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Master's Thesis

Production of PHB in a cell recycling fed-batch process using *Cupriavidus necator*

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Tulln, 2016

Acknowledgements

I would like to express my gratitude to Cornelia Haas, MSc. and Dr. Markus Neureiter for their support and supervision during the development and realisation of the process described in this thesis.

In addition I would like to thank Dr. Sabine Frühauf for helping out with material and Marcus Pruckner for providing help with the HPLC. Special thanks go to Markus Sadler for providing equipment and the constant technical support he offered as well as Ing. Wilhelm Müllner for the immense help he provided and the work he did during the modification of the reactor systems.

Furthermore I would like to thank Prof. Dr. Georg Gübitz, head of the institute, for giving me the opportunity to work at the Institute for Environmental Biotechnology at IFA-Tulln.

Finally I would like to thank my family for their support during my years of study and the process of writing this work.

Abstract

For an economically feasible production of bioplastics such as polyhydroxyalkanoates there is an urge to use cheap substrates. Such substrates often contain only a low amount of carbon, making them impractical for the use in conventional fed-batch processes. Due to these facts, new methods have to be developed to utilize such media, while nevertheless reaching high cell densities and productivities. In this work, a novel process using *Cupriavidus necator* to produce polyhydroxybutyrate (PHB), the most prominent polyhydroxyalkanoate, is proposed. This process, employing cell recycling is furthermore implemented and tested at lab scale. In various experiments the influence of fermentation conditions and process parameters were studied using mineral media. Subsequently four cell recycling fed-batch fermentation experiments were done in two different modified bioreactor systems using media with a glucose concentration of 50 g/L and hollow fibre membranes. In one of those experiments a productivity of 1.4 g PHB per litre and hour could be reached while the cell dry weight reached 32.6 g/L in only 24.3 hours, and the PHB polymer made up 92% of the cell dry weight. These results are comparable to other published processes and the data further suggest that the productivities and the PHB yield of the process can even be increased by running the process at higher cell densities and further optimising the feed strategy.

Abstract

Um einen Biokunststoff wie z.B. Polyhydroxyalkanoat zu einem konkurrenzfähigen Preis produzieren zu können, ist man bestrebt, immer billigere Substrate in der Produktion einzusetzen. Diese Substrate weisen in der Regel allerdings eine eher geringe Konzentration der Kohlenstoffquelle auf, sodass sie kaum in einem konventionellen Fed-Batch Prozesse nutzbar sind. Aus diesem Grund gilt es neue Prozesse zu entwickeln, die diese Substrate verwerten können und gleichzeitig ähnlich hohe Produktivitäten und Zelldichten wie mit konventionellen Prozessen erreichen. Im Rahmen dieser Arbeit wurde nun ein Prozess entwickelt, der dies mittels „Cell-Recyclings“ erreicht. Zur Umsetzung dieses Prozesses im Labormaßstab wurden zwei verschiedene Biorekatorsysteme z.T. stark modifiziert und mit einer Hohlfasermembran zur Rückhaltung der Zellen ausgestattet. Nach experimenteller Bestimmung unterschiedlicher Prozessparameter und Fermentationsbedingungen wurde dieser Prozess in vier „Cell-Recycling“ Fed-Batch Experimente getestet. Dies geschah unter Einsatz des Bakteriums *Cupriavidus necator*, einem natürlichen Produzenten von Polyhydroxybutyrat (PHB), dem bekanntesten Polyhydroxyalkanoat. Als Nährmedium kam ein Mineralmedium mit einer Glukosekonzentration von 50 g/L zum Einsatz. Bei diesen Experimenten konnten eine PHB Produktivität von bis zu 1.4 g/Lh und eine Zelldichte von 32.6 g/L erreicht werden. Darüber hinaus konnte in diesem Experiment in einem nur 24.3 Stunden dauernden Prozess ein sehr hoher PHB Anteil von 92 % in der getrockneten Biomasse erzielt werden. Diese Ergebnisse sind mit anderen publizierten Prozessen vergleichbar, die Daten deuten allerdings darauf hin, dass sowohl die Produktivität als auch die Ausbeute des Prozesses noch weiter gesteigert werden können, sofern der Prozess mit einer höheren Zelldichte geführt wird und man die Feed Strategie optimiert.

Contents

List of Abbreviations

PET	Polyethylen terephthalate
PBS	Polybutylen succinate
PLA	Polylactide
PHAs	Polyhydroxyalkanoates
PHB	Poly(3-hydroxybutyrate)
PHA_{SCL}	Polyhydroxyalkanoates consisting of C3 to C5 monomers
PHA_{MCL}	Polyhydroxyalkanoates consisting of C6 to C14 monomers
CoA	Coenzyme A
CDW	Cell dry weight
dd-H_2O	Double distilled water
DO	Dissolved oxygen concentration
OD	Optical density at 600 nm

1 Introduction

1.1 General information on bioplastics

Plastic has become an integral part of our everyday lives be it as bags, toothbrushes or even as tableware and up to this date almost all of these polymers are derived from petrochemical sources. For instance only 0.5 % of the traditionally plastic rich household in Austria waste consists of so-called "bioplastics" (Lorber et al., 2015).

That said, the development of bio-based and biodegradable polymers has become a big research topic since the late nineties. This development is at least in part caused by the ever dwindling crude oil reserves and the problematic accumulation of traditional plastics in our environment due to its lack of biodegradability. While there are now a lot of different polymers available that are either bio-based, biodegradable or both, high production costs remain a big hindrance for their widespread use (Ienczak et al., 2013).

When looking at the overall worldwide production of plastics in the year 2014, only 1.7 million tons were at least partly bio-based and while this number seems huge, it is relatively insignificant when compared to the total plastic production of over 300 million tons in the same year. Furthermore, only about 650.000 tons of the 1.7 million tons bio-based plastics were biodegradable. This means that, while a bio-based carbon source was used for the synthesis of all of those polymers, only the latter group can be degraded by microorganisms in the environment. The biggest part of the at least partly bio-based but not biodegradable polymers produced in 2014 was made up of "PET30". This polyethylene terephthalate is synthesized using bio-based ethylenglycol and terephthalic acid from petrochemical sources. As a result, only 30% of the carbon used for the synthesis of this polymer is bio-based. The PET30 is chemically identical with PET completely based on petrochemical carbon sources. While this polymer and comparable polymers make up a big part of the bio-based polymers, and the share is predicted to increase further, the biodegradable plastics are more interesting with respect to the development of an environmentally friendly plastic. There is a variety of polymers that fulfil both requirements of being biodegradable as well as bio-based. The most important are polylactides (PLA), polybutylen succinate (PBS) and polyhydroxyalkanoates (PHA). PLAs are, while completely bio-based, only degradable by microorganisms in industrial composting processes but nevertheless they are among the best-established bio-based polymers. They are produced by various companies and the prices, at which the polymer can be produced, reach similar levels to fossil-based polymers. While PBS is biodegradable and principally could be completely bio-based, it is today mainly derived from fossil carbon sources (Carus and Aeschelmann, 2015).

1.2 Polyhydroxyalcanoates – porperties, synthesis and producing organisms

A group of bio-based as well as biodegradable polymers are the PHAs, polyesters of high molecular weight, which are known to be constituted of a variety of more than 150 different hydroxyalkanoate monomers (Rehm and Steinbüchel, 2005). These different monomers form a range of homo- and co-polymers and the physical properties of PHAs vary accordingly. There nevertheless are some general characteristics that apply to all of them: They are thermoplastics that are insoluble in water, have quite a good resistance to hydrolytic degradation and a melting temperature in the range of 160 °C to 175 °C (Bugnicourt et al., 2014).

There are a few companies that produce PHAs on a commercial scale, but due to its rather high costs the polymers are mostly used for niche applications. There are, for instance disposable razors produced from the polymer and the US Navy decided to use a PHA polymer for the production of disposable cups that can be thrown overboard without having to think about environmental pollution (Platt, 2006).

This is possible because microbial degradation of PHAs can take place in various environments, be it in soil, compost or even in aquatic, marine environments. The degradability depends on the environment it is degraded in, the composition of the polymer and the related degree of crystallinity. While degradation of PHAs can take place under different temperatures and moisture levels, the time needed varies from a few weeks in soil to over 200 days in aquatic environments with low temperature. In any case PHAs can be completely degraded in aerobic conditions by various bacteria, leaving only carbon dioxide and water. In anaerobic conditions degradation is also possible, but in addition to carbon dioxide and water, methane is formed. The degradation process usually involves a depolymerisation catalysed by an extracellular depolymerase and the subsequent metabolization of the monomers by the bacteria (Reddy et al., 2003).

PHAs are synthesized by a wide range of prokaryotic and even eukaryotic organisms from different organic substrates making them, as mentioned before, bio-based. While many organisms are able to synthesise PHAs in low concentrations, these usually have a lower molecular weight than the PHAs of interest for the utilisation as bioplastic, and form complexes with other molecules (Rehm and Steinbüchel, 2005).

The interesting, high molecular weight PHAs are synthesised as storage compounds for carbon and energy by a range of bacteria. The synthesis of such PHAs is usually induced by a nutrient limitation of some sort, but requires excess amounts of a carbon source. Depending on the microorganism, the carbon source and the growth conditions the polymer properties vary (Khanna and Srivastava, 2005a).

The key enzymes for the production of PHAs are the PHA synthases, which polymerize coenzyme A (CoA) thioesters of hydroxyalcanoic acids into polyesters. The substrate range of these PHA synthases varies, but the enzymes can be classified as either catalysing the formation of short chain-length PHAs (PHA_{SCL}) or medium chain-length PHAs (PHA_{MCL}). The constitutes of the former group are

monomers containing three to five carbon atoms, the latter of monomers containing six to fourteen (Rehm and Steinbüchel, 2005).

The CoA thioesters utilized by the PHA synthases can be provided through different pathways producing either monomers for the PHA_{SCL} synthesis or the PHA_{MCL} synthesis. The monomers for the latter are usually formed either by degradation of fatty acids or involving the biosynthesis of fatty acids. The most common pathway for the formation of PHA_{SCL} monomers utilizes acetyl-CoA generated by the degradation of sugars. Due to the fact that the latter pathway that yields poly(3-hydroxybutyrate) is probably the most common PHA synthesis pathway and is present in a wide range of bacteria, the pathway will be described briefly (Tsuge, 2002).

As discovered during the investigation of PHA synthesis in *Cupriavidus necator,* the PHB synthesis starts with the condensation of two acetyl-CoA molecules by the enzyme β-ketothiolase. The resulting acetoacetyl-CoA enzyme is subsequently reduced by an NADPH-dependent acetoacetyl reductase forming R-3-hydroxybutyryl-CoA which is then polymerised by a PHB synthase (Pohlmann et al., 2006).

The resulting PHB polymer is probably the most common and certainly best characterized PHA and has been known since 1925. Chemically it is an isotactic homopolymer consisting of hydroxybutyrate monomers, having a relatively high degree of crystallinity thus making it rather stiff and brittle. These properties seem to make the polymer unsuitable for the usage in the packaging industry, however, the properties of the plastic can be modified with suitable additives or by blending. The resulting plastics have, compared to other bioplastics, good barrier properties and high mechanical strength. PHB is also biocompatible, which enables various medical applications. For example PHB based plastic could be used for surgical sutures that can subsequently be reabsorbed by the body, as the monomers of PHB are present in the human blood stream naturally anyway (Bugnicourt et al., 2014).

The model organism for the synthesis of PHB and in fact PHAs in general is the before mentioned *Cupriavidus necator*, a Gram-negative soil inhabiting bacterium (Pohlmann et al., 2006). While the bacterium is now known as *C. necator* it was, in the past also called *Alcaligenes eutrophus, Ralstonia eutropha* or *Wautersia eutropha* (Vandamme and Coenye, 2004).

The bacterium is a rather versatile organism that is able to survive using a lithoauthotrophic metabolism, with H_2 oxidation being the source of the required energy and CO_2 the carbon source. In addition, a heterotrophic metabolism is possible, during which a wide range of organic compounds can be used as carbon and energy source. Among those, fructose, amino acids, fatty acids, sugar acids, other organic acids and intermediates of the citric circle allow high productivities, but the bacterium lacks the enzymes to utilize glucose (Pohlmann et al., 2006). Glucose is not utilized, except by mutants currently used in biotechnological processes (Pötter et al., 2004), Under anaerobic conditions, the bacteria are also able to switch to anaerobic respiration, using for example nitrate as electron acceptor. This versatility is a result of the adaptation to life in transiently anoxic habitats such as freshwater biotopes, an adaptation that most probably also gave rise to the ability to store big amounts

of PHB under nutrient or oxygen limited conditions. PHB is stored in granules in the cytoplasm of the cells that are covered with proteins forming a layer on the granule. These so called phasin proteins supposedly are involved in the hydratation of granules and the regulation of their size as well as their number. *C. necator* is not only important for the research on the biochemistry of PHA formation, but the bacterium is also used in biotechnological PHB production processes due to its ability to reach high cell densities of up to 200 g/L in a bioreactor (Pohlmann et al., 2006).

1.3 General considerations for the biotechnological PHB production

High cell densities are important due to the fact that after nutrient limitation is imposed, bacterial growth comes to an absolute stop and the bacteria solely accumulate PHB until a considerable amount of the cells dry mass, contents of over 80% were reported, is made up of PHB and this process stops as well. Due to this phenomenon, the PHB productivity that a biotechnological process can reach is directly linked to the number of cells present in the reactor prior to the nutrient limitation, making the accumulation of a high number of cells desirable.

While this pattern of serial nutrient limitation and PHB accumulation is quite common in many microorganisms, it is by no means a necessity. There are for instance wild type strains that are able to produce PHB in a growth associated manner, examples being *Alcaligenes latus* and *Burkholderia sacchari*. There have also been experiments with genetically modified bacteria, mainly *Escherichia coli* strains containing the genes of natural PHB producers. The usage of such modified *E. coli* bacteria offers the advantage of a wider range of utilizable carbon sources and a lack of an effective degradation system for the produced PHB. Additionally, no nutrient limitation has to be induced (Ienczak et al., 2013).

As an alternative to the use of natural, microbial PHA producers and genetically engineered bacteria, there have also been attempts to use genetically engineered plants for PHA production. It has been theorized that, if the plants can be engineered in a way that would result in 20% to 40% of the dry weight being made up of PHA, the production of PHAs in plants could be economically viable. However, there are a few obstacles: The compartmentalisation of the plant cells requires that the PHA synthesis genes inserted are mainly expressed in the acetyl-CoA rich compartments of the cells, so that high productivities can be reached. Oil seed crops and especially the cells in the seeds of such plants seem to be, for instance, good candidates for PHA production due to the fact that their fatty acid synthesis also starts from acetyl-CoA. In addition to the localisation of the expression of the genes, it is also necessary to establish stable transgenes in plants that have a rather normal or at least viable phenotype (Reddy et al., 2003).

Despite these attempts to use plants for PHA production, a vast amount of biotechnological processes have been developed for the bacterial production of PHB and other PHAs, mainly co-polymers consisting of 3-hydroxybutyrate building blocks and another alkanoates for instance

3-hydroxyvalerate. These co-polymers are produced by feeding the bacteria, in addition to the regular carbon source, defined amounts of, for instance, valeric acid, that is than incorporated into the polymer. The ratio of both carbon sources subsequently influences the ratio of both monomers in the final copolymer. To achieve high cell densities most of above mentioned processes are based on two different operation modes, being either fed-batch or continuous processes.

While in both modes the PHA contents reached are usually above 60% of the cell dry weight and the volumetric productivities tend to be above 1 g / Lh these processes are usually relying on the usage of rather high sugar concentrations or other media with high carbon content. Furthermore defined media are used in many of the published processes. In line with this, the biggest cost factors in the production of PHB are substrate costs (Ienczak et al., 2013).

In addition to the substrate costs, the extraction of PHAs is the second cost-driver and is therefore also a major research topic. There are different possibilities for the extraction of the PHAs from the bacteria, with their applicability depending on the organism extracted from, the type of PHA extracted, the intracellular PHA content and the product purity required. For all methods, the cells have to be separated from aqueous environments by means of filtration or centrifugation and at least partly dried. In principal, there are four categories of methods that can be used to extract the PHA itself: solvent extraction and precipitation of the PHA, enzymatic digestion of the residual biomass, mechanical disruption of the residual biomass and the usage of high osmotic pressures to burst the cells. The best established method is the solvent extraction using mainly halogenated organic solvents, and basically consists of three steps: At first lipids are removed from the biomass using mild polar solvents. Then the PHA is dissolved by the addition of, for instance, chloroform. Due to the fact, that the chloroform does not dissolve the remaining residual biomass, and the lipids it would dissolve have been removed before, the resulting solution contains high amounts of PHA that is subsequently precipitated. This is done using a so called “PHA anti-solvent”, typically methanol, ethanol or another low molecular alcohol that reduces the PHAs solubility drastically.

While this classical method achieves high recovery yields and high product purity the method is not suitable for the industrial application because of its rather high costs due to the large amounts of solvent needed for the extraction. Furthermore, the halogenated solvents, while in principal recyclable and reusable, pose a rather high environmental as well as a health risk and as a result are problematic. That said, solvent extraction processes have been developed that do not require the use of halogenated organic solvents, but face other drawbacks. As a result there is much research needed into the reduction of costs for the downstream processing (Koller et al., 2013).

Apart from the development of cheaper extraction processes, the main focus in making PHAs economically feasible is to cut substrate costs.

1.4 Considerations for the development of a cell recycling fed-batch process

Up to 50% of the total production costs for PHB are in fact substrate costs indicating that the usage of a cheaper substrate would hugely decrease the production costs for the polymer. As a result there has been a focus on research concerning the utilization of low-value substrates for biotechnological processes. These low-value substrates can range from agricultural to industrial waste, or even surplus feedstock, but most of these substrates have the common negative feature that the concentration of the carbon source for the bacteria tends to be a rather low.

This is problematic for the usage of such substrates in most biotechnological processes. For instance in a fed-batch process, huge reactors would be needed to contain the large amounts of substrate and, as a result, fermentation volumes. Additionally, the fermentation broth would be diluted immensely solely by the addition of feed. Due to the fact that this is by no means desirable, alternative processes have to be developed which can utilize media with low carbon content without having these drawbacks (Ienczak et al., 2013).

The conventional, widely used fed-batch process for the production of PHAs mostly rely on the use of a media with a moderate carbon content, in which the bacteria are allowed to grow at the beginning and a medium with very high carbon content, that is fed to the reactor continuously. This allows high cell densities and productivities. When the productivity achieved and the PHA content in the biomass are satisfactory, the biomass and the containing PHAs are harvested. Due to the nature of PHA accumulation in most organisms, the supply of nitrogen to the reactor has to be regulated, to allow the induction of a nitrogen limitation.

To allow the usage of agricultural wastes in such processes, one could concentrate the medium to increase its carbon content significantly, but this strategy has drawbacks. Since the composition of media derived from agricultural wastes vary and are often not known at all, it is quite likely that potentially harmful substances would be concentrated alongside the carbon source for the bacteria. If such media were then used in a conventional fed-batch the harmful substance would accumulate in the reactor, potentially reaching a level at which bacterial growth is hindered.

An alternate approach would be to use media as is, but modify the process itself. There have been attempts to establish continuous processes for the PHA production, and there are some that produce results comparable to fed-batch processes, but the setup of those can be rather complex and the concentration of the carbon source used are often rather high (Atlić et al., 2011). While there would be no problem with the accumulation of potentially harmful substances in the reactor other problems arise. The polymer is accumulated in intracellular granules, making rather high residence times in the reactor necessary to accumulate meaningful amounts of PHA in the biomass. Furthermore a nutrient limitation has to be induced in most organisms to trigger the accumulation of PHA, which makes the development of a continuous process tricky. On top of these factors, the usage of substrates with low carbon content would also limit the reachable cell densities and as a result limit PHA productivities at a rather low level (Ienczak et al., 2013).

To allow high cell densities and feed rates while being able to use media with moderate carbon content and without being required to use enormous reactor volumes, one could use a form of cell recycling.
To achieve this, a filtration cartridge could be employed to hold back the bacteria in the reactor while the medium is allowed to flow through the reactor. This would enable the bacteria to metabolize the carbon source in the feed and accumulate PHAs until a high intracellular content is reached, while simultaneously excess liquid and potentially harmful substances are constantly removed from the reactor.
There have been successful attempts to utilize an external ultrafiltration membrane for cell recycling purposes to produce PHB from whey solution in a cell recycling fed-batch process. That said, the requirements for the utilisation of an agricultural waste medium were not entirely reached, because a highly concentrated whey solution (280 g/L) was used in the process (Ahn et al., 2001).
This setup could nevertheless serve as a template for the development of a process that is able of utilizing media of lower carbon content and nevertheless reach high cell densities.

2 Objectives

The aim of this work is to evaluate the potential of cell recycling in the biotechnological production of PHB with agricultural wastes or other low carbon content substrates. For this purpose a setup is to be conceived, that allows the retention of cells in the bioreactor and the reactor volume to stay constant during the fermentation. This setup will then be implemented at laboratory scale. For this purpose, a bioreactor system (0.5 L scale) will be modified in such a way as to allow a cell recycling process. The performance of this process will be tested in multiple experiments. These experiments will be performed with the PHA model organism *C. necator* and mineral media with sugar concentration between 30 g/L and 50 g/L. To assess the performance of the cell-recycling processes, the focus will be put on their volumetric PHB productivities and their PHB yield. These values will then be compared to those generated in more conventional PHB production processes, especially fed-batch processes. From this comparison it should be possible to evaluate, if the process is able to produce competitive results and further improvement is possible. If the process shows potential for the production of PHB in a bigger scale, it will be implemented on the 3 L scale and further experiments will be carried out. In this case, potential problems and starting points for further optimisation should be identified.

3 Materials and Methods

3.1 Organism and pre-culture

For all fermentation experiments, the *Cupriavidus necator* strain DSM 545, kindly provided by Martin Koller (TU Graz), was used. The cells were stored on mineral medium plates at 4° C for up to several weeks and repeatedly subcultivated on new mineral medium plates.

3.2 Medium

For all experiments a mineral medium was used. The components were prepared in three parts, which were separately autoclaved combined afterwards to form the final medium.

For the first part, the chemicals shown in **Table 1** were dissolved individually in dd-H_2O and all solutions were subsequently added into a Schott flask. The pH of the medium was adjusted to pH 7 with sulphuric acid or sodium hydroxide and after a trace elements solution (composition shown in **Table 2**) was added, the flask was filled with dd-H_2O until 80 % of the intended final volume was reached.

Table 1: **Composition of one litre of mineral medium.** All components given were dissolved in dd-H_2O. Sugar and Fe(NH_4)citrate stock solutions were autoclaved separately and added afterwards

Component	amount	
KH_2PO_4	2	g
Na_2HPO_4	4,8	g
$(NH_4)_2SO_4$	3	g
$MgSO_4 \cdot 7\ H_2O$	0,8	g
NaCl	1	g
$CaCl_2 \cdot 2\ H_2O$	0,02	g
trace elements solution (see Table 2)	5	mL
sugar stock solution (see Table 3)	100	mL
Fe(NH_4)citrate stock solution (see Table 3)	100	mL

Table 2: Composition of one litre of trace elements solution.

component	amount	
$ZnSO_4 \cdot 7\ H_2O$	250	mg
$MnCl_2 \cdot 4\ H_2O$	75	mg
H_3BO_3	750	mg
$CoCl_2 \cdot 6\ H_2O$	500	mg
$CuCl_2 \cdot 2\ H_2O$	25	mg
$NiCl_2 \cdot 6\ H_2O$	50	mg
$Na_2MoO_4 \cdot 2\ H_2O$	75	mg

The other two parts were formed by sugar and Fe(NH_4)citrate solutions, that were prepared separately as 10× stock solutions (see **Table 3**). A fructose solution was used for fermentation experiments **PHB-MB-Ib** and **PHB-MB-II**, for all other experiments a glucose solution was used for practical reasons.

The stock solutions and the mineral media were autoclaved for 20 minutes at 121 °C. To complete the medium, the salt solution described above had to be complemented with sugar and Fe(NH_4)citrate. For this purpose a volume of each stock solution equalling the volume of a tenth of the intended final volume of the mineral medium, was added.

The exact way of preparation depended on the intended usage of the media and will be described briefly in each section.

For standard fed-batches, sugar solutions with varying concentrations were used as feed (for details see **Section 3.6**). For the preparation of these feed solutions sugar was weighed into a Schott flask and dissolved in appropriate amounts of dd-H_2O. To speed up the dissolving process, the Schott flasks were heated in a water bath up to 50 °C. In all cell recycling fed-batch fermentations mineral medium containing 50 g/L of sugar, was used as a feed.

Table 3: Concentrations of the stock solutions used for varying purposes. Appropriate amounts of component were weighed in, dissolved in dd-H_2O and the supplemented with appropriate amounts of dd-H_2O until the final volume was reached.

intended use of the stock solution	concentration	component
medium for inoculum preparation	100 g / L	sugar (glucose/fructose)
medium initially in the reactor	300 g / L	sugar (glucose/fructose)
feed medium	500 g / L	sugar (glucose/fructose)
Fe(NH_4)citrate solution	0.5 g / L	Fe(NH_4)citrate

3.3 Bioreactor setup

In principle two different setups were used for the fermentation experiments. On one hand, experiments were done in the DASGIP system that was combined, for cell recycle fed-batch fermentations, with the ATF-2 systems by Refine. On the other hand, fermentations were done in the Braun Biostat system that was equipped with an external hollow fibre membrane for cell recycle fed-batch fermentations.

3.3.1 DASGIP System

The DASGIP system is a bioreactor system for up to four parallel fermentation experiments. Each reactor has a total volume of 1.5 L and is equipped with Rushton impellers, a sparger, an exhaust gas condenser and electrodes for the measurement of temperature, pH and dissolved oxygen concentration (DO) as well as a septum and a sample port.

DO, temperature and pH were automatically controlled by a bioprocess control system (DASGIP) and logged digitally on a computer. The set point for the DO value was maintained, by varying the stirrer speed up to 1200 rpm, while the rate at which pressurised air was supplied through a sterile filter and subsequently the sparger, was kept constant at 28.1 L/h. The base used to adjust the pH at the desired level was either sodium hydroxide or ammonium hydroxide. In case that addition of acid was required, hydrochloric acid was used. Both were dosed into the reactors using peristaltic pumps integrated into the control element of the DASGIP system.

The feed rate was added was controlled by the bioprocess control system.

The temperature was controlled through the “bioblock” of the DASGIP system, in which all bioreactors were inserted and which provided individual heating or cooling for all bioreactors. The exhaust gas was channelled through exhaust gas condensers, to reduce the volume loss through vaporisation, and a bottle of 2M sodium hydroxide to prohibit the contamination of the laboratory environment. Antifoam could be added, if needed, with a syringe and a hypodermic needle through a septum in the head plate of the reactor.

3.3.2 ATF-2 System and level control

The ATF-2 system is a system developed to provide a defined but alternating flow through a hollow fibre membrane and consists, in addition to the membrane, of a diaphragm pump, a control unit and a dip-tube. The system was initially developed for animal cell culture and its main aim is, to reduce the fowling of the membrane through the alternating flow. The dip tube could be inserted into one of the ports of a DASGIP bioreactor and retentate as well as feed were alternatingly pumped in and out of the reactor through it. Permeate was removed by an external peristaltic pump and stored in a waste bottle.

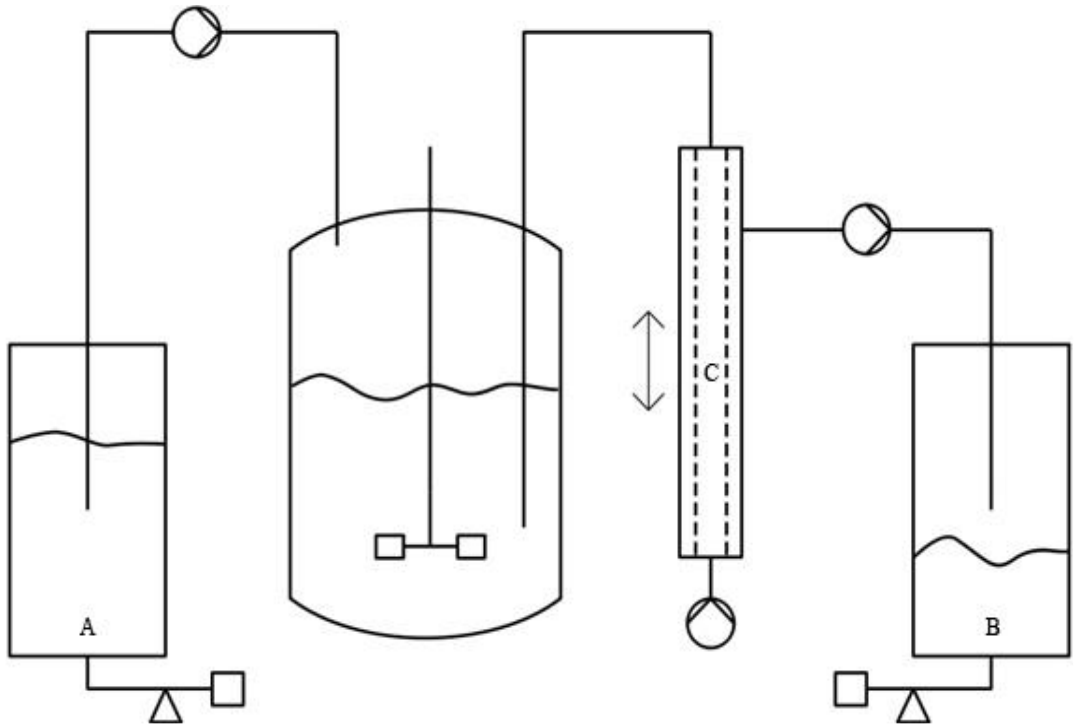

Figure 1: **Schematic of the setup for cell recycling fed-batch fermentations in the DASGIP system.** **(A)** represents the feed bottle, from which medium is pumped into the reactor using a peristaltic pump. **(B)** represents the waste bottle to which permeate is transferred using a peristaltic pump. **(C)** represents a hollow fibre membrane mounted onto a diaphragm pump, causing medium to be pumped in and out of the reactor periodically.

Two different hollow fibre membranes were used in this system. The diaphragm pump was equipped either with the CFP-2-E-4MA microfiltration membrane with a pore size of 0.2 µm or the FP-750-E-4MA with a nominal molecular weight cut-off of 750 kDa. Both membranes had a surface area of 420 cm² and were produced by GE Healthcare. Due to the availability of only one ATF-2 system, unlike the usual experiments in the DASGIP system, only one experiment at a time could be conducted using this setup.

To keep the level in the reactor steady, the amount of permeate removed from the reactor had to match the amount of feed added. To achieve this, feed and waste bottle each were placed on a scale and a control unit was constructed in house. This control unit regulated the permeate pump in a way, that kept the sum of both scale values constant, meaning that if feed was removed from the feed bottle, permeate was pumped into the waste bottle.

3.3.3 Braun Biostat

As a second bioreactor a Biostat ED 884113/6 by Braun Biotech International with a holding capacity of 5 litres was used. While the fermenter vessel itself was left in its original state, everything else was heavily modified. Big parts of the hardware of the control unit were replaced in house to allow all functions of the bioreactor to be controlled using software by Allen Bradley (Rockwell Automation).

This control software was programmed in house to accommodate the individual needs of each process and as a result allowed the variation of a high number of parameters.

For the measurement of pH, DO, pressure and temperature the fermenter was equipped with corresponding electrodes as well as an electrode for the detection of foam. Additionally, the reactor was equipped with a sparger, an exhaust gas condenser, a port at the bottom of the fermentation vessel and a number of ports for the addition of acid, base, antifoam or feed media in the head plate.

For temperature regulation, water-filled heating loops inside the reactor were used, that could either be heated or cooled by the control unit of the bioreactor. The DO was adjusted by varying the stirrer speed as well as the flow rate of the pressurised air supplied to the reactor.

The pH was adjusted by the addition of hydrochloric acid or sodium hydroxide to the reactor utilizing the corresponding peristaltic pumps integrated into the control unit. These integrated peristaltic pumps were also used for the addition of antifoam.

Antifoam was added either manually or in some fermentations a sensor was used to attempt an automated addition.

The feed rate was regulated by bioreactor's control unit. To that end the feed bottle was placed on a scale, and the weight loss was monitored during feeding.

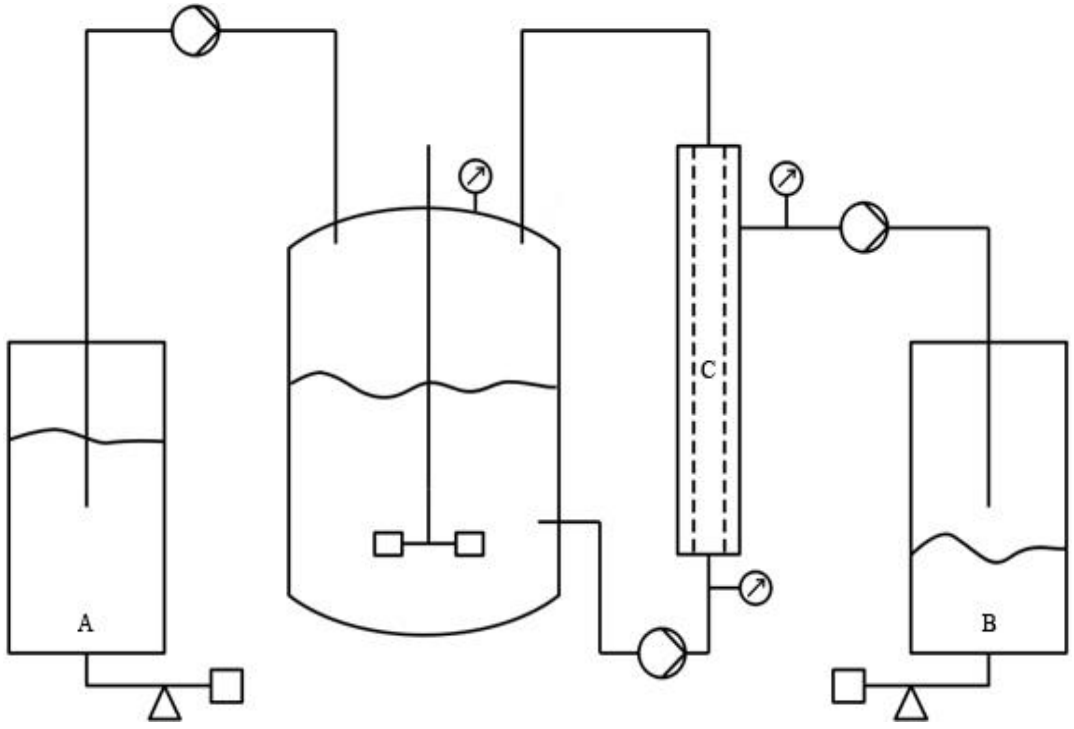

Figure 2: **Schematic of the setup for cell recycling fed-batch fermentations in the Braun Biostat system. (A)** represents the feed bottle, from which medium is pumped into the reactor using a peristaltic pump. **(B)** represents the waste bottle to which permeate is transferred using a peristaltic pump. **(C)** represents a hollow fibre through which fermentation broth is constantly pumped by a peristaltic pump. There are pressure sensors in the headspace of the reactorand on the permeate side of the membrane as well as the feed side.

For cell recycle fed-batch fermentations, the reactor was connected to the CFP-2-E-4MA microfiltration membrane. A peristaltic pump was used to draw fermentation broth out of the reactor and subsequently supply it to the hollow fibre membrane as feed. Permeate was pumped into a sterile waste bottle using a peristaltic pump. To monitor the pressure on both sides of the membrane, adequate sensors were placed on the feed side of the membrane as well as on the permeate side.

The setup for cell recycle fed-batch fermentations is shown in **Figure 2**. Similar to the setup of the DASGIP system, both feed and waste bottle were placed on scales and the control unit of the fermenter regulated the withdrawal of permeate from the hollow fibre membrane through a peristaltic pump under its control.

3.4 Preparation

3.4.1 Inoculum preparation

For the preparation of the inoculum, 160 mL of mineral medium were filled into a baffled 1L shake flask. After autoclaving, 20 mL of the sterile sugar solution and 20 mL of the sterile Fe(NH_4)citrate solution were added in a laminar flow workbench (LaminAir B 2472, Heraeus) to create a mineral medium containing 10 g/L of sugar. The shake flasks were inoculated with multiple colonies from a mineral medium plate. The shake flasks were incubated for 24 to 48 hours at 37°C (or if explicitly stated 30 °C) and 150 rpm in a shaker to reach an optical density of approximately 14, measured at 600 nm against dd-H_2O.

For fermentations in the DASGIP System, the contents of one shake flask were transferred into a sterile centrifugation bottle of appropriate size in a laminar flow workbench and centrifuged for 20 minutes at 2000 g in a Sorvall Lynx4000 centrifuge (Thermo Scientific). Under laminar flow conditions, the supernatant was discarded and the pellet subsequently resuspended in 50 mL of mineral medium. For fermentation in the Braun fermenter, two shake flasks were united into one sterile Schott flask in a laminar flow workbench and used as inoculum.

3.4.2 Bioreactor preparation (DASGIP system)

Before actual fermentations in the reactors of the DASGIP system could be started, several preparation steps were necessary.

At first, a two point calibration of the pH electrodes was done outside of the reactors using a pH 7 and a pH 4 buffer solution. The reactor vessels were assembled, meaning the stirrer, exhaust air condenser, riser tube and sparger were placed in the head plates of each reactor and filled with an appropriate amount of mineral media excluding sugar and Fe(NH_4)citrate.

The calibrated pH electrodes and the DO electrodes were subsequently placed in the reactor vessel and the whole fermenter was autoclaved at 121 °C for 20 minutes. After autoclaving, appropriate amounts of the sterile sugar and Fe(NH_4)citrate stock solutions were added.

The peristaltic pumps integrated into the DASGIP control unit were calibrated in an automated process.

To subsequently clean the lines, a CIP procedure consisting of three steps was used. At first all lines were flushed with 70% Ethanol, then with a sterile 2M sodium hydroxide solution and finally they were rinsed with sterile dd-H_2O before they were filled with acid, base or media depending what they were supposed to be used for.

The reactors were taken out of the laminar flow bench, placed in the bioblock of the DASGIP system, and all electrodes were connected to the control unit. After a minimum of six hours of conditioning in the media, the DO electrodes could be calibrated. For this purpose the stirrer was turned to maximum speed (1200 rpm), until the readings for the DO value were stable. This point was fixed as 100% DO. For the determination of 0% DO, the medium was saturated with N_2.

If the ATF-2 system was used, a dip tube was placed in a port of the reactor and sealed before autoclaving. The hollow-fibre membrane was wetted with dd-H_2O, placed on the diaphragm pump and fixated. This construct, including a tube to later connect the membrane with the dip tube, was autoclaved at 121 °C. The dip tube was connected to the reactor while sanitizing with 70% ethanol.

3.4.3 Bioreactor preparation (Braun Biostat)

Like in the DASGIP system, the pH electrodes were calibrated using a two point calibration with a pH 4 and a pH 7 buffer solution.

The DO was either calibrated leaving it in the ambient air (=100% DO) or in the same manner described for the DASGIP system in section **3.4.2**

In contrast to the DASGIP system, the sterilization of the fermenter was done with a SIP procedure. All electrodes were placed in the corresponding sockets and the reactor vessel was filled with mineral media, excluding sugar and Fe(NH_4)citrate solutions. The whole system was automatically heated to 121 °C at an overpressure of 1 bar for 15 minutes.

All solutions needed for the fermentation experiment, acid, base, antifoam, feed, etc. were filled into flasks equipped with a tube and a fitting and sterilized separately in an autoclave at 121 °C for 15 minutes. Afterwards, the fitting of the individual bottles were attached to the headplate by piercing the septa. Throughout this process, the area was sanitized with a Bunsen burner.

The mineral medium in the fermenter was then complemented with appropriate amounts of sugar and Fe(NH_4)citrate stock solutions. To compensate losses due to the sterilisation process, sterile dd-H_2O was added to the reactor as well, but the amount could only be estimated by an external scaling on the glass reactor. All solutions were added to the reactor vessel using peristaltic pumps under manual control.

The flasks containing base, feed, antifoam and, if used, acid were connected to the reactor and the corresponding tubes put into the appropriate peristaltic pump.

The hollow fibre membrane, if used, was wetted with dd-H_2O and autoclaved at 121 °C for 20 minutes.

3.5 Inoculation and sampling

3.5.1 DASGIP system

For the inoculation in the DASGIP system, a 25 mL syringe with a Luer lock fitting was filled with inoculum in the laminar flow workbench using a hypodermic needle. 5 mL of sterile dd-H_2O were also prepared for each reactor in syringes of the appropriate size.

The syringe with the inoculum was taken out of the laminar flow workbench and, after sanitizing both ends of the Luer lock fitting with 70% ethanol, connected to the rising tube of the reactor. The content of the syringe was added to the fermenter and, after sanitizing again, the syringe with the sterile dd-H_2O was used to push the inoculum remaining in the rising tube, into the fermenter. This process was repeated for each reactor.

Sampling was done using a similar method. The Luer lock fitting on the rising tube was sanitized and connected with a 5 mL syringe. 2 mL of fermentation broth were drawn up into a syringe and, due to the holdup volume of the rising tube, discarded. Using the same syringe, a sample of 5 mL was taken.

Due to the required analytical methods and the amount of samples drawn, the volume removed by sampling was relatively large compared to the working volume of the bioreactor. Therefore only one sample was drawn at each sampling point, despite the obvious drawback that duplicate measurement was not possible.

3.5.2 Braun Biostat

For the inoculum in the Braun Biostat System, a sterile flask equipped with tubing and fitting was prepared and filled with 200 mL of inoculum for the fermentation **PHB-MB_VII** and 400 mL of inoculum for all other fermentations. This was done in a in a laminar flow work bench. The inoculum flask was connected to the reactor and handled in the way described in section **3.4.3**.

3.5.3 Sample treatment

All samples were filled into 10 mL glass centrifuge tubes. For the OD measurement an appropriate amount of sample, typically 50 µL, was removed. The rest of the sample was centrifuged in an Eppendorf 5810 centrifuge for 7 minutes at 2000 g, or until a reasonably clear supernatant could be observed. The supernatant was filled into falcon tubes and frozen.

The pellet was washed twice with dd-H_2O and the supernatant resulting from this washing process was discarded, the pellet stored in a freezer.

3.6 Fermentation conditions

The fermentation conditions varied from experiment to experiment, depending on their goal, the limitations posed by the system or practical considerations.

If not stated otherwise, the sugar concentration at the start of the fermentation was 30 g glucose per litre of medium, the temperature set point 37 °C, the DO set point 20% and the pH was regulated to 7 in all fermentation experiments.

The feed strategies for fed-batch and cell recycling fed-batch fermentations were based on a constant supply of sugar and singular sugar "shots", when certain pH or DO limits were exceeded.

Detailed lists of the parameters for all fermentations can be found in **Table 4** & **Table 5**.

3.6.1 Process development on the 0.5 L scale (DASGIP system)

In all DASGIP fermentations, the gas flow into each reactor was fixed to 28.1 L/h, which equals, depending on the amount of media in the reactor, to approximately 0.9 vvm.

The sugar used in almost all fermentations was glucose, only in **PHB-MB_Ib** and **PHB-MF_II** fructose was used.

If a feed was used, a constant feed rate was set and only when a threshold of a DO of 35 % was exceeded or the pH reached a value above 7.5, an additional, defined amount of feed was added. This applies to all fermentations except **PHB-MF_II**, where no constant feed was applied and only feed "shots" were added manually, and **PHB-MBC_IV**, where the feed rate was changed during the fermentation.

3.6.2 Process development on the 3 L scale (modified Braun Biostat system)

In all fermentation experiments done in the Braun Biostat system, the starting volume was 3 litres, and the sugar used, was glucose. For fed-batch experiments a highly concentrated glucose solution was used as feed, in cell recycling fed-batch processes a mineral medium containing glucose was used. Feed was added to the reactor with a constant rate, and only if the DO rose beyond 35% a "shot" of defined size was added to immediately supply sugar. In all but the single batch process done, the nitrogen limitation was induced by switching from a NH_4OH solution to NaOH solution as base for pH-control.

Table 4: Fermentation conditions during experiments done in the DASGIP system.

experiment-code		reactor	mode of cultivation	working volume	
PHB-MB_Ib	*pH control*: 1M NaOH 1M H_2SO_4	R1 & R3	batch	500 mL	37 °C 30 g/L fructose
		R2	batch	500 mL	30 °C 30 g/L fructose
		R4	batch	500 mL	40 °C 24 g/L fructose
PHB-MF_II	*feed*: 400g/L fructose solution *feed shot:*50 mL	R1 & R2	fed-batch	425 mL	pH control: 25% NH_4OH & 1M NaOH
		R2 & R4	fed-batch	425 mL	pH control: 25% NH_4OH
PHB-MBC_IV	*pH control*: 1M NaOH *antifoam:* Fluka Antifoam A (1:10)	R1 & R3	batch	480 mL	-
		R2	cell recycling fed-batch	480 mL	*feed*: MM with / without $(NH_4)_2SO_4$ both 50 g/L glucose *feed rate:* 40 mL/h, 20 mL/h, 30 mL/h *DO feed shot*: 250 mL *membrane:* pore size of 0.2 µm
PHB-MFC_V	*pH control*: 1M NaOH *antifoam:* Fluka Antifoam A (1:10)	R1 & R3	fed-batch	400 mL	*feed*: 450g/L glucose solution *feed rate:*1,1 mL / h *DO feed shot:* 25 mL *pH feed shot:* 6 mL
		R2	cell recycling fed-batch	500 mL	*feed*: MM with / without $(NH_4)_2SO_4$ both 50 g/L glucose *feed rate:* 20 mL/h *DO feed shot:* 250 mL *membrane:* NMWC 750 kDa
PHB-MC_VI	*pH control*: 1M NaOH *antifoam:* Fluka Antifoam A (1:10)	R2	cell recycling fed-batch	500 mL	*feed*: MM with / without $(NH_4)_2SO_4$ both 50 g/L glucose *feed rate:* 20 mL/h *DO feed shot:* 250 mL *membrane:* pore size of 0.2 µm

Table 5: Fermentation conditions during experiments done in the Braun Biostat reactor.

experiment-code	mode of cultivation	conditions	
PHB-MB_VII	batch	*pH control:* 1M NaOH / 1M HCl	*stirrer speed:* 100 – 300 rpm *gas flow:* 1,5 - 3 L/min
PHB-MF_IX	fed-batch	*pH control:* 1M NaOH / 7% NH_4OH, 1M HCl *feed:* 500 g/L glucose solution *feed rate:* 12 g / h *feed shot:* 180 g	*stirrer speed:*100 – 800 rpm *gas flow:* 3-8 L/min *antifoam:* Fluka Antifoam A (1:10)
PHB-MF_IXb	fed-batch	*pH control:* 1M NaOH / 12,5% NH_4OH, *feed:* 500 g/L glucose solution *feed rate:* 12 g / h *feed shot:* 180 g	*stirrer speed:* 300 – 800 rpm *gas flow:* 9 – 11 L/min *antifoam:* Glanapon (1:10)
PHB-MC_X	cell recycling fed-batch	*pH control:* 1M NaOH / 12,5% NH_4OH *feed:* MM, 50 g/L glucose *feed rate:* 120 g / h, 240 g / h *feed shot:* 1500 g	*stirrer speed:* 300 – 800 rpm *gas flow:* 9 – 11 L/min *antifoam:* Glanapon (1:10) *membrane:* pore size of 0.2 µm

3.7 Analytics

3.7.1 Optical density

The optical density was used as an indicator for the concentration of cells in the fermentation broth. The OD was measured in a photometer against dd-H_2O at 600 nm. To stay in the linear range, the broth was diluted with dd-H_2O to measure OD values between 0,100 and 1.

3.7.2 Staining & Microscopy

For the purpose of staining the PHB in the cells with Nile blue A (Ostle and Holt, 1982), 10 µL of the cell suspension in the cuvette from the OD measurement were taken out and applied onto a clean microscopy slide. The slide was dried in the open air.

For staining the cells were subsequently fixated using a Bunsen burner. 90 µL of a one-percent solution of Nile blue A were applied onto the slide. After 10 minutes at 55 °C in a staining jar, the solution was washed off and the slide decolorized for one minute using an eight-percent solution of acetic acid. Subsequently the slides were dried in the open air. The PHB granules in the cells were stained during this process, and as a result the fluorescence of the granules after excitation with UV-light was evaluated.

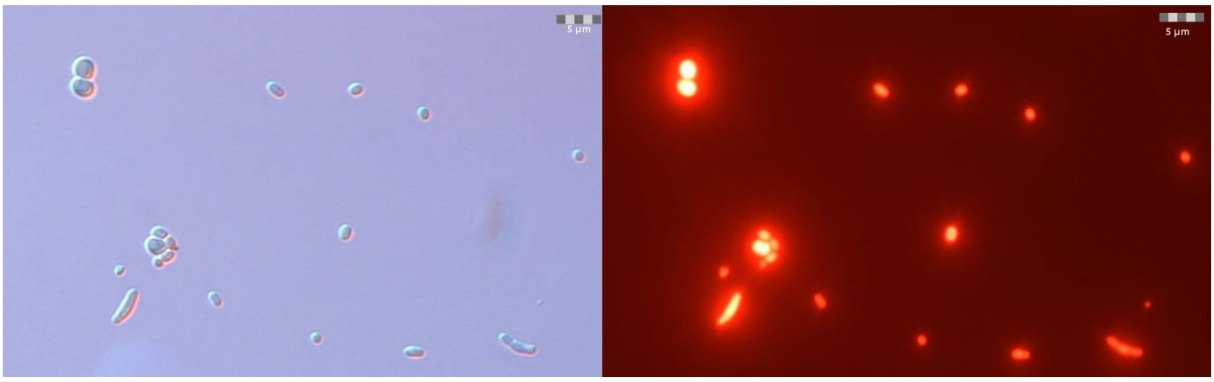

Figure 3: ***C. necator*** **containing PHB granules as seen using phase contrast microscopy (left) and fluorescence microscopy (right). Both photos were taken using sixty-fold magnification. Note that PHB granules take up most of the cell volume.**

Using an research fluorescence microscope (OLYMPUS Vanox AH BT3), equipped with the excitation filter BP 545 and the emission filter O590, the slides were viewed at a magnification of 600 in phase contrast and fluorescence mode. With a connected PC and the corresponding software a set of pictures from different spots on each slide were taken in both, phase contrast and fluorescent mode.

3.7.3 Cell dry weight

For the determination of the cell dry weight, glass centrifuge tubes, were dried at 105 °C, stored in a desiccator for cooling and weighed, using a MC1 Analytic AC 210S scale from Sartorius. These tubes were used as described in **3.5.3**.

The glass centrifuge tubes containing the pellets were taken out of the freezer and subsequently dried again at 105 °C for at least a day. After cooling down in a desiccator, the tubes were weighed to determine the weight of each pellet.

As the amount of fermentation broth used to create each pellet was known, the cell dry weight could be calculated in g CDW per litre of fermentation broth.

3.7.4 Polyhydroxybutyrate

The amount of PHB present in the cells was determined by relying on the fact that crotonic acid is formed during the acid-catalysed depolymerisation of PHB. The crotonic acid could then be separated in a HPLC system (Karr et al., 1983).

At first, two glass centrifugation tubes, each containing an amount of 10 to 20 mg of a PHB standard, were prepared. Subsequently 1 mL of concentrated sulphuric acid (98%) was added to each glass centrifuge tube containing either the dried pellets (see section **3.7.3**) or the PHB standard.

All glass centrifugation tubes were shut tightly and placed in a water bath at 90 °C, for 45 minutes or until the pellet had dissolved.

Table 6: Specifications of the HPLC setup used for the quantification of crotonic acid and sugars

Sugar	PHB
ion exchange column ION 300 (Transgenomic, Omaha, USA)	CARBOSep COREGEL 87H column (Transgenomic, Omaha USA)
45 °C	65 °C
refractive index detector (Agilent 1100, Santa Clara, USA)	refractive index detector (Agilent 1100, Santa Clara, USA)
0.005 mol / L sulphuric acid	0.005 mol / L sulphuric acid
0.325 mL / min	0.9 mL / min

After cooling, the liquefied pellet was transferred into a volumetric flask, which was subsequently filled with dd-H_2O until a final volume of 50 mL was reached. From this flask a HPLC vial was filled, after the solution had been filtered with a 0.45 µm sterile filter.

For the quantification of the crotonic acid an Agilent 1100 HPLC system was used. For details, see **Table 6**. The HPLC system was calibrated using external standards and the quantification was done using peak height. From the the amount of crotonic acid found in the PHB standards via the HPLC analysis and the amount of PHB actually weighed in a conversion factor could be calculated. This conversion factor was used to calculate a PHB concentration for each sample from the crotonic acid concentration determined for each vial.

3.7.5 Sugars

The residual sugar in the fermentation broth at the time of sampling is determined using the supernatant of the samples (see section **3.5.3**). The supernatant was stored in a freezer between centrifugation and analysis to allow simultaneous sample preparation and as a result had to be thawed before sample preparation could take place. Part of the supernatant was diluted to a sugar concentration between 10 mg/L and 1000 mg/L. The dilution was done using a sulphuric acid solution with a pH of 4, to guarantee that Carrez-precipitation would work. The Carrez-precipitation was needed to remove proteins and lipids form the sample, which could potentially harm the column.

The appropriate amount of sample was diluted in 1920 µl of sulphuric acid solution (pH4). To this mixture 40 µL of C1 solution were added, and the contents of the Eppendorf tube were mixed. After a minute of waiting, 40 µL of C2 solution were added and the contents were mixed again. The composition of both solutions for the Carrez-precipitation can be found in **Table 7**. After five minutes of waiting, the Eppendorf tubes were centrifuged with a Beckmann GS-15 centrifuge at 12500 rpm for 30 minutes. After centrifugation, the supernatant from the Eppendorf tubes was filled into HPLC vials after filtration with a sterile filter. The pellets were discarded.

Table 7: Composition of solutions C1 and C2 for the Carrez-precipitation.

	compound	concentration
C1 Solution	$K_4[Fe(CN)_6] \cdot 3\ H_2O$	5.325 g / 50 mL
C2 Solution	$ZnSO_4 \cdot 7\ H_2O$	14.400 g / 50 mL

The quantification was done using an Agilent 1100 HPLC system. For details, see **Table 6**. The HPLC was calibrated for a concentration range between 10 mg/L and 1000 mg/L of glucose and fructose using external standards. All sugar concentrations outside this range could not be quantified reliably. For the quantification of the sugar concentration in the samples, the height of the peaks was used.

3.7.6 Free ammonia

To determine the amount of free ammonia in the fermentation broth at the time of sampling, the supernatant (see section **3.5.3**) was used. Due to sample storage in a freezer, the supernatant hat to be thawed before analysis could be done.

An automated Kjeldahl distillation unit (AutoKjeldahl Unit K-370, Büchi) was used for the determination of the free ammonia content. A 30 % solution of sodium hydroxide was added automatically to the Büchi tubes containing the samples, which were subsequently heated to convert the ammonium ions into ammonia. This ammonia, distilled from the sample was transferred into a vessel where it condensed into a two percent solution of boric acid. Due to the fact, that the boric acid captured the ammonia quantitatively as ammonium ions, a subsequent titration with 0.05 M hydrochloric acid was possible (Egli, 2008).

4 Results

4.1 Process development on the 0.5 L scale (DASGIP)

4.1.1 Determination of optimal temperature for PHB production (PHB-MB_Ib)

For the determination of the optimal fermentation temperature four batch fermentations were done: one at 30 °C, one at 40 °C and two at 37 °C. For details, see **Table 4**.

The cell dry weight concentration reached in all four fermentations was relatively constant, reaching 15.7 g/L in the fermentation done at 30 °C (R2), 15.6 g/L and 15.7 g/L in the fermentations done at 37 °C (R1 & R3) and 13.2 g/L in the fermentation done at 40 °C (R4). The PHB contend also was quite steady, reaching, 69.2 % in R2, 70.4 % and 69.3 % in R1 & R3 and 62.1 % in R4. The slightly lower values for reactor 4 were a result of a lower sugar concentration compared to reactor 1, 2 and 3 due to an experimental error.

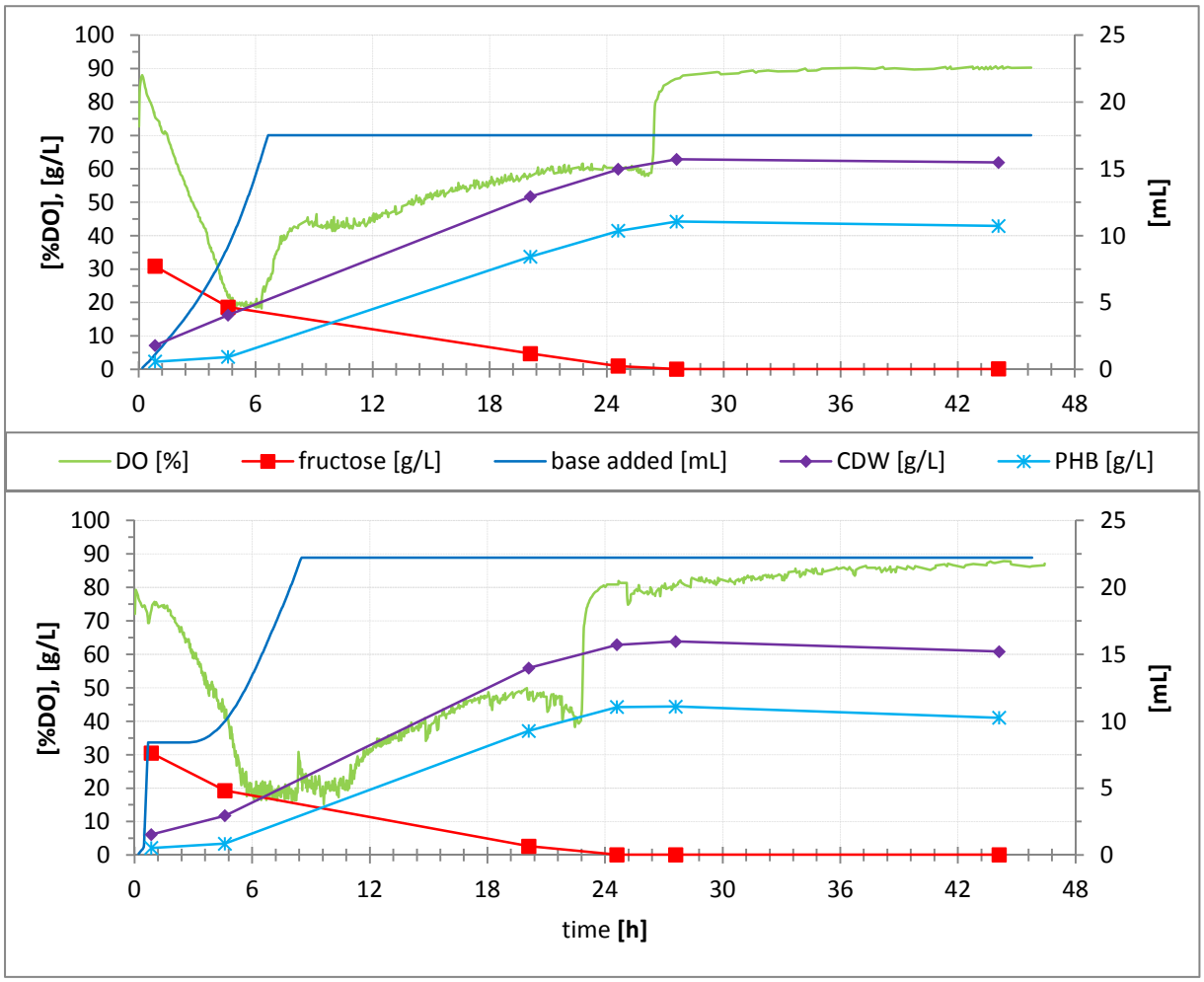

Figure 4: **The courses of a batch fermentation at 30 °C (upper diagram, fermentation PHB-MB_Ib / R2) and a batch fermentation at 37 °C (lower diagram, fermentation PHB-MB_Ib / R1).** The growth phase is shorter during the fermentation at 30 °C but the production phase is longer than in the fermentation at 37 °C, resulting in a longer duration for the overall process at 30°C.

While the amounts of PHB produced and the CDW obtained were quite similar, the time until all available sugar was consumed as well as the time needed for growth and PHB production varied considerably depending on the temperature. At 30 °C growth phase was 5.5 hours indicated, by a stop in base consumption. At 37 °C the duration of the growth phase in the two reactors was 7.5 and 6.1 hours respectively. A comparison of the course of fermentations at both temperatures can be found in **Figure 4**. At 40 °C the growth phase lasted the longest with 10 hours. The connection between the base consumption and growth lies in the fact, that the bacteria use the NH_3 in the media as nitrogen source during growth, thus the pH decreases and base has to be supplied to keep the pH stable.

The actual nitrogen concentrations were measured, but due to the gap of approximately 16 hours between the second and third sampling point, it could only be determined that after 20.1 hours of fermentation, no nitrogen was detectable in all four reactors.

The end of the production phase was reached after 26.4 hours at 30 °C, 22.9 and 20 hours at 37 °C and 23.6 hours at 40 °C. This is indicated mainly by the sharp increase of the DO concentration in the reactor, representing a sharp decline in metabolic activity, supposedly due to a lack of consumable sugars. The complete consumption of sugar is confirmed at the next sampling point after 27.6 hours.

The productivity was calculated for the sampling point closest to the maximum PHB concentration. For R1 this was the sample taken after 24.6 hours, for R2 after 27.6 hours, for R3 after 20.1 hours and for R4 24.5 hours. The volumetric productivity of PHB reached 0.45 g/Lh in R1, 0.40 g/Lh in R2, 0.53 g/Lh in R3 and 0.33 g/Lh in R4.

4.1.2 Assessment of influence of nitrogen limitation on PHB production (PHB-MF_II)

To assess the CDW and the PHB content reachable in the DASGIP system, when not under nitrogen limitation, four fed-batch fermentations were done. In contrast to R1 and R2, nitrogen limitations were not induced in R3 and R4. For details see **Table 4**. The CDW reached under both conditions was relatively similar, being 24.7 g/L, 21.8 g/L, 26.5 g/L and 21.4 g/L in R1, R2, R3 and R4. The PHB content differed greatly between nitrogen limited (R1 & R2) and not nitrogen limited fermentations (R3 & R4). In R1 64.6 % and in R2 53.7 % of the CDW were made up of PHB, but the values for R3 and R4 reached only 28.1 % and 11.1 %, respectively after 111 hours of fermentation.

The volumetric productivity of PHB resulting from these values was rather low in all reactors, being 0.14 g/Lh in R1, 0.10 g/Lh in R2, 0.07 g/Lh in R3 and 0.02 g/Lh in R4 over 111 hours of fermentation.

After 16.57 hours of fermentation the base was changed from NH_4OH to NaOH to induce a nitrogen limitation, but only after 51.07 hours of fermentation no more nitrogen was detectable in R1 and R2.

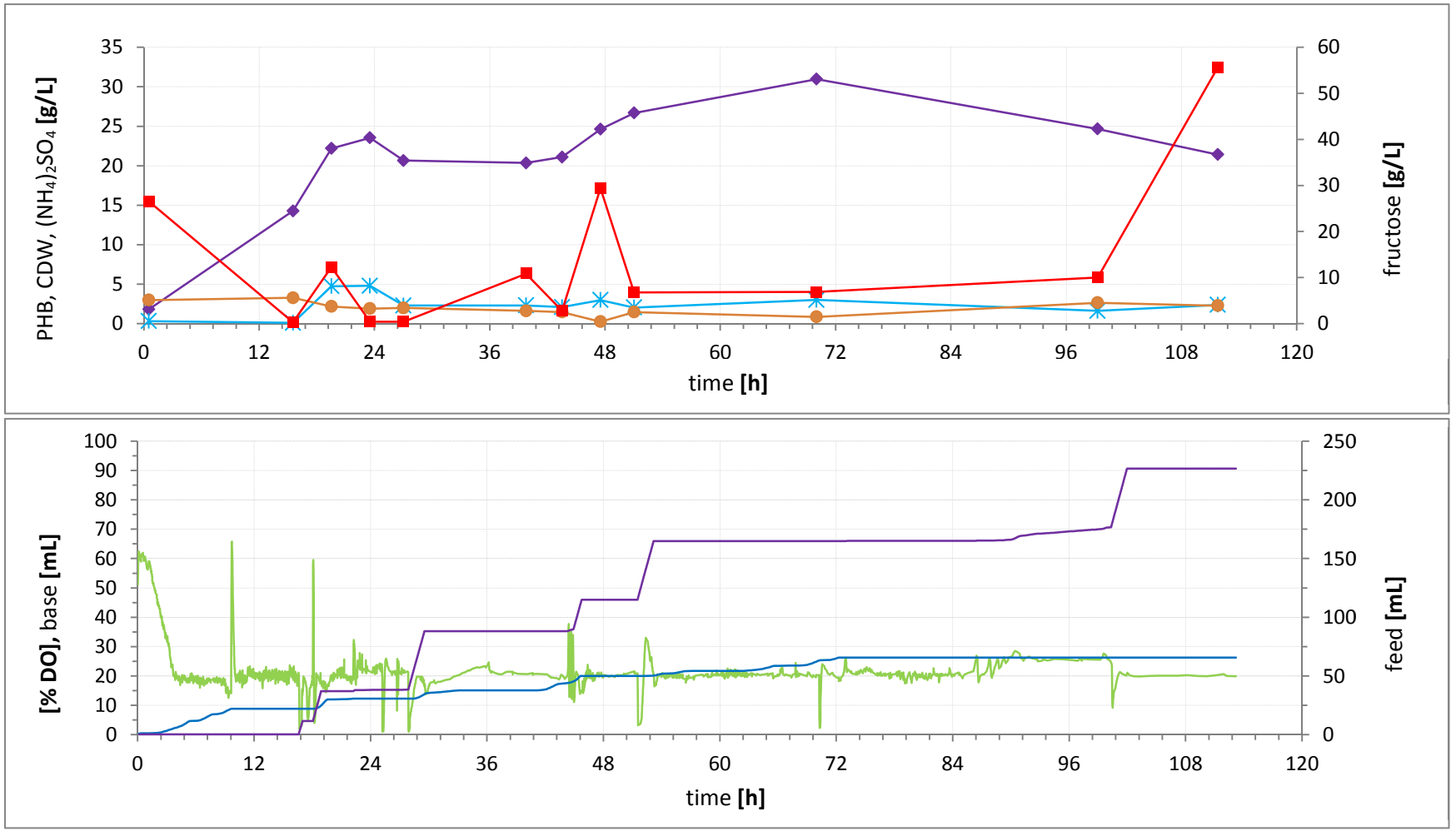

Figure 5: **Course a fed-batch fermentation without nitrogen limitation done during PHB-MCF_II (R4).** Fructose feed shots were added manually. ♦ cell dry weight concentration, × PHB concentration, ● $(NH_4)_2SO_4$ concentration, ■ fructose concentration, — amount of base added, — amount of feed added, — DO recorded

In R3 and R4 no nitrogen limitation was imposed and nitrogen was present during the whole fermentation. The fructose concentrations varied, reaching a maximum of 69.4 g/L in R1, 95.0 g/L in R2, 31.5 g/L in R3 and 55.6 g/L in R4 at the end of the fermentations and a minimum of 0.8 g/L in R1, 0.4 g/L in R2, 0.2 g/L in R3 and 0.3 g/L in R4 at various points during the fermentations. The course of an unlimited fermentation is shown in **Figure 5** using the example of the fermentation done in R4.

4.1.3 First cell recycling fed-batch experiment and batch experiments (PHB-MCB_IV)

One cell recycling fed-batch fermentation was done to assess the potential of this experimental setup in R2, while in R1 and R3 two parallel batch fermentations were done. In all following fermentations glucose was used as carbon source. For details see **Table 4**. For the batch fermentations similar results to fermentation **PHB-MB_Ib** (see section **4.1.1**) were achieved, reaching a CDW of 16.5 g/L (R1) and 16.7 g/L (R2) after a fermentation time of 20.3 h and 16.3 h respectively. The PHB content at this time also was comparable to previous results, reaching 72.8 % (R1) and 75.2 % (R3), the productivity up to this point being 0.59 g/Lh (R1) and 0.61 g/Lh (R3). In both batch fermentations no nitrogen was detectable after 16.3 hours, but the base consumption suggests that nitrogen limitation occurred after about 7.8 hours of fermentation. The PHB yields reached in the batch fermentations were 0.40 g PHB / g Glc in R1 and 0.41 g PHB / g Glc in R3.

In R2 the CDW reached a value of 36.2 g/L with a PHB content of 92.0 % after 24.3 hours of fermentation. As a result, the volumetric productivity of PHB was 1.4 g/Lh. The course of the fermentation can be seen in **Figure 7**. While the CDW concentration seems to increase after that point until it reaches 51.9 g/L after 40.3 hours, these values were, at least in part, a result of a continuing reduction of the amount of fermentation broth in the reactor due to evaporation and foaming (see **Section 5.1.3**). After 48.3 hours, the original level was approximately reached again through manual intervention, but the CDW did not increase further. Only the PHB content rose to 98.3% after 52.3 hours. The glucose concentration varied during the whole fermentation. A minimum of 4.9 g/l was observable after 16.3 hours, a maximum of 57.4 g/L at the end of the fermentation.

After about 17 hours the feed was changed from nitrogen containing mineral medium to nitrogen free, but nitrogen limitation seems to have occurred even before that. After 16.3 hours of fermentation, no nitrogen was detectable in the samples, indicating that nitrogen limitation took place in the time between 0.3 hours and 16.3 hours after inoculation and although the data is not entirely conclusive and small amounts of nitrogen definitely were supplied after that point, base consumption indicates, that a nitrogen limited environment was reached after about 7.7 hours of fermentation.

Due to the fact that the glucose concentration of the permeate could not be determined throughout the process, the yield for this process was calculated from the total amount of sugar used, being 56.9 g, and the amount of PHB produced, being 16.0 g, resulting in a yield of 0.28 g PHB / g Glc.

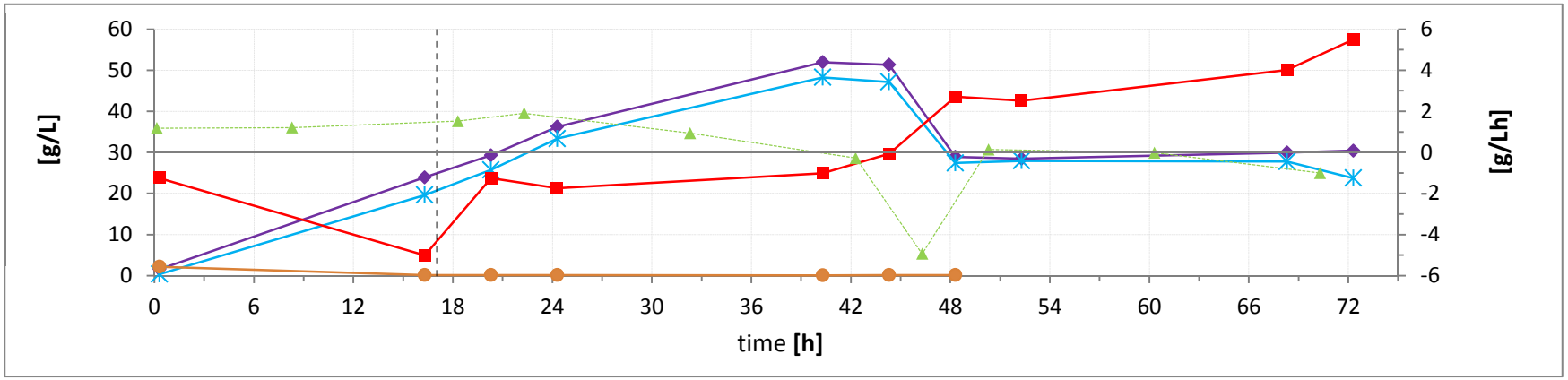

Figure 7: **Course of the cell recycling fed-batch fermentation during PHB-MCB_IV.** Change from N containing feed to N free feed at the dotted line. ♦ cell dry weight concentration, × PHB concentration, ● $(NH_4)_2SO_4$ concentration, ■ glucose concentration, ▲ PHB productivity between two sample points [g/Lh]

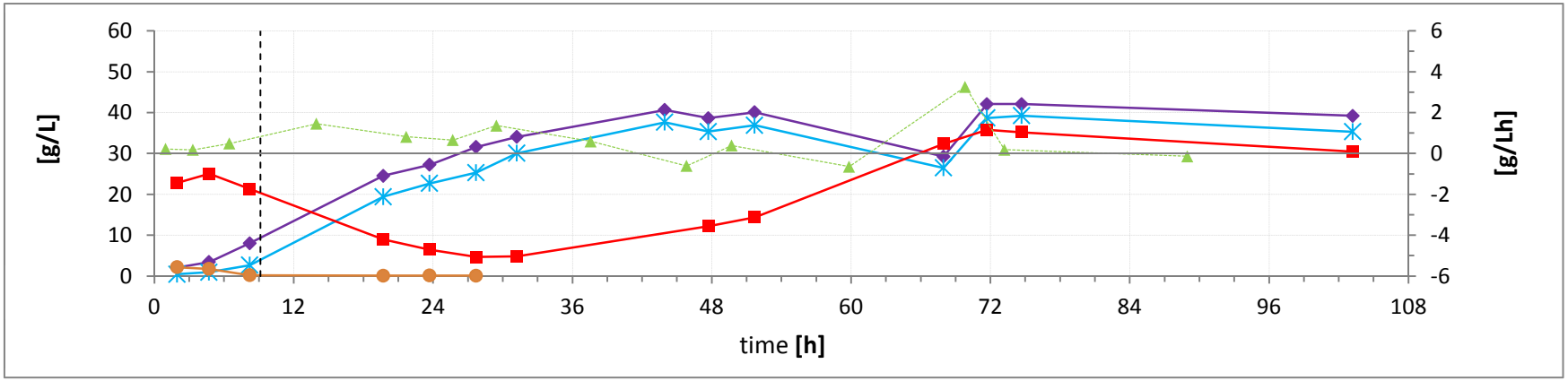

Figure 6: **Course of the cell recycling fed-batch fermentation during PHB-MCF_V.** Change from N containing feed to N free feed at the dotted line. ♦ cell dry weight concentration, × PHB concentration, ● $(NH_4)_2SO_4$ concentration, ■ glucose concentration, ▲ PHB productivity between two sample points [g/Lh]

4.1.4 Second cell recycling fed-batch experiment and fed-batch experiments (PHB-MCF_V)

To compare the performance of fed-batch and cell recycling fed-batch process, fed-batch fermentations were done in R1 and R3, and a cell recycling fed-batch process was done in R2. For details see **Table 4**. In R1 the CDW reached a maximum of 33.6 g/L with a PHB content of 73.7% after 74.7 hours of fermentation, while the CDW in R3 had reached a maximum of 56.7 g/L after only 44 hours. The PHB content in R3 at this time had reached 87.9 % resulting in a volumetric productivity of PHB of 1.13 g/Lh in R3. In R1 the volumetric productivity of PHB was 0.33 g/Lh after 74.7 hours. The course of the fermentation done in R1 can be seen in **Figure 8.** The big difference between R1 and R3 could be attributed to a faulty DO electrode in R3, which led to the automated addition of more feed to the reactor at the start of the fermentation due to the feed strategy chosen (see **Section 3.6.1**). The faulty DO electrode also caused the stirrer to permanently rotate faster during the whole fermentation, leading to a higher amount of oxygen being transferred into the fermentation broth. Nitrogen limitation could be observed after approximately 11.5 hours in R1 and 12.25 in R3, in both cases indicated by a sharp decline in base consumption at this time. This could further be substantiated by the fact, that no nitrogen could be detected in the samples drawn after 19.7 hours of fermentation, meaning, that nitrogen limitation had to have occurred before that time. For the fed-batch experiments, the PHB yield of the process was calculated as well, resulting in a yield of 0.39 g PHB / g Glc for R1 and 0.49 g PHB / g Glc for R3.

In the cell recycling fed-batch process (R2) the CDW reached after 44 hours was 40.6 g/L with 92.6 % of it being PHB. The volumetric productivity calculated from these values was 0.9 g/Lh while the process yield for the production of PHB reached was 0.28 g PHB / g Glc.

As can be seen in **Figure 6,** the glucose level during the fermentation varied between a maximum of 35 g/L and a minimum of 4.6 g/L and nitrogen limitation occurred in the period between 8,2 and 19.7 hours after the start of the fermentation. As in **PHB-MCF_IV** the limitation occurred even before the feed could be changed after 9.1 hours of fermentation, base consumption indicating, that after approximately 8.7 hours, the amount of nitrogen available to the bacteria was severely limited.

4.1.5 Third cell recycling fed-batch experiment (PHB-MC_VI)

The CDW reached in the cell recycling fed-batch process (for details see **Table 4**) was 47.1 g/L with a PHB content of 90.2 % after 44.9 hours. As a result, the volumetric productivity of PHB was 0.9 g/Lh while the calculated yield was 0.28 g PHB / g Glc.

As shown in **Figure 9** the change of feed to impose nitrogen limitation was done after 8.7 hours and the limitation actually occurred in the period between 8.2 and 20.9 hours after inoculation. A decline of base consumption after 10.5 hours indicates that after this point the cells were limited in nitrogen. The glucose concentration varied between a maximum of 34.2 g/L and a minimum of 1.8 g/L.

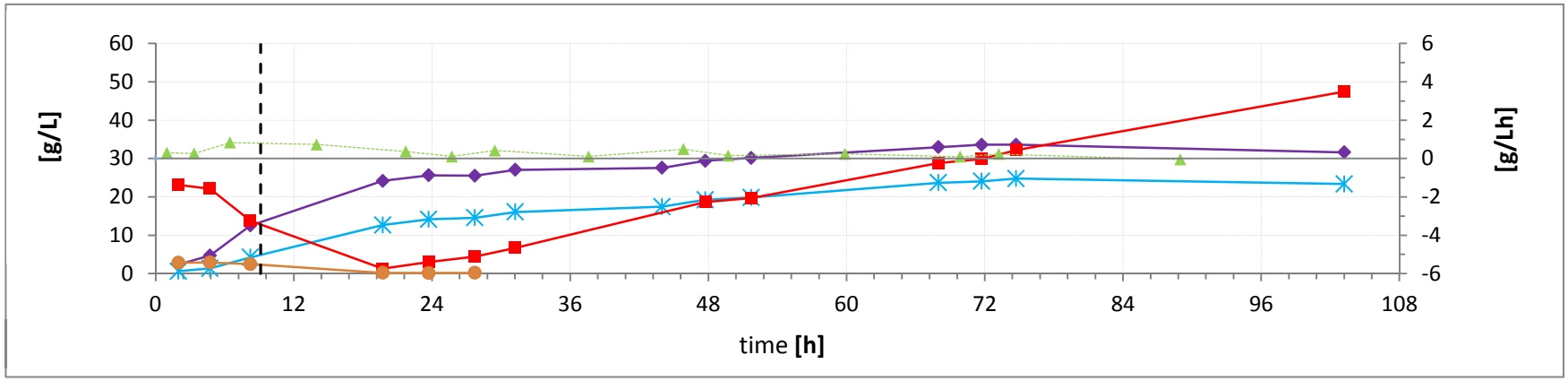

Figure 8: **Course of the fed-batch fermentation done during PHB-MCF_V (R1).** Change of the base from (NH4)OH to NaOH at the dotted line. ♦ cell dry weight concentration, × PHB concentration, ● $(NH_4)_2SO_4$ concentration, ■ glucose concentration, ▲ PHB productivity between two sample points [g/Lh]

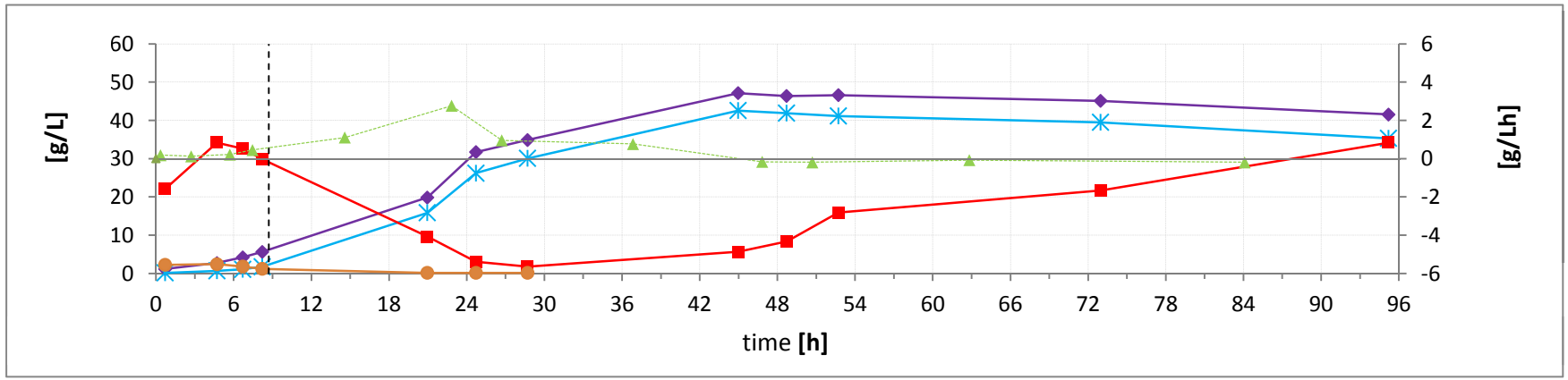

Figure 9: **Course of the cell recycling fed-batch fermentation during PHB-MC_VI.** Change from N containing feed to N free feed at the dotted line. ♦ cell dry weight concentration, × PHB concentration, ● $(NH_4)_2SO_4$ concentration, ■ glucose concentration, ▲ PHB productivity between two sample points [g/Lh]

4.2 Process development on the 3 L scale (modified Braun Biostat system)

4.2.1 Preliminary batch fermentation (PHB-MB_VII)

The first experiment in the Braun Biostat system was carried out as a simple batch fermentation (for details see **Table 4**). A CDW of 8.5 g/L with a PHB content of 76.9 % could be reached after 26.5 hours of fermentation resulting in an overall volumetric productivity of PHB of 0.2 g/Lh.

Nitrogen limitation occurred at some point between 3 and 15.75 hours of fermentation. Due to a lack of data for the amount of base added, this time span could not be narrowed down to one point of time.

Sugar was available during the whole fermentation, continually decreasing from a maximum of 22.4 g/L after 3 hours to a minimum of 6.5 g/L after 26.5 hours, when the fermentation was terminated. The overall PHB yield calculated for the process only reached 0.2 g PHB / g Glc.

4.2.2 First fed-batch fermentation (PHB-MF_IX)

An additional fed-batch process was done (for details see **Table 4**), in which the CDW reached a maximum of 62.3 g/L with a PHB content of 73.3 % after 41.5 hours of fermentation, resulting in a volumetric productivity of PHB of 1.1 g/Lh.

The sugar concentration remained at a rather low level during most of the process. The highest glucose concentration reached was 13.0 g/L after 17 hours of fermentation, but then it was constantly decreasing until a value of around 1 g/L was reached after 23 hours of fermentation. This level was maintained throughout the rest of the process. From the amount of sugar used and the PHB produced a process yield of 0.43 g PHB / g Glc was calculated.

To impose a nitrogen limitation the base used for pH control was changed from NH_4OH to NaOH after 17.8 hours of fermentation and the limitation actually occurred in the time span between 19 hours and 21 hours of fermentation. When looking at the base consumption, it can be assumed that nitrogen limitation had occurred after 19.1 hours of fermentation.

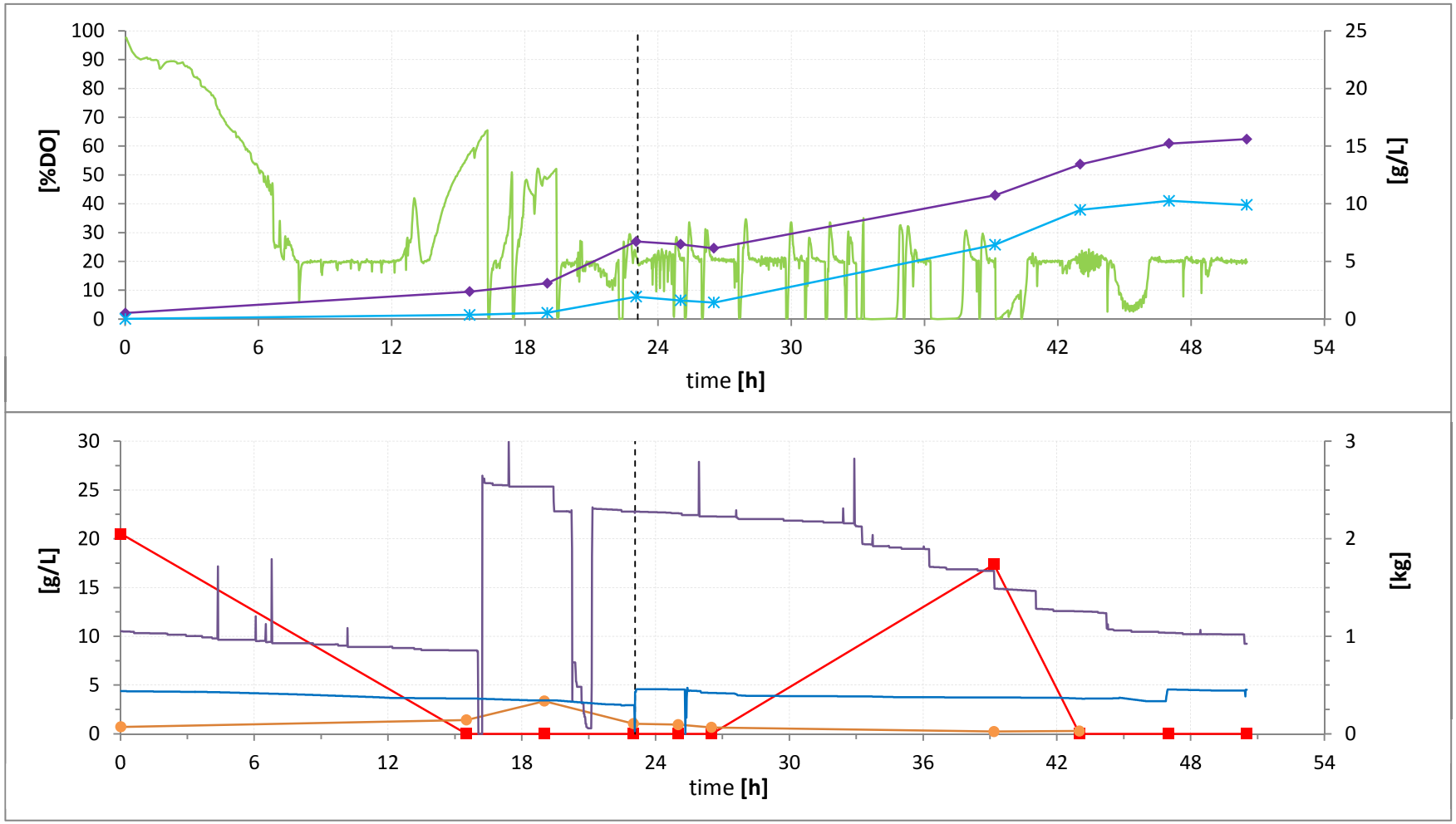

Figure 10: **Course of the fed-batch fermentation done during PHB-MF_IXb.** Change of the base from (NH4)OH to NaOH at the dotted line ♦ cell dry weight concentration, × PHB concentration ● $(NH_4)_2SO_4$ concentration, ■ glucose concentration — weight of the base bottle, — weight of the feed bottle, — DO recorded

4.2.3 Second fed-batch fermentation (PHB-MF_IXb)

The fed-batch experiment was repeated under slightly changed conditions (for details see **Table 4**) and this time yielded a DCW of 15.2 g/L with a PHB content of 67.4 % after 47 hours of fermentation. The volumetric productivity of PHB calculated was 0.2 g/Lh, the PHB yield reached a value of only 0.04 g PHB / g Glc.

To impose nitrogen limitation, the feed was changed after 28.08 hours and the nitrogen limitation occurred between that and 39.2 hours of fermentation. The determination of the glucose concentrations showed that there were only two sampling points at which sugar could be detected. As seen in **Figure 10** at the beginning of the fermentation, the glucose concentration was 20.5 g/L and after 39.2 hours of fermentation 17.3 g/L were measured. In all other samples no sugar was detected.

4.2.4 Cell-recycling fed batch fermentation (PHB-MC_X)

In a final experiment, a cell recycling fed-batch process was done in the Braun Biostat system resulting in a CDW of 44.2 g/L with a PHB content of 60.9 % after 48 hours of fermentation. The volumetric productivity of PHB reached in this process was 0.56 g/Lh.

From the measurement of the nitrogen concentrations in the samples, it can only be inferred that nitrogen limitation had to have occurred in the interval between 26 hours and 39.17 hours of fermentation.

The level of glucose available during the fermentation was relatively low throughout the fermentation. In many cases, no glucose was detectable in the samples at all, in others the level was below 3 g/L. Two notable exceptions are the sample from the beginning of the fermentation, where a glucose concentration of 21.7 g/L was determined and the sample taken after 42 hours, where the concentration was 14.7 g/L.

The slight increase in glucose concentration between the sampling points at 15.5 and 19 hours of fermentation seen in **Figure 11** is a result of a fast replacement of sugar free medium in the reactor with fresh feed medium. The prominent increase of the sugar concentration and the decrease in CDW measured at the sample point after 42 hours can be contributed to the fact, that after 40.8 hours, a feed-shot was triggered by an increase of the DO above the threshold of 35 %, and 1.5 L of feed media were added to the reactor. The volume was subsequently reduced through the membrane, but had not yet reached the original volume when the sample was taken after 42 hours.

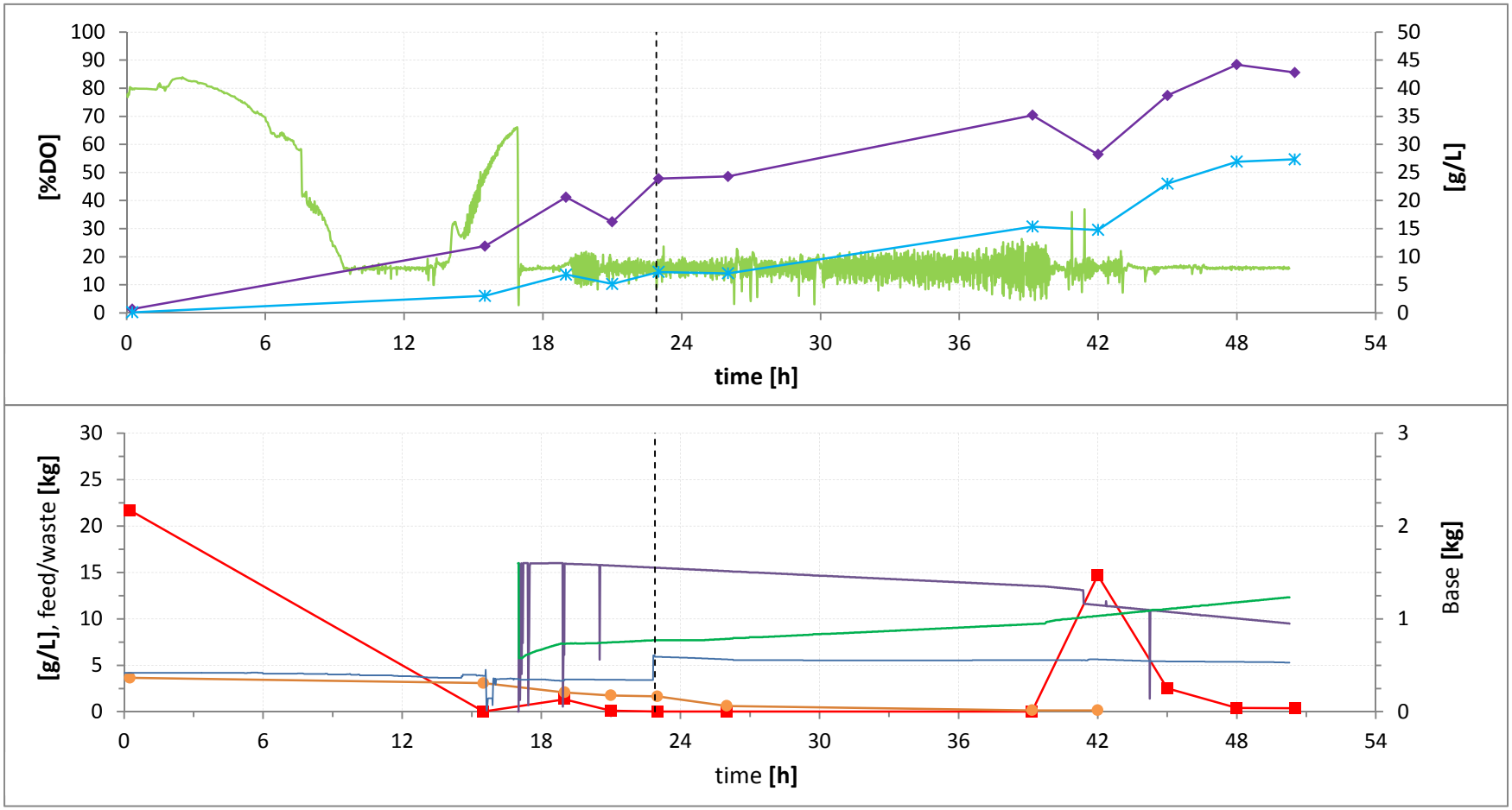

Figure 11: **Course of the cell recycling fed-batch fermentation done during PHB-MF_X.** Change of the base from (NH4)OH to NaOH at the dotted line ♦ cell dry weight concentration, × PHB concentration ● $(NH_4)_2SO_4$ concentration, ■ glucose concentration, — DO recorded, — weight of the base bottle, — weight of the feed bottle, — weight of the waste bottle
Due to lucidity reasons, the last two values are only shown from 17 hours of fermentation onward.

4.3 Summary of all results

To give an overview of the performance of the different processes used in this work, the most relevant results of the experiments are shown in **Table 8** The experiments shown in the first section were done on the 0.5 L scale (DASGIP system), the experiments in the second section on the 3 L scale (modified Braun Biostat system).

Table 8: Overview of the most important results of the experiments.

	reactor	mode of cultivation	CDW [g/L]	PHB [%]	duration [h]	PHB productivity [g/Lh]	PHB yield [gPHB/gGlc]
PHB-MB_Ib	R1	batch	15.7	70.4	24.6	0.4	-
	R2	batch	15.7	70.4	27.6	0.4	-
	R3	batch	15.6	69.3	20.1	0.5	-
	R4	batch	13.2	62.1	24.5	0.3	-
PHB-MF_II	R1	fed-batch	24.7	64.6	111.0	0.1	-
	R2	fed-batch	21.7	53	111.0	0.1	-
	R3	fed-batch	26.5	28	111.0	< 0.1	-
	R4	fed-batch	21.4	11	111.0	< 0.1	-
PHB-MCB_IV	R1	batch	16.5	72.8	20.3	0.6	0.40
	R2	cell recycle	32.6	92	24.3	1.4	0.28
	R3	batch	16.7	74.0	20.3	0.6	0.41
PHB-MCF_V	R1	fed-batch	33.6	73.7	74.7	0.3	0.39
	R2	cell recycle	40.6	92.6	44.0	0.9	0.28
	R3	fed-batch	56.7	87.9	44.0	1.1	0.49
PHB-MC_VI	-	cell recycle	47.1	90.2	44.9	0.9	0.28
PHB-MB_VII	-	batch	8.5	76.9	26.5	0.2	0.21
PHB-MF_IX	-	fed-batch	62.3	73.3	41.5	1.1	0.43
PHB-MF_IXb	-	fed-batch	15.2	67.4	47.0	0.2	< 0.1
PHB-MC_X	-	cell recycle	44.2	60.9	48.0	0.6	0.22

5 Discussion

5.1 Process development on the 0.5 L scale (DASGIP)

5.1.1 Preliminary experiments

To get a better understanding of the behaviour of the *C. necator* strain DSM 545 in the mineral medium used, a few preliminary experiments were done, investigating the optimal fermentation temperature and the effect of nitrogen limitation on the organism. Due to the fact that a change in carbon source was necessary due to logistical reasons, the effect of this change was also briefly investigated.

In **PHB-MB_Ib** the focus was on the determination of the optimal temperature for further experiments. In many published experiments with *C. necator* fermentations were conducted at 30 °C (Ryu et al., 1997; Du et al., 2001; Riedel et al., 2012; Shang et al., 2003), but higher temperatures seem to be possible as well (Cavalheiro et al., 2012).

Consequently, two fermentations were done at 37 °C, which were compared to one at 30 °C and one at 40°C (for details see **Table 4**).

For the determination of the "end point" of the fermentation two things were taken into account. On one hand it, was assumed that the time at which the sample with the maximum amount of PHB was detected was the end point of the fermentation. This is based on the assumption that, after nitrogen limitation came into effect, sugar was constantly used for PHB production. Due to the fact that the PHB contents reached were below the reported maxima, it can be assumed that PHB production only stopped after all sugar was consumed. After this end point PHB would only be degraded by the bacteria due to a low sugar environment.

On the other hand, the time at which all available sugar had been consumed was also determined using DO measurements. These two factors are linked by the fact that metabolic activity decreases when no sugar is available, leading to a sharp decrease in oxygen consumption and as a result a sharp increase in DO concentration.

The time points determined using both methods roughly correspond with each other, although there are varying deviations. These deviations can be explained by the fact that, while samples for the determination of PHB concentration were taken in rather long intervals, the DO was measured online. As a result, the durations gained by looking at the measured DO appear to be more accurate.

When looking at the results in section **4.1.1**, it is noticeable that, while almost equal amounts of PHB are produced, the total time needed to do so is longer at 30 °C than at 37 °C. This is most likely a result of a faster PHB accumulation at 37 °C than at 30 °C because data suggest that the growth phase actually is faster at the lower temperature (see **Figure 4**).

While the growth phase was 0.5 to 2 hours shorter at 30 °C, the PHB production took 3 to 6 hours longer compared to 37°C. This makes it favourable to use a process temperature of 37 °C. Due to the behaviour described above it would be interesting to use two different temperatures during the process in future fermentations, starting at 30 °C for favourable growth conditions then heating the reactor up to 37 °C during PHB accumulation.

When looking at the duration of the process, it seems as if the process at 40 °C is comparable to the process at 37 °C in speed, albeit a bit slower, but it has to be noted that considerably less PHB was produced. While this was definitely a result of a lower sugar concentration (see **Section 4.1.1** / **Table 4**) the PHB productivities are also lower throughout the process.

The lack of sugar would explain an overall lower productivity, as the amount of sugar available limits the amount of PHB that can be produced. However, productivities were also low between sampling points at the beginning of the fermentation when fructose was still available in most probably sufficient amounts. This indicates that the decreased productivity most likely results from the negative impact of the higher temperature. Therefore the temperature chosen for all following fermentations was 37° C.

The fed-batch fermentations in **PHB-MF_II** were done to compare the behaviour of the bacteria during presence and absence of nitrogen.

It is clear that the PHB content of the two reactors in which a nitrogen limitation was imposed had a higher PHB content than those in which no limitation occurred. Despite the huge impact that nitrogen limitation has on the PHB content, the results show that the *C. necator* strain DSM 545 in fact also produced PHB when no limitations were imposed. These findings are in line with data found in the literature (Du et al., 2001), although the observed PHB content, reaching a maximum of 28 % PHB, is higher than the reported 10 %.

Due to a lack of a complete analysis of the media, it is possible that this increased PHB content is a result of a limitation of a nutrient other than nitrogen. Another explanation would be a temporary limitation of nitrogen in the time between the sampling points caused by an inconsistent supply of nitrogen due to the volatility of the 25 % ammonium hydroxide solution, although this seems highly unlikely.

That said, the volumetric productivity in the fermentation without nitrogen limitation was still rather low, underlining the necessity for a nitrogen limitation.

In the experiments where a nitrogen limitation was imposed, the productivities reached were nevertheless rather low, the 0.1 g/Lh being far worse than published values like 0.42 g/Lh (Khanna and Srivastava, 2005b; Ryu et al., 1997) or even 3.14 g/Lh (Ryu et al., 1997).

This may be a result of the fact that the fructose levels in all fermentations dropped to values close to zero during growth or shortly after the nitrogen limitation was induced. The fructose limitations

mentioned were a result of the very crude feeding strategy that resulted in an unsteady fructose supply (see **Section 3.6.1**).

It is also noteworthy that the nitrogen limitation was induced relatively late in the process and the PHB content determined in both fermentations was rather low. A PHB content of only approximately 60 % could be reached, while PHB contents of up to 82 % of the dry weight are reported (Du et al., 2001; Ryu et al., 1997) and even values up to 90 % are supposed to be possible (Riedel et al., 2012).

The DO data for R1 suggest that a further increase in PHB content would have been possible, as no signs of a reduced metabolic activity can be seen, but the fact, that the sugar concentration determined was rather high suggests otherwise. A further indicator that the organism stopped accumulating PHB is the slightly decreasing PHB content observable between the last two sample points of the fermentation. In R2, the process seems to have been a bit faster than in R1, and in addition to the phenomena described above also showed an increase of the DO value at the end of the fermentation.

A limitation of nutrients other than nitrogen and sugar could be an explanation for the observed end of the PHB accumulation. Since only sugar and nitrogen were fed into the reactor this seems plausible, but could not be proven, because nutrient concentrations other than sugar and nitrogen were not measured and no trace element analysis was done.

For all further experiments glucose was used as carbon source instead of fructose. To assess, if this change has a detectable impact on the bacteria, it stands to reason to compare the results from the batch fermentations of **PHB-MCB_IV** and the relevant batch fermentations from **PHB-MB-Ib**. Apart from the different carbon source both sets of fermentations were basically done under the same conditions, for details see **Table 4**.

The CDW reached and the volumetric productivity of PHB for the fermentations done with fructose are slightly lower than for the fermentations done with glucose, but the deviations are rather small. For details see **Table 8**. This suggests that, at least for the *C. necator* strain DSM 545, the effect on the process performance due to the change in carbon source is negligible.

5.1.2 Fed-batch

The goal of this work was to establish a cell recycling fed-batch process using a medium with low carbon content. To get data for comparison, two conventional fed-batch fermentations using a highly concentrated sugar solution as feed were done in experiment **PHB-MCF_V**. A concentrated sugar feed was used, because similar processes have been reported to yield high cell densities and high volumetric productivities of PHB (Ryu et al., 1997; Shang et al., 2003).

As a result of a defective DO electrode in one of the two reactors, large amounts of sugar were supplied to the reactor at the very beginning and repeatedly throughout the fermentation. Despite this, the growth period was relatively similar in both fed-batch fermentations yielding very similar CDW and PHB content in approximately the same time. Unfortunately there were no samples drawn in the

period after the induction of the nitrogen limitation, as at the next sample point a big difference between both fermentations can be observed. The PHB content of the cells in the "regular" fermentation reached only about 52 %, whereas in the "faulty" fermentation 74 % was reached in the same amount of time and even increased further up to 91 %.

It is striking that at first the absolut amount of residual biomass, in this case defined as the difference between CDW and the amount of PHB, remained relatively constant in both fermentations (data not shown) but then decreased rapidly in the "faulty" fermentation. While a decrease of residual biomass was not uncommon in all experiments done, the degree and speed at which it took place here was much higher and cannot be explained.

The increased PHB content in the "faulty" fermentation can be explained with higher sugar content and a higher availability of oxygen during the phase after the nitrogen limitation was induced, in which a huge part of the PHB accumulation seems to be taking place. As seen in **Figure 12**, the volumetric productivity between two sample points even reached 2.51 g/Lh in the "faulty" fermentation, while it dropped to 1.13 g/Lh until the peak in PHB concentration was detected.

The data in combination with the sampling scheme furthermore suggest that the actual productivity during this phase was even higher than the values stated above, but could not be recorded.

This high productivity right after the induction of the nitrogen limitation should also be visible in a rapid decrease in sugar concentration if the feed rate stays constant during this phase. In the process that went as planned, this condition is fulfilled, and it is indeed the case that the sugar concentration drops from 13.7 g/L to 1.2 g/L in the period in question. In the "faulty" fermentation the feed rate was not constant during this phase due to the addition of a feed shot. Despite this additional sugar that was available in the sample interval, a similar drop in sugar concentration from 27.8 g/L to 2.1 g/L could be observed.

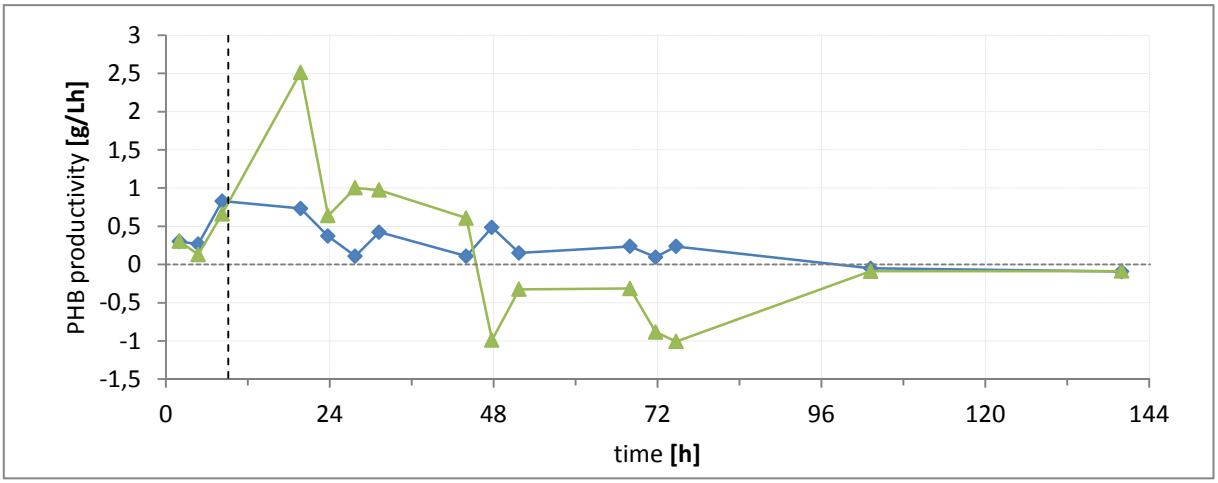

Figure 12: **PHB productivities between two sampling points for both fed-batch fermentations done in PHB-MCF_V**. The dotted vertical line represents the induction of a nitrogen limitation.
▲ PHB productivities during fermentation in R3 ♦ PHB productivities during fermentation in R3

The decrease in volumetric productivity after this phase may on one hand be a result of the increasing stress for the bacteria due to the prolonged nitrogen limitation on the other hand the quantity of PHB inside the cell may play a role. Due to an increase in size of the PHB granules inside the cell, a huge amount of the cytoplasm is filled by these granules, imposing further stress on the organism (see **Figure 3**).

The volumetric productivities of 0.3 g/Lh (R1) and 1.1 g/Lh (R3) were way below the highest values reported in literature (Shang et al., 2003; Ryu et al., 1997) but in those cases feeds with an even higher sugar concentration were used. For a process using a feed of similar concentration a productivity of 0.4 g/Lh was reported (Khanna and Srivastava, 2005b). Although this was not a fed-batch but a repeated batch, the values would be in line with the productivity reached in R1.

When looking at the PHB yields of the fed-batch experiments, one notices that the amount of PHB could be produced from one gram of glucose in R1 was comparable to the yields reached in the two batch fermentation of **PHB-MCB_VI** and the yield calculated for in R3 seems to be even higher.

All of these calculated yields are rather high, when taking into account that the maximal theoretical PHB yield for glucose is reported to be 0.48 g PHB / g Glc (Yamane, 1993). While the yields of the batches and the yield in R1 of **PHB-MCF_V**, all being approximately 0.4 g PHB / g Glc, seem – although high – plausible, the yield of 0.49 g PHB / g Glc calculated for R3 does not.

This most certainly is resulting from the way the yield was calculated and in association a problem determining the exact volume in the reactors at each point of time. The calculation of the amount of glucose added does not depend on the volume of the fermentation broth in the reactor, but the amount of PHB produced and the amount of glucose still in the reactor do due to the fact that both values were derived from measured concentrations.

This problem mainly concerned the fed-batch and cell-recycling fed-batch processes in this work. In batch processes, albeit present, the influence is not that big, due to the fact, that no big volumes are added, and volume loss is only possible through evaporation.

The starting volume and the volumes added to the reactor were of course recorded for all processes, but when higher cell densities were reached, the formation of foam started becoming a problem. This is relevant due to the fact that foam of unknown composition was pushed through the off air tubes into the bottle of NaOH through which the off air was channelled. Antifoam addition eased this problem, but due to a lack of possibilities for an automated antifoam addition, antifoam could only be added manually. The foaming possibly leads to a heightened CDW concentration in the reactor but probably does not affect the glucose concentration that much. Due to the fact that these volume losses could not be measured and no on-line measurement of the actual volume of the fermentation broth was possible, all absolute values derived from concentration based measurements have an added inaccuracy that is difficult to estimate.

Thus a solution for this problem is urgently necessary with an on-line measurement of volume or mass of the fermentation volume being one option. The other possibility would be to find a solution to the foaming problem, either by a technical solution to catch the foam and pump it back into the reactor, or by the automated addition of antifoam.

When looking at the base consumption of the process it is also striking that, in addition to the expected peak during the growth phase, the base consumption increased again, approximately when the PHB degradation began. This phenomenon is clearly visible in both fed-batch experiments from **PHB-MCF_V.** In the fed-batch fermentations from **PHB-MF_II** the beginning of a similar behaviour can be guessed, but the processes were terminated before bigger amounts of base were added to the reactors. This phenomenon was not thoroughly investigated, but HPLC analysis of both fermentations showed an unexpected peak that is thought to represent free 3-hydroxybutyrate. This assumption is based on the fact that a reference sample of pure 3-hydroxybutyrate showed a very similar retention time, but the substance could not be identified without question.

5.1.3 Cell recycling fed-batch

In total there were three cell recycling fed-batch experiments done in the DASGIP system with two different membrane systems, an ultrafiltration membrane and a microfiltration membrane. For details see **Table 4** and section **3.3.2**.

When looking at the results of all three cell recycling fed-batch processes, the first thing that stands out is the very high PHB content of approximately 90 % of the cell dry weight. When compared to other processes, the value still ranges among the highest reported, with Ryu et al. reaching 82 %, Riedel et al. reaching 73.5 % and Atlic et al. reaching 77 %. In all these processes *C. necator* was used, but the processes were either fed-batches (Ryu et al., 1997; Riedel et al., 2012) or continuous processes (Atlić et al., 2011).

While this does not apply to continuous processes the higher PHB content in comparison to fed-batch experiments is possibly explainable by the fact that all trace elements and nutrients with the exception of nitrogen were provided throughout the process and all excreted substances were permanently removed from the process, resulting in better conditions and less additional stress for the bacteria.

Another factor definitely supporting the accumulation of a high amount of PHB in this case is that the nitrogen limitation occurred very early in the process, giving the bacteria a relatively long time period in which no growth was possible, but PHB could be accumulated. The growth rates at the beginning of the fermentations are comparable to fed-batch processes, but in one of the fermentations the nitrogen limitation had already occurred way before the feed was changed, in one the limitation occurred approximately when the change was made and in only one enough nitrogen was supplied to induce the limitation by the feed change.

The described phenomenon can probably be attributed to a mismatch of the C:N ratio in the media and the actual amounts of nitrogen and glucose consumed. Supposedly not enough nitrogen was provided to allow a full utilization of the provided glucose for bacterial growth. This assumption is further substantiated by the fact that the nitrogen limitation occurred the earliest in **PHB-MCB_IV,** a fermentation in which the feed rate was higher during the first phase of the fermentation (for detailed fermentation conditions see **Table 4**).

This lack of nitrogen may on the one hand be a result of a general problem with the media composition. On the other hand there was an unknown precipitate formed during media preparation in all cases. This precipitate may have contained at least a part of the added nitrogen, reducing the concentration in the media itself. Nevertheless the question remains, why the precipitation should have been more extensive in the feed bottle than in the reactor itself. An increased amount of nitrogen in the feed definitely would be a solution for this problem, but a more elegant solution seems to be to provide the nitrogen and a nitrogen-free feed to the process separately. This may on one hand eliminate the problem of the precipitate formation in the media bottle, and on the other hand provides the opportunity to better control the amount of nitrogen supplied to the reactor.

Due to the described problem in nitrogen supply, the desired biomass concentration was not reached before the PHB accumulation started. Due to the fact that the capacity to store PHB in the cell is limited, the amount of biomass formed during the initial growth phase is one of the main factors determining how much PHB can be produced by a process and as its productivity. Nevertheless, the productivity reached in all experiments was comparable to the performance of the fed-batch fermentations.

The variation in the productivities between the first cell recycling fed-batch process (1.4 g/Lh) and the other two processes (0.9 g/Lh) can probably be explained by a difference in the feed rate at the beginning of the fermentation. In **PHB-MCB_IV** the feed rate during bacterial growth was 40 mL/h, which was later reduced while in **PHB-MCF_V** and **PHB-MC_VI** the feed rate of 20 mL/h was maintained throughout the process. It is assumed that the amount of available sugar was higher in **PHB-MCB_IV**, which resulted in a higher biomass growth, thus resulting in a higher overall productivity.

It appears that only one process using a similar setup has been published, in which a productivity of 1.1 g/Lh was reported (Ienczak et al., 2015). So the comparison was extended to other published processes using the same organism. The productivities reached in other fed-batch and continuous processes were for example 1.1 g/Lh in a fed-batch process using palm oil (Riedel et al., 2012), 1.23 g/Lh in a two stage continuous process (Du et al., 2001) and 1.85 g/Lh in a five stage continuous process (Atlić et al., 2011). That said, there are also reports of productivities way higher than those, reaching even 3.1 g/Lh (Shang et al., 2003) and 3.14 g/Lh (Ryu et al., 1997) in fed-batch processes utilizing highly concentrated glucose solutions as feed.

An interesting observation is that – like in the fed-batch experiments – the PHB productivities tend to be highest shortly after the nitrogen limitation supposedly has occurred, but later decrease and even get negative when the PHB is depolymerised and consumed by the bacteria (for details see **Figure 13**).

The pattern described above indicates that a further increase in productivity would be possible, if the phase of high productivity is optimally used. The first step to do this would be to induce the nitrogen limitation later in the process, thus letting the cells in the reactor grow to higher cell densities at the beginning of the process. This step alone would increase the productivity of the process due to the limitation posed by the intracellular polymer accumulation described above. If one now stops the process right after the phase of high productivity, the effect would be a further increased overall productivity of the process, despite a lower PHB content in the cells.

That said, the PHB contents encountered at about that point of time during the cell recycling fed-batch fermentations done in the DASGIP System usually was in the region of 80%, which is still rather high. Depending on the downstream processing necessary for the PHB extraction, it has to be weighed, whether a heightened PHB content of up to 90% but a lower overall productivity or the highest attainable overall productivityis desirable. In this work the end point was chosen as the point of time, at which the productivity was highest, but the PHB content was above 90%.

Another indicator for the performance of a process is the yield at which PHB can be produced from the substrate. Here the results from both cell recycling fed-batch processes were not as good as in the preceding fed-batch experiments.

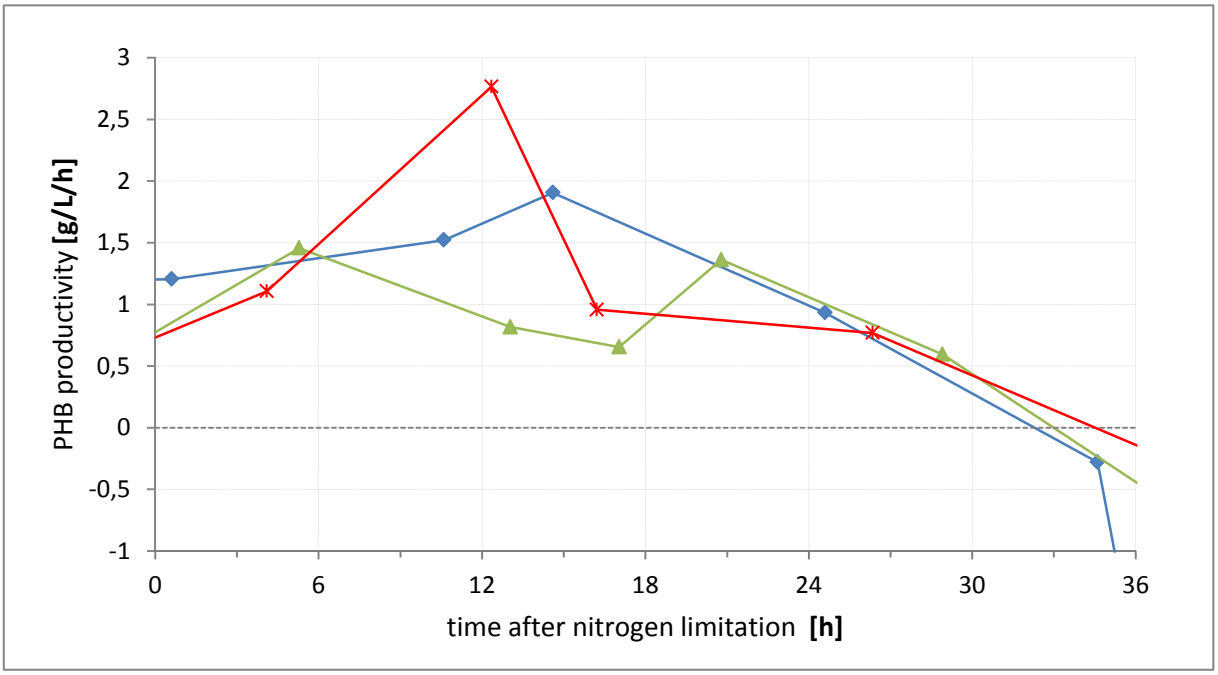

Figure 13: **PHB productivities between two sampling points over the course of fermentation time for three different cell recycling fed-batch processes.**
× PHB-MCB_IV ▲ PHB-MCF_V ♦ PHB-MF_VI.

In comparison to published processes with reported yields of 0.38 g PHB / g Glc (Ryu et al., 1997) reached in a fed-batch process and 0.36 g PHB / g Glc (Du et al., 2001) reached in a continuous process, the yield of 0.28 g PHB / g Glc reached in all cell recycling fed-batch fermentations was rather low. That said, the values were pretty much in line with the yield of 0.29 g PHB / g Glc (Atlić et al., 2011) reported for another continuous process.

The lower values in comparison to the fed-batch are probably a result of the fact that unused sugar can get out of the reactor in the cell recycling fed-batch processes used in this work as well as a continuous process. This wasted glucose is nevertheless included in the calculation for the overall PHB yield of the process, explaining the difference in yield stated above. This problem has to be addressed by optimising the feed-strategy in a cell-recycling fed-batch process or by adjusting the flow rate in a continuous process. This optimisation most probably explains the big difference between the two continuous fermentations mentioned above.

As a result of the chosen feed strategy, the glucose concentration was rather high throughout all three cell recycling fed-batch processes, only occasionally dropping below 5 g/L. The unutilized sugar was as a result constantly pumped out of the reactor vessel into the waste. Due to a lack of sampling this could not be substantiated by measurements, but a distinct discoloration of the permeate during terminal autoclaving was observable, most likely caused by Maillard reactions of the remaining glucose.

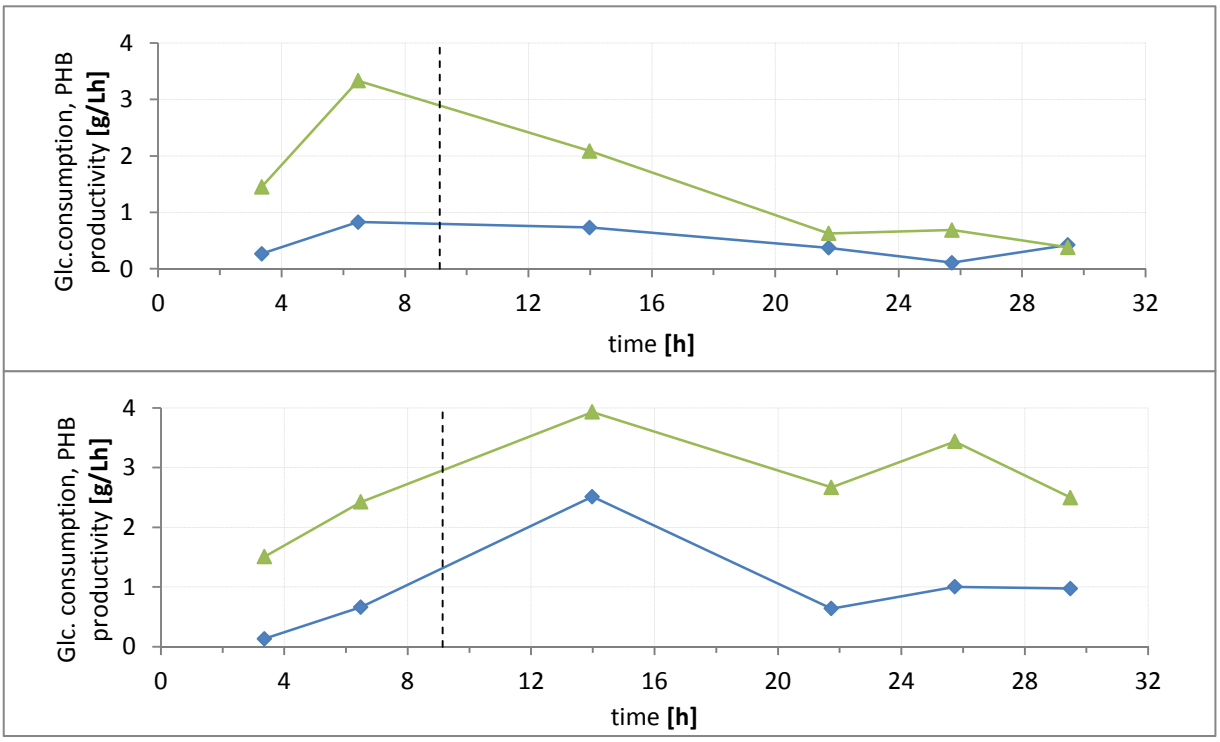

Figure 14: **Comparison of glucose consumption and PHB productivity.** The upper diagram shows values calculated for the fermentation done in R1 during PHB-MCF_V, the lower diagram shows the values for R3 during the same fermentation. The dotted line shows the time at which a nitrogen limitation was imposed. ▲ glucose consumption between two sampling points [g/Lh] ♦ PHB productivity [g/Lh]

This waste of substrate could be prevented by applying an optimised feeding strategy for this process, in a way that the glucose supplied and the glucose needed at each point of time in the fermentation is roughly equal. While the amount of glucose needed during the initial growth phase can be derived from the assumed exponential growth in this phase, the pattern after nitrogen limitation is less clear.

From **Figure 14** it can be inferred that glucose consumption follows a similar pattern as the PHB productivity. As seen in **Figure 13** the PHB productivity is rather high at first, but then decreases constantly. Correspondingly, the glucose consumption is rather high right after the nitrogen limitation is imposed, but, after a certain time the sugar consumption of the bacteria decreases. A feed based on this pattern seems possible, if the pattern was better understood and quantified, although the implementation of such a feed strategy will be rather complex. Other factors, such as the DO and the pH are also connected to growth and PHB accumulation, but the effects seen are rather small and possibly delayed, meaning that feed strategies solely based on these measurements would most probably result in extensive starvation periods. That said, feed strategies have developed for fed-batch processes combining an exponential feed and feeding based on the amount of base used during the growth phase. During the accumulation of PHB a constant feed rate is applied, (Mozumder et al., 2014), but this is not conclusive with the observations made during the fermentations done in this work. As shown in **Figure 14,** the glucose consumption is not constant after nitrogen limitation, making the feeding strategy problematic for the direct application in a cell recycling fed-batch process without adaptation. Despite the drawbacks the feed strategy chosen, a constant feed rate supplemented with defined "shots" in case the DO or the pH rose beyond a certain threshold (for details see **Table 4**) was used for all further experiments as well.

When comparing both membranes (see section **3.3.2**), the choice of membrane does not seem to influence the process extensively. The DCW, PHB content, productivity and yield are in a similar range in all three fermentations.

Like in the fed-batch fermentations, the formation of foam was problematic and the manual addition of antifoam could at best ease the problem. During varying periods of time, the foaming in all three fermentations was strong enough to push parts of the foam out of the reactor creating the same problem described above (see **Section 5.1.2**)

Despite the usage of antifoam, no problems with clogging of the membrane or the removal of permeate could be noticed during all fermentations.

5.2 Process development on the 3 L scale (modified Braun Biostat system)

5.2.1 Preliminary experiment

Due to the fact that the Braun Biostat reactor was completely overhauled and the control unit changed prior to the start of the experiments, the first fermentations were mainly used to get acquainted with the controls. During these first preliminary fermentations certain data could not be recorded or critical control parameters could not be maintained at the desired level throughout the fermentation process.

For one of these fermentations (data of the other fermentations not shown), a batch process labelled **PHB-MB_VII**, there are, for instance, no records of the DO levels in the reactor, the speed of the stirrer or the amount of base added throughout both processes. This fermentation yielded results that were significantly worse than those of the DASGIP batch fermentations. The time needed to reach a CDW approximately half as high as in the DASGIP system, was approximately six hours longer.

The difference in productivity can probably be attributed to a difference in the inoculum used. In both fermentations the content of one shake flask (200 mL, see **Section 3.4.1**) was used to inoculate the medium, but due to the much larger reactor volume in the Braun Biostat system, the initial concentration of cells in the reactor was much lower and the process therefore slower. It is possible, that a CDW comparable to the DASGIP system could have been reached, but the process was terminated before this could be determined. The fact that the process was terminated too early is further substantiated by the fact that the PHB content was still rising between the last two sampling points and sugar was still present in the reactor when the last sample was drawn.

The overall yield was considerably lower than in batch processes done in the DASGIP system, but this is most likely a result of the residual sugar in the reactor due to the early termination of the process.

5.2.2 Fed-batch

Due to the flawed data collected in the previous fed-batch experiments in the Braun Biostat reactor (data not shown), two additional fed-batch experiments were done when the problems were solved. As a result, DO data as well as stirrer speed and the amount of base used by the process could be recorded during these experiments (**PHB-MF_IX** and **PHB-MF_IXb)**. For both fermentations the conditions were very similar (see **Table 4** for details): a concentrated glucose solution was used as feed, nitrogen was provided using NH_4OH to control the pH and the inoculum was prepared the same way, but the results were rather different with the later fermentation performing considerably worse than the former.

The big difference between the two fermentations seems to be the course of the growth phase at the beginning of the fermentation. While in the fermentation **PHB-MF_IX** 18.2 g of biomass were accumulated during the first 17 hours, the amount accumulated in **PHB-MF_IXb** during the first 15.5 hours was only 2.38 g. An accurate comparison of the values at the same time of the fermentation process is not possible due to a difference in the sample scheme, but it can be inferred from the

recorded data that bacterial growth stopped after about 12 hours of fermentation. This is indicated by a rising DO concentration, a rapid drop of the stirrer speed, and a stop in consumption of NH_4.

A difference in the amount of oxygen supplied could influence the growth, and there had indeed been a difference between the two fermentations. Some minor changes in the DO control were made during **PHB-MF_IX** and between the two fermentations, on the one hand by raising the limits imposed for stirrer speed and gas flow. On the other hand, the way the DO was maintained was by prioritizing a DO control through changes in stirrer speed over a change in gas flow. This was done due to frequent problems with foaming when the gas flow spiked. Raising the upper limits for stirrer speed and gas flow mentioned above was necessary to allow the process control to sustain the set DO values during the process, especially the growth phase. As a result, the DO control worked significantly better in **PHB-MF_IXb** than in the **PHB-MF_IX** fermentation, during which the DO was allowed to drop to zero over an extended period of time.

While these changes certainly have an effect on the fermentations, they cannot explain the significantly worse performance in **PHB-MF_IXb**.

Due to the fact that an insufficient oxygen supply could more or less be ruled out, the most likely explanation is a lack of glucose.

This assumption is further substantiated by the fact, that at after 15.5 hours of fermentation no glucose could be detected and that every time glucose was subsequently supplied to the process, the DO suddenly dropped, respectively the stirrer speed spiked. While this seems conclusive, some other factors concerning the glucose supply and consumption do not.

When looking at the weight recorded from the scale the feed was placed on, one notices that the rate at which feed seems to have been added to the reactor was higher in **PHB-MF_IX** than in **PHB-MF_IXb**, although they should have been the same. While the set point was a feed rate of 12 g feed / h, the rate reached in **PHB-MF_IX** over the first 12 hours of fermentation was approximately 14.4 g / h, while in **PHB-MF_IXb** it was 13.2 g / h. This corresponds to a feed rate of 5.9 and 5.4 g Glc / h respectively, when using a feed density of 1.21 kg / L for the calculation.

This difference can be attributed to difficulties controlling the exact amounts of feed added with the peristaltic pumps and the control parameters used. The differences stated affected bacterial growth without any doubt and may, to a certain extent, be responsible for the slower growth in **PHB-MF_IXb** but they do not explain the huge difference in the biomass yield.

In **PHB-MF_IX** a biomass yield of about 0.4 g CDW / g Glc was reached during the first 17 hours, but the yield reached in **PHB-MF_IXb** was drastically lower (only about 0.03 g CDW / g Glc during the first 15.5 hours). The different time periods for which the yield was calculated were caused by the different sampling scheme.

While a rough comparison to the biomass yield calculated from fed-batch experiments done in the DASGIP reactor (data not shown) would suggest that the value of 0.4 g CDW / g Glc is more

reasonable for a working process than a value in the scale of 0.03 g CDW / g Glc, it can only be speculated on the reasons for this discrepancy.

One could presume, that due to some unknown inhibition, growth was hindered and the sugar was solely used to sustain the living bacteria. However, this would neither explain the rapid response to the feed shots that contained nothing except sugar, nor does it seem likely, that the amounts of glucose needed to sustain viability are that high. Another option that cannot be verified by the data recorded, is that the sugar concentration in the feed was – due to some unknown reason – not as high as it was supposed to be.

The induction of PHB production by a change of base used for pH control worked rather well in both fermentations and the difference in NH_4 concentration of the base used did not have a big impact. In both fermentations similar fractions of the biomass were made up of PHB after a similar amount of time, albeit the total amount of PHB produced and therefore the PHB productivity obviously was lower in **PHB-MF_IXb** due to the problems accumulating biomass during the growth phase discussed before.

A comparison of the overall performance of both fermentations to the ones reached in the DASGIP system and to other published results show that the productivity of 1.1 g/Lh reached in **PHB-MF_IX** is in line with the value reached in one of the DASGIP fed-batches (**PHB-MCF_V** / R3, see **Section 4.3**) and other published results (Riedel et al., 2012). The overall PHB yield is also quite similar for the DASGIP processes and **PHB-MF_IX**.

The productivity of 0.3 g/Lh reached in **PHB-MF_IXb** is also comparable to a result reached in the DASGIP system (**PHB-MCF_V** / R1), but lower than most productivities reported for other fed-batches (for details see **Section 5.1.2**). While this can be explained by the low cell density reached in the process, there is no explanation for the low overall PHB yield. According to calculations, the overall yield for this process is only 0.04 g PHB / g Glc being almost one order of magnitude lower in **PHB-MF_IXb** than in **PHB_MF_IX**.

The only likely explanation is that the glucose concentration of the feed used in **PHB_MF_IXb** was way below the concentration it was supposed to be, which would also be in line with the indications stated earlier.

Like in the DASGIP fermentations, the generation of foam was problematic and could not sufficiently be controlled by the addition of antifoam. The resulting volume loss in the reactor due to the fact that foam was pushed out of the reactor through the off air tubes creates the same problem with all concentration based measurements mentioned before (see **Section 5.1.2**).

Due to the drastic difference between the two fermentations in both systems, the lack of repetitions and the mentioned problem concerning the volume loss, no conclusive results can be gained from the comparison of the performance of both systems.

5.2.3 Cell recycling fed-batch

As a conclusion of the work, one cell recycling fed-batch fermentation (**PHB-MC_X)** was performed in the Braun Biostat reactor. When comparing the results of this fermentation to those done in the DASGIP system (see **Section 4.3**) the CDW reached is similar but the time needed and the PHB content were lower, resulting in a lower PHB productivity.

The longer process time is, at least in part, a result of the difference in inoculum size (see Section **3.4.1**) in comparison to the reactor volume. While the ratio of those factors used in the DASGIP system results in shorter process time, the smaller inoculum size used in the Braun Biostat system is probably closer to what would be used in an actual industrial process. Furthermore, there was no feed added during the initial 17 hours of fermentation, resulting in total depletion of glucose after about 13.7 hours of fermentation, as shown by the base consumption and the DO values measured and a starvation period thereafter. While a feed amount equal to the reactor volume was added after 17 hours of fermentation to provide a big amount of glucose in a short amount time, this – while certainly benefiting renewed bacterial growth – could not compensate the problems caused by the starvation. The volume added was removed slower than feed was added in this period due to limitations in the rate at which permeate could be removed without causing a high trans-membrane pressure. The original volume was nevertheless reached before the next sampling point and should – as a result – not have any impact on concentration based measurements.

The imposition of a nitrogen limitation occurred without problems, so the lower PHB content may be a result of the fact that during the crucial starting period of the PHB-accumulation phase (see **Section 5.1.2**) the amount of glucose available was limited. This is most likely a consequence of the feed rate being too low, but the gathered data nevertheless suggests that a prolonged fermentation would potentially have yielded a higher PHB content. This can be inferred from the fact that the PHB content was still rising and there was still metabolic activity when the fermentation was terminated.

It can be concluded that a higher feed rate during the whole process would be advisable for future processes.

Despite the described phases of limitation, a sample drawn from the permeate bottle at the end of the fermentation showed that the permeate nevertheless had a glucose concentration of about 3 g / L. A lack of possibility to sample the permeate at adequate points of time makes an overall assessment of the feed strategy problematic, but it is obvious that, like in the DASGIP system (see **Section 5.1.3**) significant amounts of sugar were washed out of the reactor before they could be metabolized. Due to the same problems it comes to no surprise that the overall PHB yield in the Braun Biostat fermentation was, albeit a bit lower, similar to the yield calculated for the DASGIP system (see **Table** 8).

Like in the previous processes, the formation of foam was a problem and could not be controlled by the automated, or manual for that matter, addition of antifoam. A technical solution for the volume loss problem (see **Section 5.1.2**) was attempted by channelling the foam through the off air tubes into

a big sterile vessel, where the foam was allowed to collapse and subsequently as much liquid as possible was pumped back into the reactor. Nevertheless, volume loss was problematic, reducing the volume at one point from initially 3 litres to about 1.5 litres. The loss could not be compensated through the automated addition of feed, only through manual addition of feed without simultaneous removal of permeate. This is a result of the fact that there was no measurement of the volume of fermentation broth in the reactor, but the level was merely controlled by removing the same amount of liquid through the membrane as added.

Despite all technical efforts, all concentration based measurements during the fermentation were – again – flawed and the technical solution attempted, while easing the problem somewhat, also has drawbacks. For one, there was further complexity added to the setup and – of more importance – for the other, a part of the fermentation broth was outside of the reactor at almost all times, where no control of temperature, pH or DO was possible. From this it can be concluded that other solutions for the problem should be conceived, the easiest of which would involve some sort of volume measurement and an automated level control based on that measurement. This would, at least in cell recycling fed-batch processes, allow the control unit to judge the amount of volume loss due to foaming and consequently to compensate it by the addition of appropriate amounts of feed. While this would definitely result in the loss of a fraction of the biomass, this solution seems preferable to an attempt to catch the lost volume and return it by yet another pump to the reactor. That said, the most elegant solution would most probably be to find an antifoaming agent that in fact suppresses the foaming to an unproblematic degree and additionally does not interact negatively with the membrane.

Despite the usage of considerable amounts of antifoam, no problems with clogging of the membrane could be observed in this single fermentation, but this should by no means indicate that such problems could not occur at higher cell densities or during longer processes. While pressure sensors were placed at the feed side and at the permeate side of the membrane, the trans-membrane pressure was only monitored superficially (data not shown). While the trans-membrane pressure was not problematic during regular operation, the quick removal of big amounts of media at one point posed a problem. In order to prevent damaging the membrane, the trans-membrane pressure has to be kept in a certain range during the whole process, indicating that, using this exact setup, the removal of permeate could in the future pose an upper limit to the feed rates possible.

As a result, further investigation is needed to better understand the possibilities and limitations posed by the membrane under various conditions, and ideally be able to implement the trans-membrane pressure measured into feed control. This would on one hand give online data on the condition of the membrane and on the other hand help lengthen its lifetime.

Only after these things are better understood, an adaptation to an agricultural waste medium or another waste medium seem reasonable, because their potential heterogeneity and the potentially heightened amount of solids in the medium might cause further problems for the filtration.

6 Summary and Conclusion

In this work a process for the production of a PHA in a cell recycling fed-batch fermentation using a hollow fibre membrane could be established at laboratory scale. This was done using *C. necator*, a natural producer of PHB, the most prominent PHA. The amount of PHB that could be accumulated in the cells was as high as 92% and it was possible to achieve relatively high cell densities of up to 47 g/L with a medium of low carbon concentration. The performance of this process was tested in a total of four different cell recycling fed-batch fermentations done in two different setups and bioreactors. As a reference, several fed-batch and batch fermentations were also done in both systems. Those additional fermentations indicate that fermentations using *C. necator* DSM 545 should be carried out at a temperature of around 37°C and that a difference in performance of processes using fructose and those using glucose is not observable. Furthermore, the type of membrane used, ultrafiltration or microfiltration, does not seem to influence performance of the process.

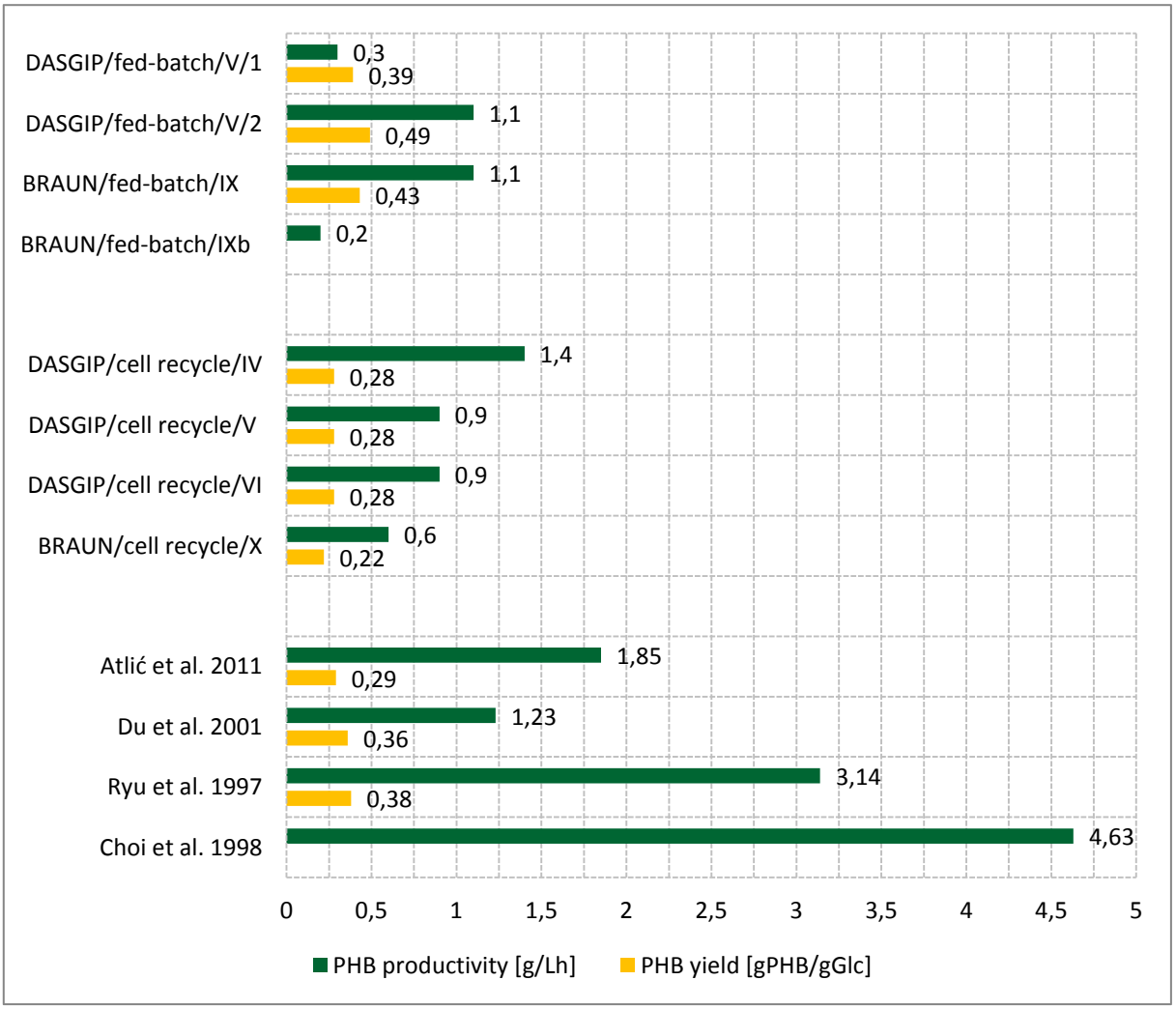

Figure 15: **Comparison of PHB productivity and PHB yield.** The organism used by Atlic et al., Du et al. and Ryu et al. was *C. necator*, Choi et al. used a genetically modified *E. coli*. Atlic et al. and Du et al used a continuous process, Ryu et al. and Choi et al. a fed-batch process. The carbon source used in all four processes was glucose.

The PHB productivities achieved in the cell recycling fed-batch fermentations were in a range between 0.6 g/Lh and 1.4 g/Lh while the yields achieved varied between 0.22 g PHB / g Glc in the Braun Biostat system and 0.28 g PHB / g Glc in the DASGIP system. The productivities achieved in the DASGIP system were comparable to productivities gained from fed-batch experiments in both systems and to productivities being reported in literature for similar processes (Ienczak et al., 2015).

The results of the Braun Biostat cell recycling fed-batch fermentation were worse than those of the DASGIP fermentations, but this is explainable by a variety of difficulties due to the preliminary nature of the experiments in that system and does not indicate that experiments should not be done on a bigger scale.

Furthermore the results generated in both systems indicate that, by optimising the process developed, a further increase in productivity is possible. The points that need most effort for the optimisation of the process are the ideal timing of the nitrogen limitation or – to be more precise – the ideal cell densities at which the limitation should be induced and the improvement of the feed strategy.

An optimisation of the feed strategy should additionally have a positive impact on the PHB yield of the process. The yields in cell-recycling fed-batch processes were lower than the yields calculated for the fed-batch and batch fermentations in this work and other yields reported in literature. This is a result of the fact that significant amounts of sugar were flushed out of the system and into the waste. To minimize this waste of sugar it could for instance be attempted to implement a feeding strategy based on a model of the process, or an online glucose sensor could be used.

While the problems mentioned and some other minor issues need to be addressed before this setup can be used on a bigger scale or with an actual agricultural or industrial waste medium, the results indicate that the process works well at lab scale. The process offers an attractive option for the production of PHB and shows big potential for further optimisation. While the productivities reached in this work may be below the values reached using engineered *E. coli* strains (Choi et al., 1998), the performance of other fermentations using *C. necator* seem to be achievable. The big advantage to these conventional processes is that the process developed does not rely on the use of an expensive medium with high carbon content but is in fact able to utilize a cheaper carbon source with lower carbon content.

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