{\max}$	Modelled maximum biomass specific PHB production rate
$\widetilde{q}_{PHB,2}^{feast}$	Modelled biomass specific PHB production rate
t	Time of simultaneous presence of ammonium and acetate
$\widetilde{t}^{*}$	Modelled time at which PHB production would not occur anymore
α	Exponent of inhibition term
β	Exponent of inhibition term
$\widetilde{\mu}^{\max}$	Modelled maximum biomass specific growth rate
DO	Dissolved oxygen
HRT	Hydraulic residence time
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
SBR	Sequencing batch reactor
SRT	Sludge residence time
TSS	Total suspended solids

CHAPTER 7

General Discussion

General Discussion

When considering all the results obtained during this PhD study, a few interesting points remain to be discussed that concern mixed culture PHA production in general. A point that leads often to discussions is the definition and existence of steady state for this kind of mixed cultures. Another point relates to the actual subject of this thesis – the key parameters which influence the PHA storage capacity of a mixed culture. Further the issue of up-scaling of the mixed culture process will be considered. Finally suggestions for future research are made at the end of this chapter.

Steady state in PHA producing SBRs with open mixed cultures

In most of our SBR studies it was defined that steady state was reached when for at least five days (i) the concentration of total suspended solids (TSS) at the end of the cycle, (ii) the measured sludge residence time (SRT) and (iii) the length of the feast phase as indicated by the dissolved oxygen (DO) changes were constant. This definition of steady state relates to operational stability, not microbial stability, with the purpose to compare different SBR runs at a similar stage of operational development. The purpose was not to find a true long-term steady state, where no changes would be observable over a much longer period of time (e.g. months or years). A true long-term steady-state may not exist for this kind of microbial communities. The continuous selective pressure will likely always lead to changes in the microbial composition and performance of the reactor. Even if a pure culture was obtained, mutations and continued selection would probably lead to significant changes with time.

If different operational conditions for SBR operation are to be compared, a practical definition of the time point at which SBR cycle measurements can be made is needed. We found that our steady state definition was suitable for judging the impact of operational parameters on reactor behaviour and comparing different SBRs. Observed yields and reaction rates showed significant differences between different operating conditions at the point that the SBRs were stable over a period of at least 5 days.

If also the impact of operational parameters on microbial parameters such as the maximum PHA storage capacity is to be investigated, longer time frames of reactor operation may be required. For example it was found that a nitrogen limited culture operated at 0.5 d SRT showed a stable reactor operation over several months judging from the length of the feast phase, but the culture's PHB storage capacity decreased from about 2 to 0.1 Cmol/Cmol biomass within 3.5 months (Chapter 3). A carbon limited culture on the other hand showed an improvement of the maximum PHB production capacity with time when a SBR was operated for a long time (> 1 year) under the same conditions (Chapter 5). It remains thus difficult to define at which time point a sufficient microbial stability is reached that will allow to evaluate the impact of operational parameters on maximal PHA storage capacities. It may be useful to measure the maximal PHA storage capacity at the point when operational SBR stability is reached and again a certain number of SRTs later, to investigate at least whether the PHA

storage capacity decreases, increases or remains unchanged. Considering that a true steady state might never be reached, such a pragmatic approach would at least allow to observe a trend.

Factors influencing maximum PHB contents

The maximum cellular PHB content in acetate-fed feast-famine cultures reported previously was 65 wt% which is equivalent to a PHB fraction of 2.2 Cmol/Cmol (PHB per active biomass) (Serafim et al., 2008a). The culture enriched for this thesis (Chapter 5 and 6) was able to store up to 89 wt% PHB which is a PHB fraction of 9.6 Cmol/Cmol, so more than 4 times as much PHB per amount of biomass. A remaining question is thus which key parameters led to the latter extraordinary culture with such a high PHB storage capacity.

Serafim et al. (2004), who reached the 65 wt% PHB, worked in the SBR enrichment step with a longer SRT, the same cycle length, varying pH, a lower reactor temperature and with a different limitation compared to our most successful SBR conditions (Table 7.1). For PHB production Serafim et al. (2004) used a different feeding strategy, ammonium was present for longer, the pH was variable and the temperature was lower compared to our most successful fed-batch experiment (Table 7.1). There are thus differences in most process parameters and it is difficult to identify the most relevant parameters simply from this comparison. We investigated the influence of individual parameters on the PHB production in this thesis (Chapter 3 and 4) and the results may help identify the key parameters required for process optimization.

Table 7.1. Comparison of experimental conditions for PHB production.

	Serafim et al. (2004)	**This study (Chap. 5 & 6)**
SBR operation		
SRT [d]	10	1
SBR cycle length [h]	12	12
pH [-]	not controlled	7
Temperature [°C]	22	30
Limiting substance	nitrogen source	carbon source
Fed-batch reactor operation		
Feeding strategy	pulse feeding	continuous feeding
Presence of nitrogen source [h]	1	0
pH [-]	not controlled	7
Temperature [°C]	22	30
Max. PHB content [wt%]	65	89
Max. PHB fraction [Cmol/Cmol]	2.2	9.6

When comparing nitrogen and carbon limited SBR cultures at different SRTs ranging from 0.5 – 4 days, we found that the SRT within this range did not have an effect on the maximum PHB storage capacity of the cultures, but nitrogen limitation in the SBR did have a negative impact in most cases (Chapter 3). The SBRs in this study were operated with 4 h cycle length and at 20°C

and the maximum storage capacity ranged from PHB fractions of about 1.5 to 3 Cmol/Cmol, with the highest values reached by cultures that were carbon limited in the SBR.

When the cycle length of an SBR operated at 1 d SRT with carbon limitation and a reactor temperature of 20°C was increased from 4 h to 12 h, we observed that the reactor took a long time (about 4 months) to reach steady state. The steady state with 12 h cycle length was characterized by a much higher acetate uptake rate compared to the 4 h steady state (1.17 versus 0.63 Cmol/Cmol/h) and a higher PHB production rate (0.66 versus 0.35 Cmol/Cmol/h) (unpublished results). Also PHB contents reached at the end of the feast phase were significantly increased in the SBR with longer cycles (52 versus 14 wt%) (unpublished results). The culture from the SBR with 12 h cycles had an increased PHB storage capacity of up to 3.2 Cmol/Cmol even with ammonium limitation (continuous presence of ammonium at low concentrations) in the fed-batch stage (unpublished results). A fed-batch experiment with ammonium starvation was conducted but PHB analysis unfortunately failed in this experiment, however, TSS samples and material balances indicate that the culture may have stored more than 80 wt% PHB (>4.5 Cmol/Cmol) (unpublished results).

Considering these results it seems that the combination of a long SBR cycle length with a short SRT (small number of cycles per SRT) and carbon limitation is one of the keys for improved selection for high capacity PHB storing bacteria. The competition for acetate was apparently stronger in the SBR with the longer cycle length, since acetate uptake rates increased considerably. The culture was also forced to store a larger amount of PHB at a high rate to be able to grow throughout the longer famine phase. Organisms with a small PHB storage capacity and low acetate uptake rate were therefore probably eliminated from the system. The low SRT contributes to this selective pressure by demanding a high amount of biomass production per cycle and thus also a high amount of PHB storage. Carbon limitation in the SBR ensures that the culture can produce the majority of the new biomass in the famine phase, promoting PHB storage further, while nitrogen limitation would lead to more growth in the feast phase and hence less need for PHB storage.

The PHB storage capacity of the SBR culture was probably further increased by increasing the reactor temperature to 30°C. A SBR of 4 h cycle length, 1 d SRT and operated under carbon limitation at 30°C, which was inoculated from the 20°C SBR with 12 h cycle length, produced a culture with a PHB storage capacity of about 6 Cmol/Cmol (Chapter 4). Higher reactor temperatures during the SBR enrichment led to higher reaction rates and a change in competition and selective pressure in favour of fast PHB storing organisms. The reactor temperature is thus likely also a key parameter in selecting high capacity PHB storing bacteria.

Another important factor is time: we found that the storage capacity of a culture maintained under the same conditions in the SBR (1 d SRT, 12 h cycle length, carbon limited, 30°C) over more than a year increased its storage capacity from initially less than 80 wt% (4.5 Cmol/Cmol) to almost 90 wt% (9.6 Cmol/Cmol) within that time (Chapter 5).

From the results obtained so far it seems thus that a combination of a fairly low SRT with a long cycle length, carbon limitation, a slightly elevated temperature of 30°C and a long operation time is a good strategy for enriching efficient PHB storing bacteria with a high PHB storage capacity.

The optimization of the culture enrichment step is clearly of major importance for the enrichment of a high capacity PHB storing culture. But it is also relevant to ensure optimal conditions in the PHB production step in order to reach the maximum PHB storage capacity of the enriched culture. We have compared pulse feeding of acetate with continuous feeding for the same SBR culture and found no differences in PHB production rates and maximum PHB contents reached in fed-batch experiments (unpublished results). The temperature at which the PHB production was performed influenced PHB production rates, but not the maximum PHB content for the same culture (Chapter 4). A factor that does have a major negative impact on reaching the maximum PHB contents in fed-batch experiments is the presence of all essential nutrients (Chapter 6). Nutrient starvation seems to be the most important factor in this step.

By optimizing the discussed parameters in the culture enrichment and PHB production step we were able to improve PHB storage capacities and specific production rates considerably.

Considerations for up-scaling and practical implementation

Before the developed process can be scaled-up, volumetric productivities need to be optimized. The biomass concentrations applied in the lab scale experiments were very low, often below 1 g/l of active biomass. This means that also the volumetric PHA productivities and volumetric PHA concentrations were quite low, despite of the high specific PHB production rates. It will thus be a challenge to improve volumetric productivities in the future by increasing biomass concentrations, thereby making the system more economically viable. Biomass concentrations in the SBR can simply be increased by supplying more substrate per SBR cycle. The true limitation particularly in larger scale will then likely lie in the transfer of oxygen into the system – oxygen uptake rates in the feast phase can be as high as 0.9 mol/Cmol/h. Assuming a reasonable maximum oxygen transfer rate of 0.18 mol/l/h (van 't Riet et al., 1991) this would mean a maximum biomass concentration of about 5 g/l if the extremely high rates observed in lab scale could be maintained in large scale. Since these high oxygen uptake rates only occur briefly, i.e. during the feast phase and at the beginning of the fed-batch experiment (unless growth is permitted), it might be possible to allow oxygen limitation during these stages in order to be able to work at higher biomass concentrations. However, if oxygen becomes too limiting, the feast-famine culture may change to a culture dominated by phosphate or glycogen accumulating organisms (Mino et al., 1998). The influence of oxygen limitation on PHB production in feast-famine cultures would therefore be another important parameter to investigate in the future.

Assuming a maximum biomass concentration of 5 g/l and an average biomass specific PHB production rate of 1.2 g/g/h (Chapter 5) for the mixed culture PHB production process, a volumetric PHB productivity of up to 6 g/l/h could be reached in the fed-batch step. In pure cultures of recombinant bacteria a volumetric productivity of 2.5 g/l/h has been reported (Anon., 2002). The biomass concentrations in pure culture processes are considerably higher than the 5 g/l assumed for the mixed culture process, however, the specific rate of PHB production is lower. While volumetric productivities of the mixed culture process could thus be optimized to exceed those of pure culture processes, the amount of PHA per reactor liquid volume would be lower due to the lower biomass concentrations. This will require a relatively larger amount of water to be removed during downstream processing in the mixed culture process. A settling step could be used as a first step for concentrating the biomass.

For scaling-up of the mixed culture PHA production step potentially larger reactors could be used than for the pure culture process, since the mixed culture process is an open unsterile process. The reactors could be much simpler, similar to those used in wastewater treatment. Reactor sizes would, however, be limited by the oxygen mass transfer that can be achieved with a reasonable amount of energy expenditure and possibly by the heat transfer area if heating or cooling was needed.

An important aspect is also the selection of a suitable waste stream as a substrate for the mixed culture PHA production process. Apart from a suitable composition (see Chapter 3 and 6) the waste stream needs to be available in large enough amounts without to much variation in supply throughout the year.

Suggestions for future work

One parameter which still requires investigation is the impact of the reactor pH. One option is to control the pH at different values and to compare the obtained steady states and maximum PHA production capacities in order to find the optimal pH for the enrichment step. Another option is to investigate if the SBR could be operated without pH control, i.e. with varying pH. It would be an advantage if the SBR could be operated without pH control, since the costs for acid and base solutions for pH control could be saved. If the effluent from an anaerobic reactor producing fatty acids was used as a substrate, the pH of the feed stream would be fairly low (Temudo, 2008). The mixed culture in the aerobic SBR would need to tolerate low pHs at least for a short time. With the uptake of fatty acids and conversion into PHA and biomass, the pH would increase during the feast phase (Serafim et al., 2004).

Another parameter that requires investigation is the effect of oxygen limitation during the feast phase. If biomass densities are to be increased in order to improve volumetric productivities, oxygen limitation will likely be a problem as discussed above. If reactors are operated with a strictly anaerobic feast phase, likely phosphate or glycogen accumulating organisms will be enriched (Mino et al., 1998; Reis et al., 2003). But it is not completely clear what will happen under oxygen limiting conditions. Satoh et al. (Satoh et al., 1998) investigated the effect of

oxygen limitation (microaerophilic conditions) on cultures originally enriched under anaerobic/aerobic conditions and found an improvement of PHA contents. A similar study would be useful for aerobic feast-famine cultures.

In this thesis acetate has been used as the sole carbon source, leading to pure PHB. Pure PHB is not a very versatile material; co-polymers with other monomer units usually have more desirable properties. It would thus be useful to use other pure fatty acids or alcohols and mixtures thereof as a substrate in fed-batch experiments in order to investigate what kind of polymers could be produced. The next step would then be to enrich the SBR culture with a different sole carbon source or mixtures of different carbon sources. Finally the aim would be to use real (fermented) wastewaters.

Further points that require research are the scale-up of the process and identification of suitable waste streams.

A subject that might be of interest for researchers working on pure cultures of natural PHA producers could be to investigate if growing pure cultures in a feast-famine SBR might help to improve the rate of PHA production in these cultures. Usually these cultures are grown up to the desired amount of biomass for full scale production in several (fed-)batch steps in which only growth is required. If these cultures would be grown in steps of feast-famine SBRs, they could potentially be better 'prepared' for the final PHA production step, i.e. the cell metabolism might be better optimized for PHA production. If such pure culture feast-famine SBRs could be operated without contamination for a long time, the pure culture could possibly also be optimized for more efficient PHA producers through spontaneous mutations and natural selection.

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Curriculum Vitae

Katja Johnson was born as Katja Schmid in Peine, Germany, on 19 December 1976. In 1996 she finished her school education at Gymnasium Groß Ilsede and moved on to study process engineering at Hamburg University of Technology. During this time she did a research internship at the Institute of Biotechnology I at Hamburg University of Technology on anaerobic treatment of wastewater from the fish industry and another research internship at Unilever Research in Vlaardingen, the Netherlands, on improving the process for the production of extra virgin olive oil. She finished her studies with a thesis on examination of transport mechanisms in immobilised cell culture systems and graduated as Diplom-Ingenieur in 2001.

Katja worked for about 2.5 years at Danisco in Niebüll in the very north of Germany as a research and development project manager for probiotic cultures before moving to Delft University of Technology, department of Biotechnology, for her PhD studies. She performed the research presented in this thesis under the supervision of prof. dr. ir. Mark van Loosdrecht and dr. ir. Robbert Kleerebezem in 2004.

In August 2009 she returned to the food industry, working at Danone in the Netherlands as a product technologist for medical nutrition.

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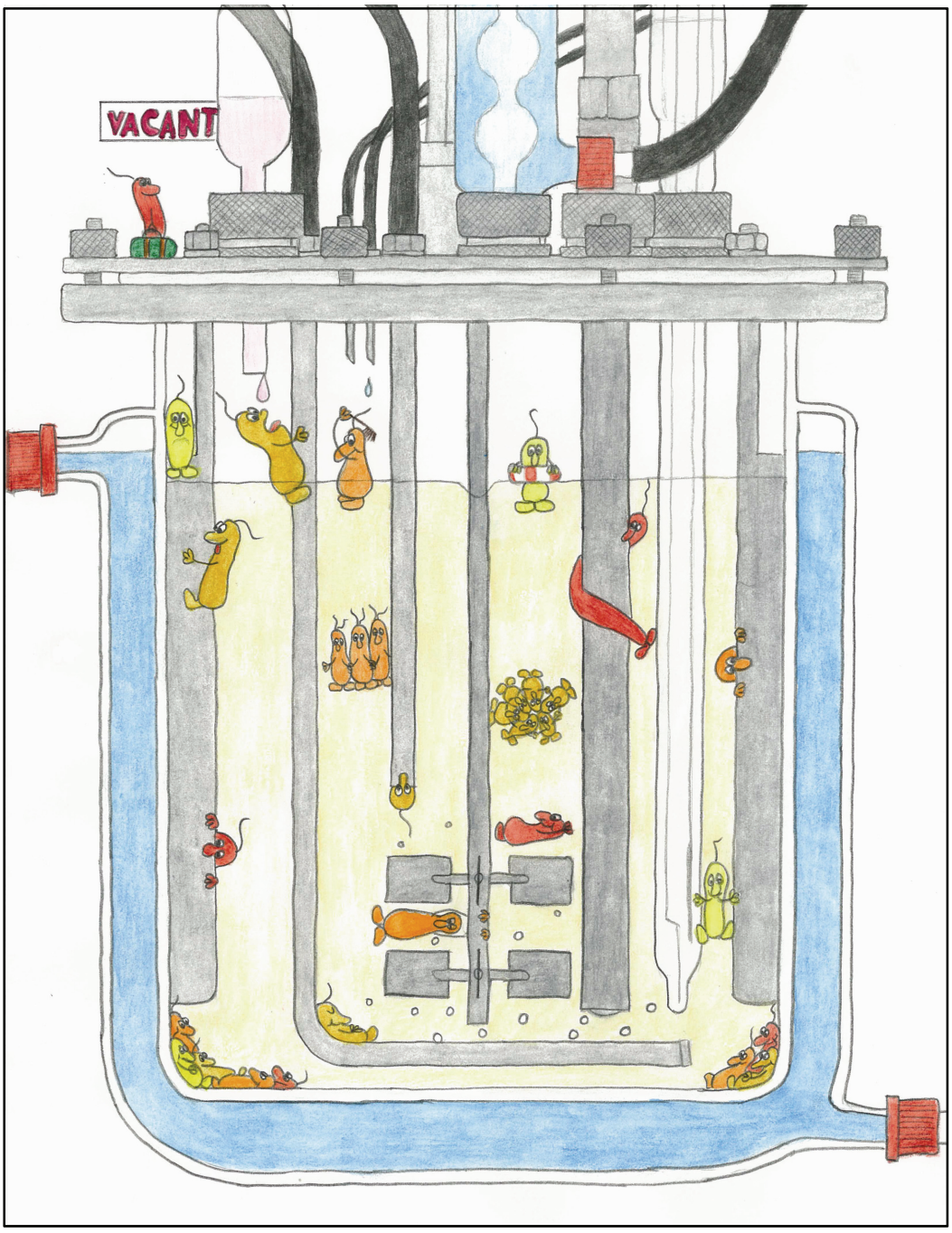
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