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Master's thesis

Bacterial production of polyhydroxyalkanoates (PHAs) from chicory roots

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Abstract

Polyhydroxyalkanoates (PHAs) are biobased and biodegradable polymers, and represent an interesting alternative to petroleum based conventional plastics in the future. While their thermoplasticity and the big variety of usable monomers lead to extremely versatile materials, their biggest drawback are still the high production costs. As the raw materials were determined as some of the biggest cost factors, cheap waste products are investigated for the usage as fermentation substrate.

In this master's thesis, a fermentation process for P(3HB), based on a chicory root hydrolysate, was investigated and optimized. The fructan inulin was extracted from chopped and dried chicory roots and enzymatically hydrolysed to fructose and glucose. This hydrolysate was used as fermentation substrate for the biotechnological production of P(3HB) using three different strains of *Cupriavidus necator* (DSM 428, 531, and 545).

By changing the pre-culturing conditions and supplementing nitrogen, the volumetric productivity of *C. necator* DSM 428 could be increased by more than an order of magnitude. Urea, ammonium sulfate, soy peptone, and casein peptone were tested as nitrogen sources. Soy peptone gave the best results, leading to a twentyfold increase of the volumetric productivity to $0.27\,\mathrm{g\,L^{-1}\,h^{-1}}$. In a direct comparison without any nutrient supplementation, DSM 428, 531, and 545 showed productivities of 0.07, 0.01, and $0.20\,\mathrm{g\,L^{-1}\,h^{-1}}$, respectively. Within two days of fermentation, the most promising strain (DSM 545) produced $11.1\,\mathrm{g\,L^{-1}}$ P(3HB) and a cell dry weight of $14.2\,\mathrm{g\,L^{-1}}$, corresponding to a PHA content in dry weight of 78 %. The detoxification of the fermentation substrate using charcoal or laccase did not further improve the fermentation performance, which indicates that a detoxification of the chicory root hydrolysate is not required.

To sum up, it can be said that chicory roots are a promising raw material for the production of PHAs through bacterial fermentation.

Abstract

Polyhydroxyalkanoate (PHAs) sind biologisch abbaubare Polymere, deren Produktion auf erneuerbaren Ressourcen basiert. Aus diesem Grund stellen sie eine interessante Zukunftsalternative zu konventionellen Kunststoffen aus Erdöl dar. PHAs sind infolge der Vielzahl an nutzbaren Monomeren in ihren Eigenschaften sehr flexibel. Angesichts hoher Produktionskosten werden PHAs derzeit allerdings nicht im großen Maßstab hergestellt. Da ein großer Teil dieser Kosten auf das Konto von Rohmaterialien geht, wird mittlerweile versucht diese durch Abfallstoffe zu ersetzen.

In dieser Masterarbeit wurde ein biotechnologischer Prozess, basierend auf einem Chicorée-Wurzeln-Hydrolysat, untersucht und optimiert. Das Fruktan Inulin wurde aus den Wurzeln extrahiert und enzymatisch zu Fructose und Glucose hydrolysiert. Dieses Hydrolysat wurde anschließend als Substrat für die biotechnologische Produktion von P(3HB) durch *Cupriavidus necator* genutzt, wobei drei verschiedene Stämme des Bakteriums zum Einsatz kamen (DSM 428, 531 und 545).

Durch die Änderung der Vorkulturbedingungen und die Supplementation von Stickstoff konnte die Produktivität von *C. necator* DSM 428 um mehr als eine Größenordnung erhöht werden. Der Zusatz von Harnstoff, Ammoniumsulfat, Soja-Pepton und Casein-Pepton als Stickstoffquelle wurde untersucht. Die besten Ergebnisse wurden durch den Zusatz von Soja-Pepton erzielt, wobei eine Verzwanzigfachung der Produktivität auf $0.27\,\mathrm{g\,L^{-1}\,h^{-1}}$ erreicht wurde. In einem direkten Vergleich, und ohne Stickstoff-Supplementation, erzielten DSM 428, 531 und 545 Produktivitäten von 0.07, 0.01 bzw. $0.20\,\mathrm{g\,L^{-1}\,h^{-1}}$. DSM 545, der vielversprechendste Stamm, produzierte innerhalb von zwei Tagen $11.1\,\mathrm{g\,L^{-1}}$ P(3HB) und eine Zelltrockenmasse von $14.2\,\mathrm{g\,L^{-1}}$, was einem PHA-Anteil von 78 % entspricht. Durch die Detoxifizierung des Substrates mittels Behandlung durch Aktivkohle oder Laccase konnte keine Steigerung der Produktivität erreicht werden. Dies lässt darauf schließen, dass eine Detoxifizierung des Chicorée-Wurzeln-Hydrolysates nicht notwendig ist.

Zusammenfassend kann gesagt werden, dass Chicorée-Wurzeln einen vielversprechenden Rohstoff für die bakterielle Produktion von PHAs darstellen.

Contents

Glossary

C. necator	*Cupriavidus necator*
E. coli	*Escherichia coli*
CDW	Cell dry weight
ddH_2O	Double-distilled water
DO	Dissolved oxygen
H_2SO_4	Sulfuric acid
H_3BO_3	Boric acid
HPLC	High performance liquid chromatography
IEC-HPLC	Ion exchange high performance liquid chromatography
KH_2PO_4	Potassium dihydrogen phosphate
LB	Lysogeny broth
NH_3	Ammonia
NH_4OH	Ammonium hydroxyde
NH_4Cl	Ammonium chloride
$(NH_4)_2SO_4$	Ammonium sulfate
OD_{600}	Optical density at 600 nm
P(3HB-3HHx)	Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)
P(3HP)	Poly-3-hydroxyproprionate
PHA	Polyhydroxyalkanoate
PHB	Poly-3-hydroxybutyrate = P(3HB)
PHO	Poly-3-hydroxyoctanoate
PLA	Poly lactic acid/Polylactide
RI	Refractive index
TKN	Total Kjeldahl nitrogen
WCB	Working cell bank

1. Introduction

1.1 Overview

1.1.1 What are plastics?

Plastics are high-molecular-weight polymers and for the most part synthetic, petroleum derived materials. Due to their physical properties they are normally categorized as thermoplasts, duroplasts, and elastomers (Domininghaus et al. 1972).

Although the plastic production in Europe decreased from 58 to 57 million tons between 2011 and 2012, the global production increased during the same time from 280 to 288 million tons. In Europe, 39.4 % of the plastic produced today is used as packaging material, 20.3 % in buildings and constructions and 8.2, 5.5, and 4.2 % in automotives, electronics, and agriculture, respectively. The most common plastic products used are polyethylene (PE-LD, PE-HD), polypropylene (PP), polyvinyl chloride (PVC), polyethylene terephthalate (PET), and polyurethane (PUR) (PlasticsEurope 2013).

1.1.2 Properties of conventional plastics

Compared to materials like metal, wood or paper, plastics have advantageous mechanical and chemical properties for certain applications. They are normally easily processable, lightweight, cheap, water resistant, and durable. In addition, they are extremely versatile and there are different specialized plastics for nearly every purpose (Domininghaus et al. 1972).

Plastics replaced many traditional materials during the last decades for many applications. People are in contact with synthetic polymers every day. Despite all these advantages, previously described plastics suffer from some drawbacks:

Petroleum based: Most conventional plastics are based on petroleum, a non-

regenerative, finite resource. It cannot precisely predicted how long petroleum will be available in large quantities, but estimates see an exhaustion of the most crude oil sources for the actual century. It is thought that due to the increasing expenditures the oil price is going to increase on long-term (Miller & Sorrell 2014; Owen et al. 2010).

Carbon dioxide: Crude oil stores carbon since million of years. As conventional plastics are based on crude oil, their CO_2 cycle is not closed. Their combustion or degradation releases CO_2 in the atmosphere, where it reinforces the climate change (Montzka et al. 2011).

No degradation: Most conventional plastics are not biodegradable within a reasonable timeframe, which in the end leads to an accumulation of plastics in the environment. About 60 years ago, in the 1950s, mass-production of plastics began. Today plastic macro- and micro-fragments are ubiquitous on earth and accumulate almost everywhere; in soil, lake beds, in the sea, even on remote Antarctic islands, in tropical sea beds, and in bird nests (Andrady 2011; Barnes et al. 2009).

1.1.3 "Bioplastics"

It is thought that one or more of the issues addressed in Section 1.1.2 in future could be eliminated by the usage of "bioplastics". But, what are "bioplastics"?

The word "bioplastic" describes a multitude of materials which are - in contrast to conventional plastics - biobased, biodegradable, or both (Koller et al. 2010; European-Bioplastics 2014a). This means that a material called a bioplastic may be:

Biodegradable (but not biobased)**:** Petroleum based plastics which degrade in nature within a short time frame. E. g.: Petroleum based PBS (Tokiwa et al. 2009).

Biobased (but not biodegradable)**:** Plastics produced from regenerative resources which do not degrade in nature. E. g.: Biobased PE (Ethylene comes from ethanol which was produced by fermenting sugar) (Shen et al. 2010).

Biobased and biodegradable: Plastics made from regenerative resources which degrade in nature within a short time frame. E. g.: PHAs like PHB or PHV (Koller et al. 2010).

The production capacities of bioplastics increased from 1.02 million tons in 2010 to 1.40 million tons in 2012 and are thought to reach 6 million tons in 2017. Most of the growth is probably going to the account of biobased (not biodegradable) plastics. These biobased plastics can easily replace conventional plastics in all market segments, whereas biodegradable materials are estimated to be a growing niche market for short-lived applications, like packaging material for fresh produce (European-Bioplastics 2014b).

1.2 Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHAs) are biobased and biodegradable polymers, and they represent a possible alternative to conventional plastics. Due to a large number of usable monomers, they have a great versatility and range in their properties from thermoplastic to elastomeric. However, high production costs and other limitations are preventing a large-scale production of PHAs today.

1.2.1 History

Already in 1926, the French chemist Maurice Lemoigne, while working at the Lille branch of the Pasteur Institute, discovered the polyhydroxybutyrate polyester in form of intracellular granules in *Bacillus megaterium* (Lundberg 2009).

Almost 50 years later, Wallen and Rohwedder discovered a new PHA by chloroform extraction from activated sludge. The PHA heteropolymer consisted of 3-hydroxyvaleric and 3-hydroxybutyric acid. Compared to PHB, the newly discovered P(3HB-3HV) had a much lower melting point and was soluble in hot ethanol (Wallen & Rohwedder 1974).

Nowadays, more than 300 PHA producing organisms are known (Volova et al. 2008), and more than 150 building blocks (i. e. hydroxyalkanoic acid monomers) have been identified (Steinbuechel & Lutke-Eversloh 2003).

1.2.2 Physiological significance

Physiologically, PHA granules serve as energy and carbon storage. The nearly insoluble macromolecule allows microorganisms to store large energy quantities in form of highly reduced carbon, without significantly affecting the osmotic pressure of the cell (Anderson & Dawes 1990).

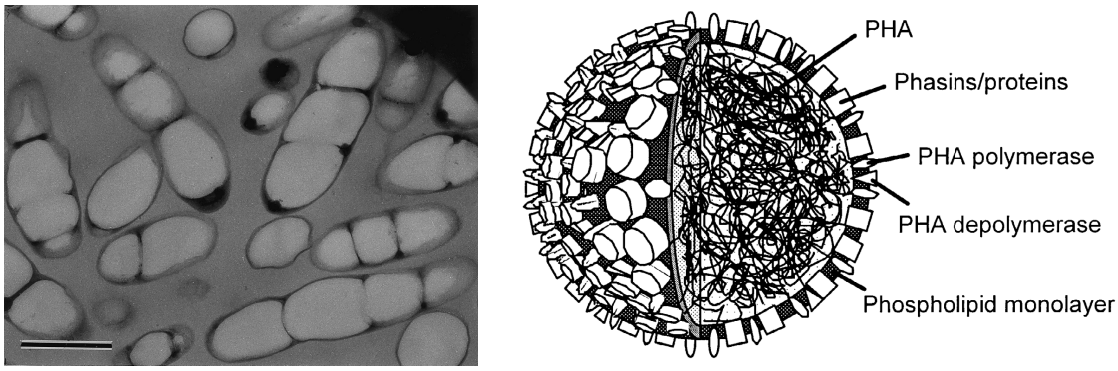

Figure 1.1: Left: TEM image of *C. necator* containing large amounts of PHA (90 % of the CDW; bar: 0.5 µm). Source: Sudesh et al. (2000, p. 1507) - Right: Scheme of a PHA granule. Source: Zinn et al. (2001, p. 8)

Cells usually accumulate PHA under unbalanced nutrient conditions, in a medium containing an excess of carbon where another nutrient is limiting (Agnew & Pfleger 2013). However, it was shown that some bacteria are able to accumulate PHA under non-limiting conditions too (Keshavarz & Roy 2010).

Figure 1.1 shows a transmission electron microscopy image of PHA granules in *C. necator* as well as a composition scheme of a single granule. It consists of a PHA core that is covered by a phospholipid monolayer, interspersed with phasins (structural proteins), PHA polymerases, PHA depolymerases, and other proteins of unknown function (Zinn et al. 2001). PHA polymerases are directly bound to the PHA chains, in contrast to depolymerases (Koller et al. 2013). The key enzymes in PHA biosynthesis are the PHA synthases PhaC, which can be classified into four groups. They use coenzyme A thioesters of hydroxyalkanoic acids to catalyse their polymerization into PHAs, while releasing CoA (Rehm & Steinbüchel 2005; Ushimaru et al. 2014).

Although the described PHA storage granules ($>$ 1000 monomers) were found only in certain prokaryotes, shorter PHAs ($<$ 150 monomers) were found in all living organisms. In contrast to storage PHAs, they are not sequestered within cytoplasmic granules, but complexed to cellular macromolecules, and therefore termed cPHAs (Reusch 2002).

1.2.3 Chemical structure and classification

Most PHAs are polyesters of aliphatic hydroxyalkanoic acids. Their general structure is shown in Figure 1.2. The most common PHAs are poly-3-hydroxybutyrate

1.2.4 Properties and material applications

1.2.4.1 Mechanical properties

Different PHAs vary widely in their mechanical properties. The chemical monomeric structure, like length of side chain or type of functional group, determine melting point, glass transition temperature, and crystallinity of the polymer. These in turn define possible applications of the material (Akaraonye et al. 2010). PHAs can range from stiff and brittle thermoplastics to rubber like elastomers (Ojumu et al. 2004).

Some scl-PHAs, like P(3HB), are highly crystalline, stiff and brittle plastics, with mechanical characteristics similar to those of some conventional plastic like polypropylene (Padermshoke et al. 2005; Verlinden et al. 2007). Unfortunately, their low elasticity makes them nearly useless for most applications, as the material breaks at an elongation of 5 % (Table 1.1). On the other hand, another scl-PHA, P(4HB), is relatively strong and flexible and has a high elongation to break.

Mcl-PHAs typically behave more like thermoplastic elastomers. They have a low crystallinity and tensile strength but high elongation to break values (Akaraonye et al. 2010).

Copolymers are a possibility to alter and fine-tune PHA material properties. As more than 150 hydroxyalkanoic acids are known today, numerous combinations of them are conceivable. Table 1.1 shows, how mechanical properties of P(3HB) based copolymers like P(3HB-20%3HV) or P(3HB-16%4HB) dramatically differ, compared to the P(3HB) homopolymer.

Also blends of PHAs with other materials are under investigation. Of great interest are combinations of PHAs with other biodegradable materials. Bhatt et al. (2008) showed how different rubbers behave when they were blended with 5 to 15 % mcl-PHAs. Applications ranging from PHA filled elastomers to rubber reinforced thermoplastics are imaginable. Salemi et al. (2007) showed how biopolymeric blends based on PHA and their use as paper coating material enabled waterproof and more durable papers for the use in agriculture.

P(3HB), the best described PHA, has good UV resistance, but low resistance to acids and bases. A low oxygen permeability makes the material suitable for the packaging of oxygen-sensitive products (Chakraborty et al. 2012).

Table 1.1: Comparison of the physical properties of specific PHAs and two commonly used synthetic polymers. Source: Akaraonye et al. (2010, p. 734)

Polymer	**Melting temperature** (°C)	**Glass transition temperature** (°C)	**Young's modulus** (GPa)	**Elongation to break** (%)	**Tensile strength** (MPa)
P(3HB)	180	4	3.5	5	40
P(4HB)	53	-48	149	1000	104
P(3HB-20%3HV)	145	-1	1.2	50	20
P(3HB-16%4HB)	150	-7	-	444	26
P(3HB-10%3HHx)	127	-1	-	400	21
P(3HB-6%3HD)	130	-8	-	680	17
Polypropylene	176	-10	1.7	400	34.5
Polystyrene	240	100	3.1	-	50

1.2.4.2 Biodegradability

As extracellular PHA depolymerase producing bacteria are very common in nature, PHAs possess a high biodegradability. However, the rate of degradation depends on the activity of these enzymes, which in fact varies from polymer composition and crystallinity, as well as from environmental conditions like temperature, moisture level, and pH (Akaraonye et al. 2010).

Another biobased an biodegradable plastic is polylactic acid (PLA). In contrast to PHA, it is today already produced on a large scale, and it has been shown that it does degrade during industrial high-temperature composting within a short period of time. However, PLA's high glass transition temperature between 45 and 120 °C makes it relatively resistant to microbial degradation under environmental conditions (MatWeb 2014; Tokiwa et al. 2009).

1.2.4.3 Biocompatibility

PHAs have good biocompatibility, which makes the material interesting for medicinal applications. Polyhydroxyoctanoate (PHO) was investigated for the use as heart valve scaffold (Sodian et al. 2000), and P(3HB-3HHx) was applied as scaffold for cartilage, osteoblast, and fibroblasts (Chen et al. 2005).

1.3 Production of polyhydroxyalkanoates

1.3.1 Production methods

During the last decades, numerous methods for PHA production were investigated. The most flexible approach is the production by microbial fermentation. A variety of organisms and strains on a multitude of different substrates were already examined for PHA production. The addition of precursors to the fermentation media modifies the composition of the final product. On the other hand, costs for carbon source and purification are relatively high, and energy as well as equipment costs are not negligible (Gumel et al. 2013; Zinn et al. 2001; Castilho et al. 2009).

1.3.1.1 Microbial fermentation

Generally, PHA producing bacteria can be divided into two groups. The first group produces PHA under unbalanced growth conditions, when carbon is available in excess, but another essential nutrient like N, P, Mg, K, O, or S is limited. The second group produces PHA in a growth-associated manner, without any nutrient limitation. Most strains of *Cupriavidus necator* and methylotrophic bacteria belong to the first group, whereas *Alcaligenes latus*, *Azotobacter beijerinckii*, and recombinant *Escherichia coli* belong to the second. This fact has to be considered when creating fermentation and feeding strategies (Lee & Park 2002).

Cupriavidus necator, earlier known as *Ralstonia eutropha*, *Wautersia eutropha*, or *Alcaligenes eutrophus* (Vandamme & Coenye 2004) is one of the best described PHA producing organisms. *C. necator* is a soil inhabiting, gram-negative, mesophilic, rod shaped bacteria with 2 to 10 peritrichous flagella (Makkar & Casida 1987). Depending on environmental conditions and physiological state, cell morphology can vary between short, nearly spherical and more elongated shapes. *C. necator* can live under facultative anaerobic and facultative chemolithoautotrophic conditions (Heinzle & Lafferty 1980). Different strains were isolated and characterized. *C. necator* H16 (DSM 428) can grow and produce P(3HB) on different carbon sources like fructose, gluconate, even-numbered fatty acids, and vegetable oils. However, *C. necator* H16 and closely related strains cannot consume glucose (Orita et al. 2012). DSM 545 is a glucose-consuming mutant of H16, which makes it interesting for the fermentation of glucose comprising substrates. Furthermore, *C. necator* DSM 545 seems to perform well under nutrient sufficient conditions (Berezina 2013).

Alcaligenes latus produces P(3HB) during its growth phase. The ability to consume sucrose makes it a candidate for fermentations based on cheaper substrates, like molasses (Lee & Park 2002).

Several *Pseudomonas sp*, such as *Pseudomonas putida*, *Pseudomonas aeruginosa*, or *Pseudomonas chlororaphis*, are able to produce PHAs. They incorporate longer monomers and produce mcl-PHAs (Furrer et al. 2007; Muhr et al. 2013).

Other organisms used for the production of PHAs are genetically engineered *Escherichia coli*. PHA synthase genes from *C. necator* were isolated and introduced into *E. coli* already back in 1995 (Lee & Chang 1995; Antonio et al. 2000). Further, the production of other PHAs than P(3HB) in *E. coli* was studied, like the recombinant production of P(3HP) (Wang et al. 2013).

1.3.1.2 Genetically modified plants

As bacterial fermentations suffer from high raw material costs (see Section 1.3.4), transgenic plants were created with the capability to produce PHAs. Although PHA yields are still low and field cultivation of genetically modified plants may be uncertain due to legal restrictions, a PHA production in plants would have some advantages especially for large-scale production, such as no need for expensive raw materials and fermentation equipment, no energy-consuming sterilization, and straightforward up-scale (Bohmert-Tatarev et al. 2011).

1.3.1.3 Enzymatic *in vitro* synthesis

The advantages of enzymatic *in vitro* synthesis are stereoselectivity, chemoselectivity, regioselectivity, and enantioselectivity. These ensure well-defined properties of the product. Furthermore, the separation of an enzyme catalysed reaction mixture is straightforward, and reactions typically take place under ambient reaction conditions. However, the enzymatic *in vitro* synthesis of PHAs has some drawbacks: Typically, organic solvents are required for achieving high enzyme (lipase) activities. Furthermore, due to the increasing free polymer concentration during the process, the viscosity of the medium increases, which leads to diffusion limitations and low molecular weight polymers (Gumel et al. 2013).

1.3.2 Drawbacks and problems

Despite some undisputed advantages of PHAs, PHA-based products are serving today only a niche market. Too high production costs are preventing a widespread usage of PHA as material of choice for bulk products. The costs for raw materials and product purification were determined as the crucial cost factors for a reasonable large-scale production. As PHA accumulation occurs under aerobic conditions, high amounts of carbon get lost through cellular respiration. Less than 50 % of the consumed carbon is directed towards biomass and PHA formation (Yamane 1993).

Other problems regard the processing of certain PHAs. The homopolymer P(3HB) already starts to degrade at temperatures near the high melting point of 180 °C. Random chain scission reactions lead to a reduction of the number–average degree of polymerization within minutes by 50 %. Higher temperatures cause an exponential increase of the degradation rate (Shaked et al. 2009; Aoyagi et al. 2002). This makes the use of melt extrusion technology difficult. However, such mechanical limitations can be overcome by producing copolymers of 3-hydroxybutyrate with 3-hydroxyvalerate, 3-hydroxyhexanoate, or 4-hydroxybutyrate, which have lower melting temperatures (Table 1.1) (Volova et al. 2008).

On the other hand, the production of PHA copolymers in most cases requires the addition of precursors to the medium. These again, raise the production costs.

1.3.3 Companies

Due to all of these problems, only few middle- to large-scale PHA production sites exist today. Chen (2009) summarizes several of them. For obvious reasons, the exact composition of the produced PHAs stays a trade secret in many companies. Nevertheless, most of them seem to produce copolymers like P(3HB-3HV), P(3HB-4HB), or P(3HB-3HHx).

One PHA producer is the American company Metabolix Inc. Metabolix produces PHA based performance additives, as well as films and bags and sells them under the name Biopol. Biopol assets were bought from Monsanto in 2001 (Metabolix 2001, 2014).

Bio-on is a company from Italy and produces PHA based automotive parts, beverage bottles, electronic parts, food packaging materials, fibres, and pharmaceutical products. The products are traded under the name MINERV-PHA™(Bio-on 2014).

Beside other European and US enterprises, mainly Chinese companies are active

in the PHA business. One of the larger ones is Tianjin Green Bio-Science. The company produces 10 000 t PHA (raw and packaging materials) per year (GreenBio 2014).

1.3.4 Microbial production from inexpensive carbon sources

The amount of microbially available energy and carbon directly influences the theoretical amount of the desired final product (PHA). Therefore, cheap carbon and energy sources are crucial for the reduction of the total production costs. As about 50 % of the entire costs go on the account of raw materials, cheaper starting materials could improve economic viability (Koller et al. 2010; Castilho et al. 2009).

Nowadays, a multitude of possible waste or surplus products were already investigated for the usage as fermentation starting materials. Some of them are described in the following paragraphs.

1.3.4.1 Surplus whey

As a by-product of the cheese production, large amounts of whey accrue every year. In 2008, 186 million tons of whey were produced globally. The biggest whey producers are the EU and the USA with a total market share of 70 % (Affertsholt 2009). Mostly, hydrolyzed whey permeate is used as substrate for fermentations, as many PHA producing organisms cannot use lactose as carbon source (Koller et al. 2005).

Koller et al. (2005) investigated the use of lactose as carbon source for PHA production, by first separating crude whey into permeate (lactose fraction) and retentate (protein fraction). Subsequently, the permeate was concentrated to a lactose concentration of $200\,\mathrm{g\,L^{-1}}$, which represents the upper solubility limit of lactose in water. A highly osmophilic, non-specified organism was used for the fermentation. Because of the strain's inability to directly use lactose, the disaccharide was cleaved enzymatically with β-galactosidase to its monomers, glucose and galactose.

Also the direct use of lactose as carbon source is possible, shown by Ahn et al. (2001). A recombinant *E. coli*, harbouring PHA synthase genes from *Alcaligenes latus*, was used as organism in a fed-batch process with cell-recycle system.

1.3.4.2 Waste lipids

Waste lipids from food processing industry, slaughterhouses, edible oil processing industry, dairy products industry, and oil mills, as well as waste cooking oils and animal fats are available in large quantities today. A part of them are used for the production of soap or biodiesel (Wang et al. 2007). However, the biggest part of such fats and oils has to be discarded, leading to high disposal costs and environmental pollution (Nikodinovic-Runic et al. 2013).

As these waste lipids contain lots of energy and carbon, their use as raw material for the biotechnological production of PHAs was investigated. Cooking oils, tallow (Taniguchi et al. 2003), olive mill effluents (Dionisi et al. 2005), palm oil and palm oil related products (Loo et al. 2005), and many more waste materials of this category were examined for the use as fermentation substrates (Koller et al. 2010; Nikodinovic-Runic et al. 2013).

1.3.4.3 Waste glycerol from the biofuel production

Due to the European biofuel policy, the production amount of biodiesel increased strongly during the last decade. As result of the transesterification process, triglycerides of vegetable oils and animal fats are converted to more volatile and less viscous methyl- or ethyl esters. The remaining glycerol rich phase is an energy and carbon rich waste product with potential as starting material for fermentations of different biotechnological products (Meher et al. 2006; Bozbas 2008; Ibrahim & Steinbuechel 2009).

1.3.4.4 Lignocellulosic wastes

Lignocellulosic materials constitute the most abundant renewable resource on earth. Theoretically, the main wood components cellulose (β-1,4-D-glucan), hemicellulose (irregular polymers of D-arabinose, D-xylose, D-mannose, D-glucose, D-galactose, and sugar alcohols), and lignin (complex polyphenols) could provide an almost unlimited source of valuable starting materials for different processes. Enormous effort has been invested in the development and establishment of diverse biorefinery strategies during the last decades. However, to make the previously mentioned monomers accessible, pretreatments are needed. Many of them (e. g. pretreatments with dilute acid or at high temperatures) lead to the release of inhibitory substances, impairing the fermentation performance, and making the use of detox-

ification processes unavoidable (Section 1.4.2) (Sun & Cheng 2002; Koller et al. 2010).

Radhika & Murugesan (2012) used a water hyacinth based hydrolysate as sole carbon source for the bacterial fermentation of P(3HB) via *C. necator*. Acid and enzymatic hydrolysates were produced and fermented. Also a detoxified acid hydrolysate was used as carbon source. Acidic, detoxified acidic, and enzymatic hydrolysates resulted in maximal PHB concentrations of 2.0, 3.7, and 3.2 $\mathrm{g\,L^{-1}}$, respectively.

1.3.4.5 Starch

Also starch-based fermentation substrates were investigated. Kim (2000) and Halami (2008) investigated the fermentation directly on soluble starch, whereas Koutinas et al. (2007) used a wheat hydrolysate as basis of a fermentation media (Castilho et al. 2009). Also hydrolyzed corn starch was tested out (Choi & Lee 1999; Koller et al. 2010).

1.3.4.6 Chicory roots

This master's thesis describes the use of chicory root based hydrolysates as fermentation substrate for the production of PHAs.

Chicory (*Cichorium intybus*) belongs to the family *Asteraceae*, subfamily *Cichorioidea*, and tribe *Lactuceae*. They form tap roots which contain large amounts of carbohydrates, mainly inulin (Figure 1.4). Inulin is a polysaccharide, more precisely, a β-(2,1)-fructan. It consists of one glucose molecule and 2 to 60 fructose subunits. Because of its β-configuration at the anomeric C 2, inulin-type fructans are classified as non-digestible carbohydrates. They resist intestinal digestive enzymes (Roberfroid 2005).

The cultivation method of chicory depends on the purpose for which it is grown and on the cultivar used. The most highly specialized method is the biennial cultivation for the witloof chicory cultivar (Figure 1.5), which proceeds in three stages; field production, storage and forcing (Ryder 1999; Corey et al. 1990).

During the field production stage, starting at the end of spring, crops are precision-seeded in well drained, not to stony or compact soils. Wrong soil conditions would impair the development of the carbohydrate rich tap roots. The soil nitrogen content should not be excessively high, as this may lead to undesirable high leaf growth, decreasing the wanted accumulation of dry matter in the tap root. The crops are grown for 70 to 100 days and are harvested between late summer and mid autumn.

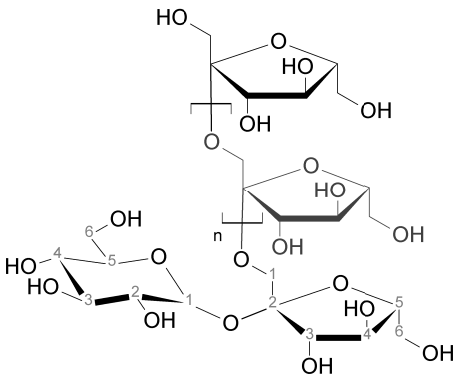

Figure 1.4: Structural formula of inulin. Source: Fisch (2006)

Leafs are mowed several centimetres above the crown. Roots are lifted and trimmed to an uniform length (Corey et al. 1990).

Following harvest, the roots are stored for several weeks at 0 to 2 °C and 95 to 98 % relative humidity. During this stage, two important things happen to the roots: First, the storage carbohydrate inulin partially breaks down to oligo- or monomeric sugars, and secondly, the vegetative bud differentiates to a floral meristem (Corey et al. 1990).

During the forcing, the third and last stage, the floral meristem elongates. Different cultivation methods were used in the past. Nowadays, in large-scale production, roots are forced hydroponically and in the dark. Forcing takes 20 to 25 days at temperatures between 13 and 22 °C. The end product is a chicon, a small white head

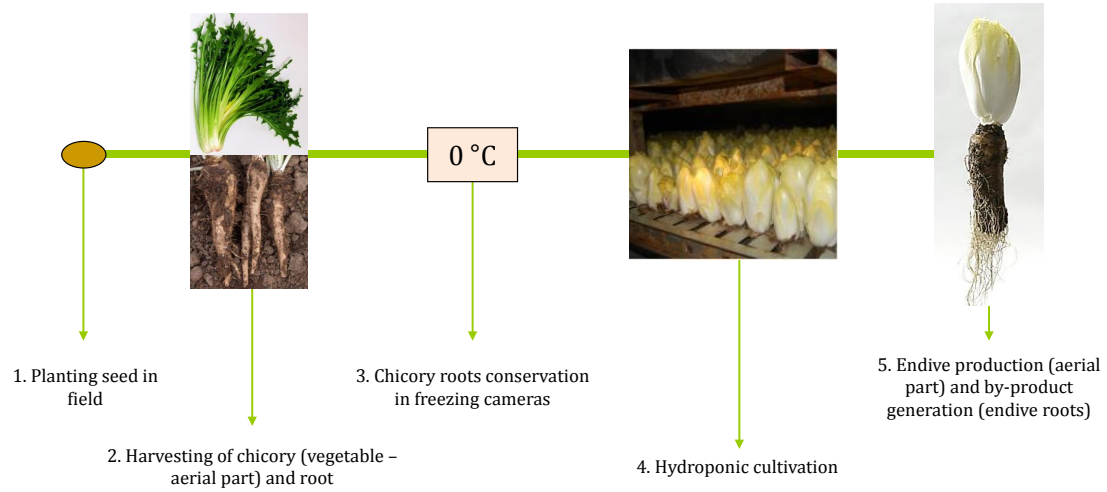

Figure 1.5: Endive production scheme. Source: Tratamiento Subproductos Agroalimentarios (trasa s.l.)

of leaves, ringed with yellow-green regions (Ryder 1999; Corey et al. 1990).

The remaining roots are a waste product, but they still contain considerable amounts of inulin and other carbohydrates. They can be used as animal feed and in biogas plants.

1.3.5 Nutrients

Besides carbon, microorganisms require a range of nutrients. In case these nutrients are not provided by the substrates, they must be supplied. However, for PHA production most organisms need the limitation in one nutrient except carbon. A balance between bacterial growth and PHA accumulation has to be found. Often, two-stage processes are used. At the first stage, fermentation conditions are optimized for bacterial proliferation until the desired cell density is reached. At the second stage, fermentation conditions are changed. The cell division should be hindered, and energy and carbon should be diverted to PHA accumulation as much as possible (Ramsay et al. 1990; Cavalheiro et al. 2012a).

1.3.5.1 Nitrogen

One of the nutrients which are getting a lot of attention in the field of PHA research is nitrogen. A great number of scientific publications are dealing with different nitrogen sources and supplementation strategies. It should be noted that the correct nitrogen source and the amount of supplementation strongly depend on the nitrogen content and the bioavailability of the nitrogen already present in the raw fermentation media.

There are a variety of usable nitrogen sources, all having their pros and cons. Defined nitrogen sources, like NH_4OH, $(NH_4)_2SO_4$, NH_4Cl or urea, are cheap, pure, and have constant quality. Complex nitrogen sources like peptone or yeast extract contain amino acids and short peptones. These can lead to a faster and better PHA accumulation (Mahishi & Rawal 2002; Lee & Chang 1994). However, some complex nitrogen sources often are quite expensive and, especially for the production of bulk products, not the first choice. The third possibility is the use of cheap waste or surplus materials, though substrates like corn steep liquor vary in their compositions, impairing reproducibility and overall performance (Mahishi et al. 2003).

Chanprateep et al. (2008) studied the newly isolated strain A-04 of *C. necator* for the production of P(3HB-4HB). The researchers examined butyric acid and γ-

hydroxybutyric acid as carbon sources and added different amounts of nitrogen in form of $(NH_4)_2SO_4$, achieving various carbon to nitrogen (C:N) ratios. Nitrogen deficiency (C:N = 200) led to the highest specific production rate of P(3HB). However, nitrogen-sufficient conditions (C:N between 4 and 20) gave the highest specific production rate of P(4HB). This means that P(4HB) production in *C. necator* A-04 was growth associated, while P(3HB) was only produced under nitrogen limitation.

Cavalheiro et al. (2012a) used a two-stage process: During the exponential growth phase, HCl and NH_4OH were used for pH control. Once the targeted cell concentration was reached, NH_4OH was replaced by KOH, leading to nitrogen limitation and promoting PHA accumulation. Main carbon source in the experiments were glucose or glycerol rich phase.

Compared to cultivations in basal medium, amino acid supplementation increases the production of P(3HB) in recombinant *E. coli*. A possible explanation for this could be end-product inhibition of different amino acid producing biosynthetic pathways and a carbon flux change towards PHA accumulation (Mahishi & Rawal 2002).

Garcia et al. (2013) investigated the production of P(3HB-3HV) from crude glycerol (main carbon and energy source) and rapeseed meal hydrolysates. The rapeseed meal hydrolysate contained up to $2\,060\,mg\,L^{-1}$ free amino nitrogen (FAN) and $304\,mg\,L^{-1}$ inorganic phosphorus. It was supplemented in varying amounts. The best results ($15.1\,g\,L^{-1}$ CDW and $7\,g\,L^{-1}$ PHA) where obtained with an initial FAN content of $460\,mg\,L^{-1}$. At initial FAN concentrations lower than $250\,mg\,L^{-1}$ glycerol consumption was incomplete, whereas FAN contents higher than approximately $500\,mg\,L^{-1}$ led to higher microbial growth but reduced PHA accumulation.

1.3.5.2 Phosphorus

In theory, each essential nutrient could be limited to trigger PHA accumulation through nutrient limitation. Often, inorganic salts, like NH_2PO_4 or KH_2PO_4, are used to control the level of phosphorous in the medium.

Lee et al. (2000) used phosphorus limitation to promote PHA production. At mcl-PHA fermentations with *Pseudomonas putida* a PHA content of 18.7 % and a PHA concentration of $32.3\,g\,L^{-1}$ were achieved. Through the reduction of the initial KH_2PO_4 concentration, the PHA content and concentration increased to 51.4 % and $72.6\,g\,L^{-1}$, respectively.

1.3.5.3 Oxygen

Different DO concentrations seem to affect PHA accumulation. The DO in turn is influenced by stirrer speed and aeration rate.

Cavalheiro et al. (2012a,b) investigated the effect of oxygen limitation during the PHA accumulation phase in a two-staged process. PHA accumulation was promoted through nitrogen starvation. Fermentations at lower oxygen content (DO = 2 %) showed significantly lower PHA productivities compared to higher DO fermentations (DO = 20 %). The fermentations were carried out with glycerol as main carbon source. As organism *C. necator* DSM 545 was used.

1.4 Optimization of waste based bioprocesses

Compared to fermentations on defined substrate media, bioreactions on complex waste or surplus materials generally make the method development process and optimization more complicated. The lack of specific data, how a certain organism or strain performs on a selected media, can transform a biotechnological process into a trial & error game. Data about the exact media composition or the bioavailability of nutrients usually are missing as likewise, making the solution of optimization problems laborious and time-consuming. Furthermore, fermentation substrates from waste materials normally have fluctuating properties, as the starting materials for media production are of natural origin and variable. This leads to reproducibility problems. Last but not least, some required pretreatments can generate toxic or microbe-inhibiting substances in the media, decreasing volumetric productivity and destroying economic suitability.

1.4.1 Fermentation strategy and process conditions

As at most other biotechnological processes, first experiments are usually done in shaking flasks and laboratory scale batch reactors. For larger scale, fed-batch and continuous reactor strategies are needed.

One problem of waste or surplus material based fermentation media often are unsuitable substrate concentrations. Hence, additional preparation steps are sometimes required. Feed solutions for fed-batch processes often require high sugar contents. If the substrate concentration is too low, preliminary cross-flow filtration and concentration steps can be used (Koller et al. 2005). An alternative to con-

centration steps are continuous cell-recycle systems. Periodically, a small part of fermentation broth is removed from the reactor and pumped to an external membrane where the depleted medium is separated from the cells. These in turn are pumped back into the reactor which is refilled with fresh medium (Ahn et al. 2001).

Feeding regimes can change process course and productivity. Hafuka et al. (2011) tested three different feeding regimes (1-pulse, stepwise, and continuous). It could be shown that 1-pulse feeding led to the highest cell concentration, but stepwise and continuous feeding gave the best PHB yield.

As many bacteria produce PHA under nutrient limitation (Section 1.3.1.1), the type of bacteria has to be considered when developing fermentation strategies. The bacteria used in this work, *Cupriavidus necator*, is usually cultivated at neutral pH conditions and at 30 °C (Xu et al. 2010). However, also the cultivation at 37 °C seems to be promising (Spoljaric et al. 2013).

1.4.2 Detoxification of substrates

Depending on the used substrates and substrate pretreatments, toxic substances can inhibit microbial growth and impair the fermentation performance. Especially harsh pretreatments, like acid hydrolysis of lignocellulosic materials, promote the formation and release of such substances. Well known inhibitors are sugar derived furan derivatives, like furfural and 5-hydroxy-methyl-furfural (HMF), aliphatic acids, like acetic, formic, and levulinic acid, as well as lignin derived phenolic compounds (Larsson et al. 1999; Jönsson et al. 2013; Nikodinovic-Runic et al. 2013).

Different detoxification methods are known today. The most important are treatment with activated charcoal, overliming, and enzymatic detoxification.

Activated charcoal: Especially phenols derived from lignin degradation can be removed by the treatment of hydrolysates with activated charcoal. The treatment efficacy strongly depends upon the used hydrolysate:charcoal ratio. Reported values are in the range from 50:1 to 10:1. Also parameters like time, pH, and temperature affect the performance. After the adsorption process, all solid compounds have to be removed from the substrate (Mussatto & Roberto 2001; Parajó et al. 1996; Canilha et al. 2004).

Meinita et al. (2012) showed that the treatment of a red seaweed (*Kappaphycus alvarezii*) hydrolysate with activated charcoal resulted in decreased HMF and

levulinic acid concentrations. However, also a part of the sugars (glucose, galactose) were adsorbed.

Treatment with lime: During the process called "overliming", lime (CaO) or hydrated lime ($Ca(OH)_2$) is added to a hydrolysate, resulting in a pH increase up to 9-11. Although this is a highly exothermic reaction, further heating is normally required to maintain a temperature of 40 to 60 °C over a period of time (up to 90 min). Subsequently, the solids (mainly gypsum) are removed from the substrate.

Overliming is reported to decrease the toxicity of acid hydrolysates and to improve their fermentability. However, the exact mechanism is still not completely understood today (Mohagheghi et al. 2006; Purwadi et al. 2004). Some well-known inhibitors like furfural and HMF are reported to decrease in their concentration during the pretreatment by forming complexes with calcium ions which degrade. Other toxic substances, like acetic acid and phenols, seem to be not or only slightly affected by overliming. A drawback of this pretreatment is that also sugars are partially degraded (Millati et al. 2002; Purwadi et al. 2004).

Enzymatic detoxification: Laccases and peroxidases are used for the enzymatic detoxification of lignocellulosic hydrolysates. It was shown that these enzymes are able to decrease the concentration of monophenolic compounds in the substrate, resulting in an improved fermentability (Jönsson et al. 1998).

Laccase is a copper-containing oxidase which catalyses the one-electron oxidation of many phenolic and non-phenolic substrates. It uses oxygen as electron acceptor that is reduced to water (Casa et al. 2003). The formed phenoxy radicals polymerize and lead to less toxic high-molecular-weight products (Jurado et al. 2009). Different peroxidases, like horseradish peroxidase or lignin peroxidase, catalyse the oxidation of various substrates with hydrogen peroxide. The reaction products undergo further non-enzymatic reactions leading also to less toxic polymers (Jönsson et al. 1998; Wagner & Nicell 2002).

Ion exchange resins: Anion exchanger, cation exchanger, and resins without charged groups were examined for substrate detoxification prior fermentation. All three types of resins were able to decrease the concentration of furans and phenols. However, only the use of anion exchange resins at pH 10 yielded a decrease of aliphatic acids (Nilvebrant et al. 2001).

Membrane filtration: Newer studies are investigating the use of nanofiltration techniques. By using appropriate cut-offs, sugars (retentate) can be separated from smaller molecules (permeate) like aliphatic acids and furans (Stoutenburg et al. 2008; Weng et al. 2010).

Beside the methods described above, there are many other detoxification techniques, like a biological method using *Trichoderma reesei* (Okuda et al. 2008), or combinations of described pretreatments, like overliming and charcoal detoxification (Tripathi et al. 2012).

2. Objectives

This work aims to establish a PHB[1] production process which is based on the fermentation of a chicory root hydrolysate, by using different strains of *Cupriavidus necator*.

An already existing standard protocol should be modified in order to obtain a viable bioprocess which permits high product yields, short process times, and a good reproducibility. Although the main goal is the maximization of the PHB productivity, economic viability should be kept in mind, enabling a possible use of the process in larger scale. To achieve these aims, different bacterial strains, pre-culturing methods, cell origins, nutrient additives, and process conditions have to be investigated:

- A substrate extraction and hydrolysis process has to be performed and evaluated. A sufficient amount of hydrolysate should be prepared for the subsequent fermentation experiments.
- An already existing fermentation protocol should be executed and evaluated. Subsequently, different experiments should be carried out in order to reduce the process duration and to enhance the conversion of substrate to PHB.
- Three different *C. necator* strains (DSM 428, 531, and 545) should be compared so as to select the most promising one.
- It should be determined, whether detoxification of the hydrolysate can improve the fermentation process.

[1] PHB and P(3HB) are used synonymously.

3. Materials and methods

3.1 Hydrolysis of chicory roots

3.1.1 Starting material

Chopped and dried chicory roots (*Cichorium intybus*, Figure 3.1) from Navarra (Spain), provided by Tratamiento Subproductos Agroalimentarios (trasa s.I.), were used as starting material for the preparation of the fermentation substrate. For this purpose, an already established protocol for extraction and hydrolysis for 3 L was scaled-up to 60 L.

3.1.2 Extraction and hydrolysis

54 kg of RO water were heated up in a stirrable vessel (Figure 3.2) to 55 °C. After the water reached the target temperature, 11 mL of enzyme solution (Table 3.1) and 6 kg of dried and milled chicory roots were added to the hydrolysis vessel. The first sample was taken after 30 min. Further samples were taken hourly.

At each point in time, duplicates were taken, diluted 1:10 with ddH_2O, and used

Figure 3.1: Dried and chopped chicory roots

Figure 3.2: Stirrable hydrolysis reactor and cider press

for HPLC sugar analysis (Section 3.4.3). Another sample was taken to measure the pH value of the hydrolysate.

After six hours of hydrolysis, the temperature control was switched off. The reactor was moved into a 10 to 15 °C cool room where the hydrolysate cooled down over night. The reaction was still ongoing, as enzymes were neither removed nor deactivated.

3.1.3 Clarification

After 18 h of cool storage, the liquid phase was separated from the solid phase through a cider press (Figure 3.2). Subsequently, the liquid phase was centrifuged for 30 min at 4 000 g by a Sorvall Lynx 4000 centrifuge with a F10-4x1000 LEX rotor in $4 * 1$ L centrifugation bottles.

The supernatant was further clarified by a vacuum filtration through a laboratory

Table 3.1: Enzyme solution for hydrolysis

Manufacturer	Megazyme
Product name	Fructanase mixture (purified) (20 mL) E-FRMXLQ
Enzymes	Mixture of exo- and endo-inulinases with a catalytic activiy of 2 000 U mL^{-1} and 200 U mL^{-1}, respectively Contaminated with minor amounts of other enzymes: α-galactosidase (0.15 U mL^{-1}), β-glucanase (0.35 U mL^{-1}), pectinase (< 0.30 U mL^{-1})
Solvent	50 % glycerol with 0.02 % sodium azide

filter (110 mm filter paper circles, S&S 595), before the hydrolysate was portioned in 2 L and 10 L PE bottles and stored at −18 °C.

3.1.4 Detoxification

Because substrate preparation steps like extraction and hydrolysis were executed at relatively mild conditions, detoxification was only tested at a later stage of the process optimization. Two methods were examined, both of them independently and immediately prior to reactor and medium autoclaving.

Activated charcoal: 2 g activated charcoal per 100 mL of media were added to the hydrolysate. The reaction took 60 min and occurred at room temperature. Subsequently, solids were separated by two centrifugation and decanting steps. The centrifugation was carried out at 4000 and 8 000 g, for 10 and 20 min, respectively.

Laccase pretreatment: To remove lignin derived monophenolic compounds from the media, a pretreatment with laccase was tested out. The enzyme was produced in-house through *Trametes hirsuta* strain IMA2002 and was available as unpurified fermentation broth. 5 mL of this solution were added to the fermenter. The enzyme solution had an activity of 273 U mL^{-1}. The specific enzyme activity was determined to be 34 U mg^{-1}. As the reaction consumes oxygen, the course of the reaction was monitored with a DO electrode. After one hour at room temperature, under continuous stirring and airing, the reaction was essentially over.

3.2 Shaking flask experiments

For the evaluation of different nitrogen sources, a shaking flask experiment was carried out. The selected strain DSM 428 was taken from the working cell bank (−80 °C). After thawing, a LB-agar containing Petri dish was inoculated with the cells and incubated for 3 days at 30 °C. A 250 mL shaking flask containing 50 mL LB medium was inoculated with one colony from the Petri dish and incubated for 24 h at 30 °C in a Infors HT incubation shaker at 150 rpm.

After incubation, 250 mL shaking flasks containing 50 mL hydrolysate with different nitrogen additives were inoculated with 1 mL of the inoculum suspension. Used

nitrogen additives were NH_4OH, $(NH_4)_2SO_4$, casein peptone, and urea. Also negative samples (hydrolysate without nitrogen supplementation) and a control sample (LB medium) was made. Furthermore, each experiment was done with and without trace elements supplementation.

The initial pH was set to 6.8 with 1 M HCl or NaOH. During the experiment, the pH value was corrected to 6.8 $\pm$ 0.5 once a day. Measurement values were OD_{600} and NaOH or HCl consumption, respectively.

3.3 Fermentation experiments

The first fermentations were carried out according to an already existing standard protocol. Since this protocol did not work satisfactory, in subsequent fermentations modified parameters and process conditions were investigated, with the attempt to improve the overall fermentation performance. The standard protocol is described in the following sections.

Table 3.2 shows an overview of all fermentation cycles done, including all major changes compared to the standard protocol. Latter will be described in detail in following sections. Only experiments which gained knowledge are shown in the results and discussion section (Section 4).

3.3.1 Fermentation system

The fermentations were carried out in a DASGIP bioreactor system (Figure 3.3), consisting of up to four fermenter vessels, each possessing stirrer, exhaust gas condenser, as well as electrodes for temperature, DO, and pH. Four experiments were performed simultaneously.

3.3.2 Bioreactor preparation

Several preparation steps were needed before each fermentation run. First, the peristaltic pumps had to be calibrated with the used fluids (acid and base). Subsequently, the pH electrodes were calibrated by using pH 7 and pH 4 buffer solutions.

Next, the reactors had to be assembled. Main parts were: Vessel, stirrer, top lid, pH electrode, DO electrode, exhaust gas condenser, medium inlet connectors, and sterile filter for air supply.

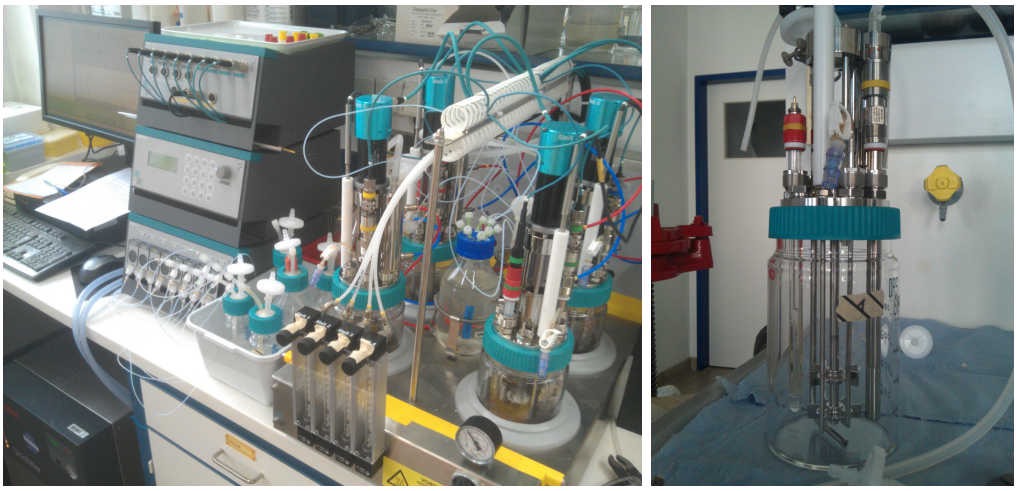

Figure 3.3: DASGIP bioreactor system

After filling in the reactor with medium and in some cases adding additives (no additives for standard protocol), all reactors were autoclaved as a whole at 121 °C for 20 min. Afterwords, all connections were closed densely, and all electrodes as well as stirrers were connected to the main station. As the DO electrodes needed about six hours to equilibrate, stirrer and aeration ran over night, enabling DO electrodes calibration at the next day. That was carried out at maximal stirrer speed (1 200 rpm) with air (100 %) and nitrogen (0 %).

As the hydrolysate had a pH of approximately 4.5, pH control of the reactor was turned on some hours before inoculation.

3.3.3 Fermentation parameters

The pH was regulated by addition of 1 M HCl and 1 M NaOH solutions (standard protocol). For some experiments, the NaOH solution was replaced by 1 M NH_3. HCl and NaOH were autoclaved at 121 °C for 20 min, NH_3 was sterilized by filtration. Acid and base were pumped in the fermenter vessels by peristaltic pumps. While the pH value for most experiments was set to be fixed at 6.8 or 7.0, some experiments were made with non-fixed pH value at the beginning of the fermentation (starting pH was always 6.8 or 7.0).

Sterile air was used to introduce oxygen into the system. The aeration rate was kept constant at 11 $L\,h^{-1}$. The DO concentration was regulated by the stirrer speed (400 to 1 200 rpm) and was set to a minimal DO concentration of 20 %. The temperature set point was 30 °C.

Table 3.2: Fermentation experiments overview

Cycle	System	Reactor	Strain	Changes to standard protocol
PHB-VII	CSTR	1	428	
		2	428	Clones 4 and 5 from PHB-VI reactor 2
		3, 4	531	
PHB-VIIb	CSTR	1	428	Clone 5 from PHB_VI reactor 2
		2	428	Clone 4 from PHB-VI reactor 2
		3	428	Inoculum plate from PHB-VI reactor 2
		4	428	Lyophilisate from PHB-VI reactor 2
PHB-VIII	Shaking flasks		428	Different nitrogen sources with and without trace element addition
PHB-IX	CSTR	1 - 4	428	Hydrolysate used for resuspending the inoculum cell pellet was adjusted to pH 7 before the resuspension More clones (10-30) for each inoculum flask
		1	428	Clone 5 from PHB-VI reactor 2
		2	428	Clone 4 from PHB-VI reactor 2
		3	428	Inoculum plate from PHB-VI reactor 2
		4	428	WCB 130529 from PHB-VI reactor 2
PHB-X	CSTR	1 - 4	428	Base pH control only Comparison of NH_3 to adjust the pH initially and NaOH Cell origin: WCB 130529 More clones (10-30) for each inoculum flask
		1	428	Base: NaOH
		2	428	Base: NH_3 for whole fermentation
		3, 4	428	Base: NH_3 for initial pH adjustment, NaOH for remaining fermentation
PHB-XI	CSTR	1 - 4	428	Comparison of NH_3 to adjust the pH initially and NaOH All clones from WCB 130529 More clones (10-30) for each inoculum flask
		1, 2	428	Base: NaOH for pH adjustment
		3, 4	428	Base: NH_3 for initial pH adjustment, NaOH for remaining fermentation
PHB-XII	CSTR	1 - 4	428	Minimal medium pre-culture I Acid/base control, pH 7.0 Different concentrations of $(NH_4)_2SO_4$
PHB-XIII	CSTR	1 - 4	428	Minimal medium pre-culture I Different nitrogen sources: Urea, $(NH_4)_2SO_4$, soy peptone, casein peptone
PHB-XIV	CSTR	1 - 4		Minimal medium pre-culture II Different nitrogen sources (same as in PHB-XIII)
		1, 2	428	
		3, 4	545	
PHB-XV	CSTR	1 - 4		Minimal medium pre-culture II
		1, 2	545	Laccase treatment
		3, 4	545	Charcoal treatment
PHB-XVI	CSTR	1 - 4		Minimal medium pre-culture II
		1, 2	531	
		3	545	Charcoal treatment
		4	545	Laccase treatment

Several experimental data could be monitored on-line during the fermentation: pH, temperature, DO, pH, stirrer speed, and added volume of acid and base.

3.3.4 Organism and pre-culture

As fermentation organisms three different strains of *Cupriavidus necator* were tested; DSM 428, DSM 531, and DSM 545. Different pre-culture conditions were studied. The strains DSM 428 and 531 were available as working cell bank, whereas the strain DSM 545 was a gift from Martin Koller (TU Graz), and was stored at 4 °C on minimal medium plates.

3.3.4.1 Organism origin

The existing standard protocol started with strains from a working cell bank (WCB), created in May 2013. This WCB was produced from an overnight LB medium shaking flask culture; 100 µL of glycerol where added to the 900 µL of cell suspension, mixed, incubated for one hour, and deep-frozen (−80 °C). Other cell origins studied were:

- Cells which were stored for several weeks on LB medium plates or minimal medium plates.
- Cells which were stored for several months and longer on minimal medium plates.
- Lyophilized cells from a previous fermentation.
- Cells from exponential growth phase from a previous fermentation.

Different cell origins and pre-culturing conditions with various combinations thereof were examined. The two most important ones are described below:

Standard protocol: The selected strains were taken from the working cell bank (WCB, −80 °C). After thawing, LB-agar containing Petri dishes were inoculated with the cells and incubated for 2 to 3 days at 30 °C. 250 mL LB medium containing 1 L shaking flasks were inoculated with one colony from the Petri dishes. They were incubated for 24 h at 30 °C in a Infors HT incubation shaker at 150 rpm.

Modified protocol: The selected strains were cultured at minimal medium plates for several generations and stored on such plates at 4 °C. Minimal medium containing Petri dishes were inoculated with colonies from the plates and incubated for 3 days at 30 °C. No single clones were used. After incubation, 250 mL minimal medium containing 1 L shaking flasks were inoculated with colonies from the plates and incubated for 36 to 48 h at 30 °C. Table 3.5 shows the minimal medium's composition.

3.3.4.2 Inoculum preparation

After incubation, the optical density at 600 nm was measured. The content of two flasks was united in a sterilized 1 L centrifugation bottle. After some optimizations enhancing the cell growth in the shaking flask the inoculum volume could be halved. Thenceforth, only one shaking flask was used per fermenter (see Section 4.3.5).

The cell suspension was centrifuged at 2 000 g for 20 min. The supernatant was discarded. The cell pellet was resuspended in 25 mL hydrolysate whose pH was adjusted to 7.0 previously with sterile NaOH (1 M). The resuspended cell pellet was used as inoculum for the fermenters by injecting the cell suspension through the sample port.

3.3.5 Medium

The chicory root hydrolysate (Section 3.1) was thawed in a water bath at 60 °C under manual periodic shaking. A volume of 475 mL hydrolysate was filled in each of the four fermenter vessels. Additives, like $(NH_4)_2SO_4$, urea, or peptone, were added at this point prior autoclaving. The reactors, including electrodes for pH and DO, where autoclaved as a whole (as described in Section 3.3.2).

3.4 Off-line analytics

3.4.1 Sampling

During the fermentation, 2 mL of fermentation broth were aspirated in a sterile 5 mL syringe and discarded. Subsequently, 5 mL of fermentation broth were sampled and placed into glass centrifuge tubes. The sample was used for OD_{600} measurement, sugar (fructose, glucose), CDW, PHB, TKN and ammonia quantification as well as for microscopy.

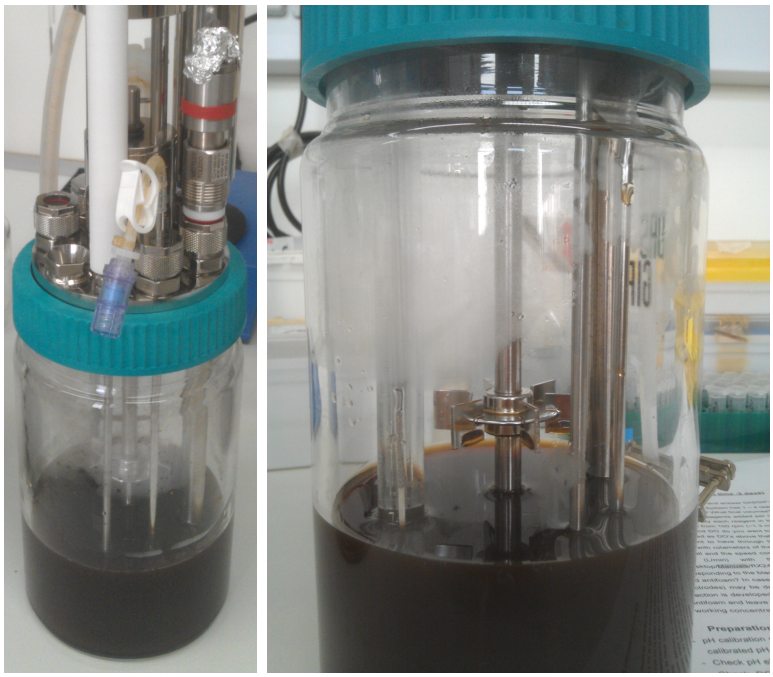

Figure 3.4: Hydrolysate filled reactor vessel with two stirrers. The bottom stirrer (not visible) disperses the blown in air while the upper stirrer mechanically destroys foam which forms during fermentation.

Each sample was centrifuged in an Eppendorf 5810 centrifuge for 10 min at 2 000 g. The supernatant was used for sugar as well as ammonia and TKN quantification. The cell pellets was washed two times with 5 mL ddH_2O and used for CDW and PHB measurement (Figure 3.5).

3.4.2 Optical density

The optical density (OD_{600}) of the fermentation broth was measured at 600 nm against ddH_2O in a Hach Lange DR 3900 photometer. Therefore, the sample was diluted to appropriate turbidity with ddH_2O.

Table 3.3: Carrez clarification reagents

C1 solution	5.325 g of $K_4[Fe(CN)_6] * 3\ H_2O$ dissolved in a volume of 50 mL ddH_2O
C2 solution	14.400 g of $ZnSO_4 * 7\ H_2O$ didssolved in 50 mL ddH_2O

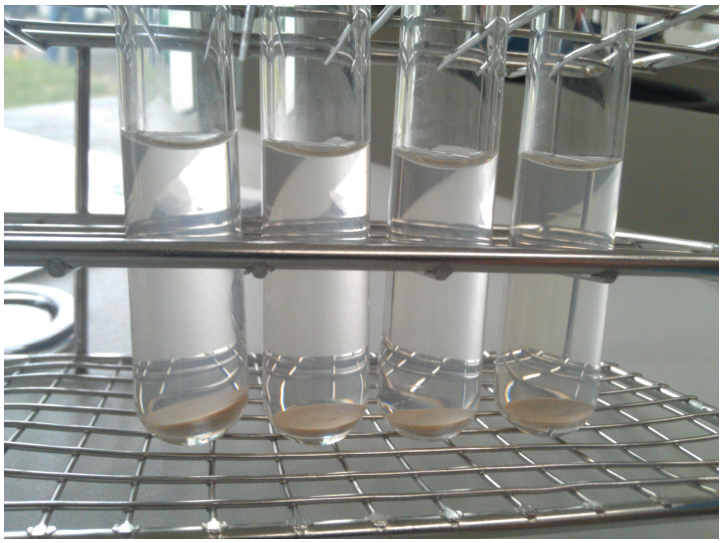

Figure 3.5: Washed cell pellets

3.4.3 Residual sugar quantification

Fructose and glucose concentrations from the fermentation broth supernatant and the hydrolysate were quantified by ion exchange HPLC (IEC-HPLC, Table 3.4). For the quantification of the total (not just monomeric) sugar content during the hydrolysation of the chicory roots, an acidic hydrolysis in H_2SO_4 (4 %) was carried out over night. Therefore, 44.5 µL of H_2SO_4 (49 %) were added to 500 µL sample. The following day, the samples were neutralized with 1 M NaOH and quantified by HPLC.

Table 3.4: HPLC parameters for sugar and crotonic acid quantification

	Sugars	Crotonic acid
HPLC model	Agilent 1100 series	Agilent 1100 series
Column	ICSep ION-300	CARBOSep COREGEL 87H
Mobile phase	H_2SO_4 (0.005 M)	H_2SO_4 (0.005 M)
Flow	0.325 mL min^{-1}	0.325 mL min^{-1}
Temperature	45 °C	65 °C
Detector	Refractive index (RI)	Refractive index (RI)
Calibration	Peak height	Peak area

Table 3.5: Minimal medium composition (plates and broth). Substances marked with asterisk (*) were autoclaved separately.

	Minimal medium plate ($g\,L^{-1}$)	Minimal medium broth ($g\,L^{-1}$)
Na_2HPO_4	4.8	4.8
KH_2PO_4	2.0	2.0
$(NH_4)_2SO_4$	3.0	3.0
$MgSO_4 * 7\ H_2O$	0.8	0.8
NaCl	1.0	1.0
$CaCl * 2\ H_2O$	0.02	0.02
Trace elements solution SL6	5.0	5.0
Agar-agar	14	-
NH_4Fe(III)citrate*	0.05	0.05
Fructose*	10	10

3.4.3.1 Sample preparation for HPLC

Since macromolecules like proteins and lipids from the sample would impair the column's performance, a Carrez clarification was carried out (Gaub 1983).

40 µL of C1 solution (composition see Table 3.3) were mixed with 50 µL sample and 1 870 µL ddH_2O in 2 mL reaction tubes. Next, 40 µL C2 solution were added to the reaction tube. After shaking, the samples were placed for 30 min in the refrigerator, where the macromolecules could precipitate. The samples were centrifuged in a laboratory centrifuge (BECKMAN GS-15 with F2402 rotor) at 12 500 rpm for 30 min. The supernatant was separated from the precipitate, filtered through a 0.45 µm laboratory filter and analysed by HPLC.

3.4.4 Dry weight

The cell pellet in the glass centrifuge tube was dried for 24 h at 105 °C and stored for one day in a desiccator. The dry weight was measured with an analytical balance (MC1 Analytic AC 210S from Sartorius).

3.4.5 PHB quantification

The type of PHB analysis accomplished during this work relies on the quantification of crotonic acid which is formed during chemical depolymerization of PHB (Karr et al. 1983).

1 mL of concentrated H_2SO_4 (97 %) was added to the dried cell pellet. The samples' centrifugation tubes were tightly closed and placed in a 90 °C hot water bath for at least 30 min. If after 30 min the sample tubes still contained solid particles, the reaction time was extended up to an hour. The same procedure was carried out with two tubes containing pure PHB. They were used to calculate a correction factor.

After hydrolysis, all sample tubes were let cool down to room temperature. Subsequently, the whole content of each sample tube was transferred into a 50 mL volumetric flask and filled up with ddH_2O. The solution was filtered through 0.45 µm sterile filter and analysed by HPLC (Table 3.4).

3.4.5.1 Calculation of productivity and yield

Only the main fermentation data were used for the calculation of productivity and yield. The pre-culture was not taken into account. Also Section "measurement errors" on page 39 has to be considered.

The volumetric productivity Q_P was calculated according to equation 3.1; PHB_{end} and PHB_{start} are the final and initial PHB concentrations, Δt is the total fermentation time.

$$Q_P\ (\mathrm{g\,L^{-1}\,h^{-1}}) = \frac{PHB_{end} - PHB_{start}}{\Delta t} \tag{3.1}$$

The yield $Y_{P/S}$ was calculated according to equation 3.2; $Sugar_{start}$ is the sum of fructose and glucose concentrations at the fermentation start, $Sugar_{end}$ is the sum of fructose and glucose concentrations at the end of the fermentation.

$$Y_{P/S}\ (\mathrm{g\,g^{-1}}) = \frac{PHB_{end} - PHB_{start}}{Sugar_{start} - Sugar_{end}} \tag{3.2}$$

3.4.6 Microscopy

The diluted samples from the OD_{600} measurement (Section 3.4.2) were used for microscopy after the OD measurement. 2 samples, 10 µL of each, were placed

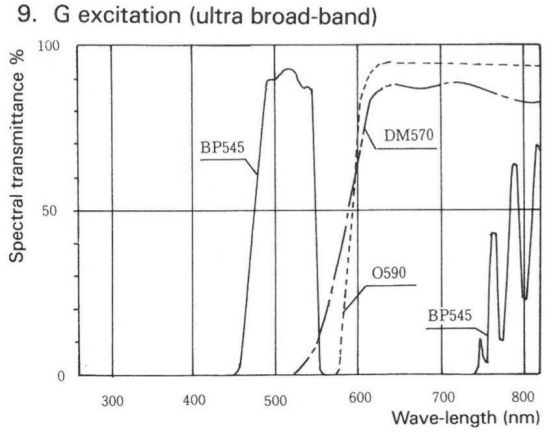

Figure 3.6: Spectral transmittance of different fluorescence microscopy filters (BP545 was used for the excitation light, DM570 for the emitted fluorescence). Source: Olympus (n.d., p. 27)

and distributed on a microscope slide. After the cell suspension dried at room temperature, the bacteria were heat fixed by passing several times through the flame of a Bunsen burner.

After the microscope slide cooled down, the PHB granules inside the cells where stained. The microscope slide was covered with 90 to 100 µL 1 % aqueous Nile blue A fluorescent dye. After 10 min of incubation at 55 °C in a moist atmosphere, excess dye was removed with tap water and decolourized by placing the slide for 1 min into 8 % aqueous acetic acid solution (Ostle & Holt 1982).

The now stained PHB granules were made visible through fluorescence microscopy. Nile blue A has an absorption and emission maximum in water at 635 nm and 674 nm, respectively (Jose & Burgess 2006). The samples were viewed in an Olympus VANOX AHBT3 microscope in bright field, phase contrast, and fluorescence mode. For fluorescence microscopy the excitation light was filtered through a BP545 filter, whereas the emitted fluorescence had to pass through a DM570 filter (Figure 3.6).

3.4.7 Nitrogen quantification

Total Kjeldahl nitrogen (TKN) and free ammonium where quantified from fermentation supernatant samples. Both ammonium and TKN were quantified using the AutoKjeldahl Unit from Büchi (Egli & Büchi Labortechnik 2008).

3.4.7.1 Ammonium

An AutoKjeldahl Unit K-370 from Büchi was used for the determination of the ammonium content. 5 g of sample were transferred in Büchi sample vials. The following steps were done automatically by the device:

- Distillation of ammonia after addition of NaOH (30 %) to the sample.
- Capture of ammonia as ammonium in boric acid (H_3BO_3, 20 g L^{-1}).
- Titration with HCl (0.05 M).

3.4.7.2 Total Kjeldahl nitrogen

2 g of sample were transferred in sample vials and diluted with 5 to 10 mL ddH_2O. After addition of a catalyst tablet[1], 20 mL H_2SO_4 (97 %) were added to the sample. The degradation of the organic material was carried out by the Büchi Digest Automat K-438. The nitrogen derived ammonium was quantified by the AutoKjeldahl Unit K-370 from Büchi as described before (section 3.4.7.1).

[1]Brand name "Kjeltabs". Content per tablet: 3.5 g K_2SO_4 and 0.4 g $CuSO_4 * 5\ H_2O$.

4. Results and discussion

4.1 Chicory root hydrolysis

After separating solid from liquid phase through the cider press, 39.93 kg of fluid and 19.43 kg of press cake could be obtained from the 6 kg dried chicory roots (5.39 kg of dry matter[1]) and 54 kg RO water. The press cake contained 3.17 kg of total solids, meaning that 41 % of the initial dry content was extracted through the process. The distribution of dry matter is shown in Figure 4.1. A considerable amount of monomeric sugars was lost due to the high water content of the press cake (83.7 %).

The values represented in Figure 4.2 shows measured sugar concentrations during ongoing hydrolysis. After hydrolysis, filtration, and autoclaving, 92 % of the total

[1]The dry matter content of the dried chicory roots was determined to be 89.8 %.

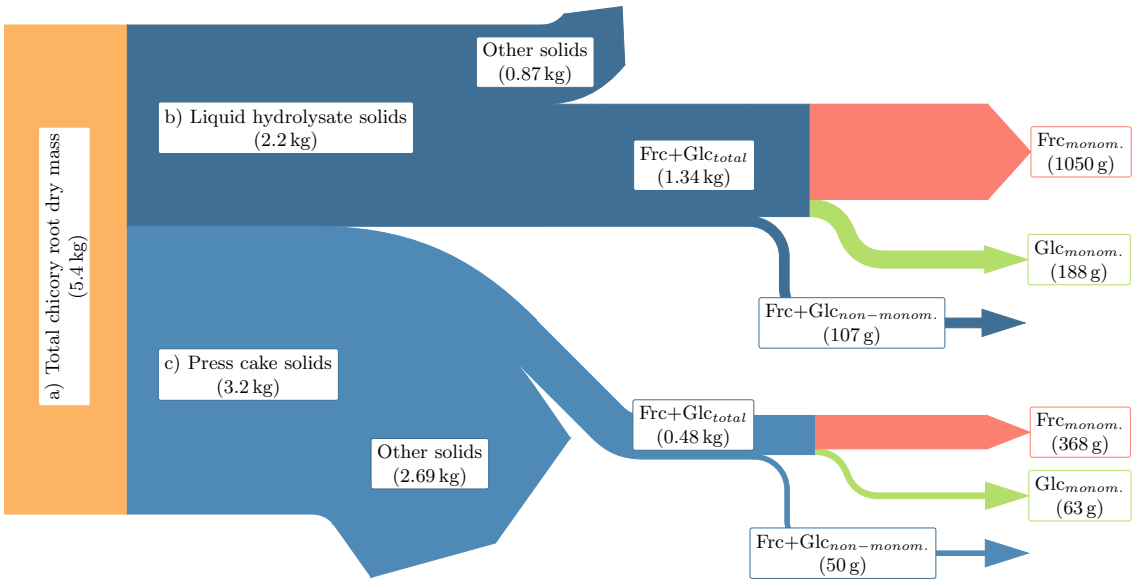

Figure 4.1: Distribution of solid masses during chicory root hydrolysis (a), and composition of liquid hydrolysate (b) and press cake/waste (c).

extracted fructose ($29.0\,\mathrm{g\,L^{-1}}$) was monomeric ($26.7\,\mathrm{g\,L^{-1}}$). The same percentage is true for the glucose content ($5.2\,\mathrm{g\,L^{-1}}$ total, $4.8\,\mathrm{g\,L^{-1}}$ monomeric).

In summary, liquid phase[2] contained 1.050 kg of monomeric fructose and 189 g of monomeric glucose beside some other sugars in negligible amounts (not shown here).

4.1.1 Discussion

A more effective press machine/technique would increase the total sugar yield by decreasing the water (and solved sugar) content from the press cake (Figure 4.1).

Figure 4.2 shows the importance of the long over-night cool-down phase with ongoing hydrolysis as well as autoclaving for the achievement of high conversion rates. It can also be seen, that the press-cake still contained a notable amount of sugars, resulting from its high water content.

[2] Assuming a density of $1.015\,\mathrm{kg\,L^{-1}}$.

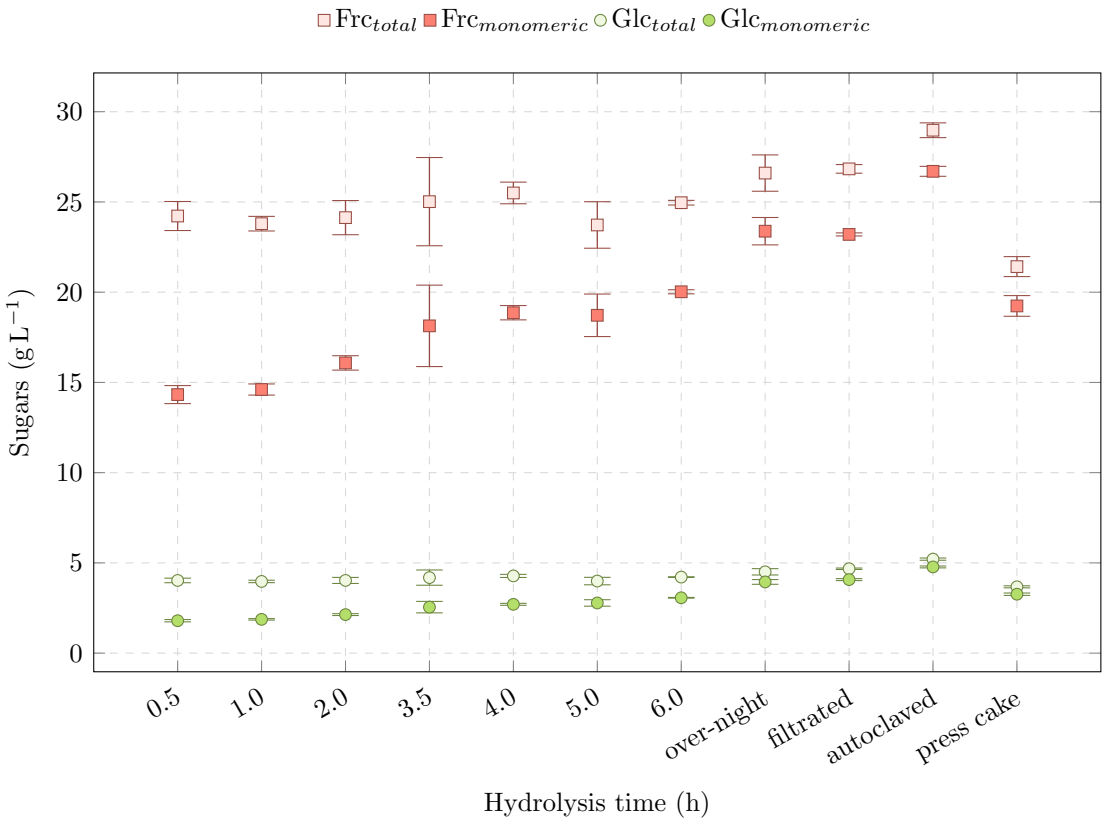

Figure 4.2: Hydrolysis: Sugar concentrations at different times, at the next day, after filtration, autoclaving, and in the press cake/waste.

The error bars are representing the standard deviation between the duplicates. As the errors for glucose and fructose concentration are of similar size for each time, inhomogeneous samples because of imperfect solid-to-liquid ratios could explain these errors.

4.2 Shaking flask experiments

The shaking flask experiments were aborted after five days, as except the LB medium control flasks, no other flask showed microbial growth. For all hydrolysate flasks, no increase in optical density (OD_{600}) could be observed.

The small inoculum volume as well as not precisely controllable process conditions could explain why bacteria did not grow on the hydrolysate. Aeration may have been insufficient or not constant enough. No information can be obtained whether one of the used additives shortens the lag phase or enables higher cell densities.

4.3 Fermentation experiments

Presented data: Each fermentation shown in this section is represented in form of two figures (plots). The upper figure displays the data measured off-line; cell dry weight (CDW), PHB concentration, fructose and glucose content, as well as optical density (OD_{600}). The figure below shows on-line data; pH, temperature, dissolved oxygen concentration (DO), amount of acid and base added to the medium.

Nomenclature: Each single fermentation makes part of a fermentation cycle, as four fermentations were carried out simultaneously, and is identified by following identification string: PHB-[Cycle no.]-[Strain no.]-[no.]. E. g.: PHB-XIV-428-2 describes the second fermentation of fermentation cycle PHB-XIV which was carried out with strain DSM 428.

Measurement errors: The concentrations shown in the fermentation plots must be handled with caution. Beside normal random errors, there are some systematic errors which are not negligible. On the one hand, the addition of acid and base changes the total reactor volume and decreases the sugar, PHB, and CDW concentrations. On the other hand, ongoing evaporation lead to an increase of those values. Furthermore, probe sampling leads to step-wise removal of reactor content.

By measuring the reactor volume at start and end as well as all feeds and drains, the overall error can be estimated to be between 5 and 10 % (overestimation) at the end of fermentation. Due to ongoing evaporation, error becomes higher the longer the experiment runs.

4.3.1 Evaluation of the standard protocol

The first fermentations were carried out according to the existing standard protocol as described in Section 3.3. Four fermentations were executed, two of them using *C. necator* DSM 428, and the other two using strain DSM 531. Three of these fermentations are shown in Figures 4.3, 4.4 and 4.5.

These first fermentations were done to get an impression of the current state. Results of this fermentation cycle were used to decide which strain to use on following fermentations.

4.3.1.1 PHB-VII-428-1 / Standard protocol

Figure 4.3 shows that after almost 8 days of fermentation a CDW of 3.8 g L^{-1} and a PHB concentration of 1.7 g L^{-1} were reached. This corresponds to a PHB content in dry weight of 43 %. No glucose was consumed, and also 80 % of the fructose stayed untouched. In line with the other fermentations of this cycle, the lag phase was quite long (up to 3 days).

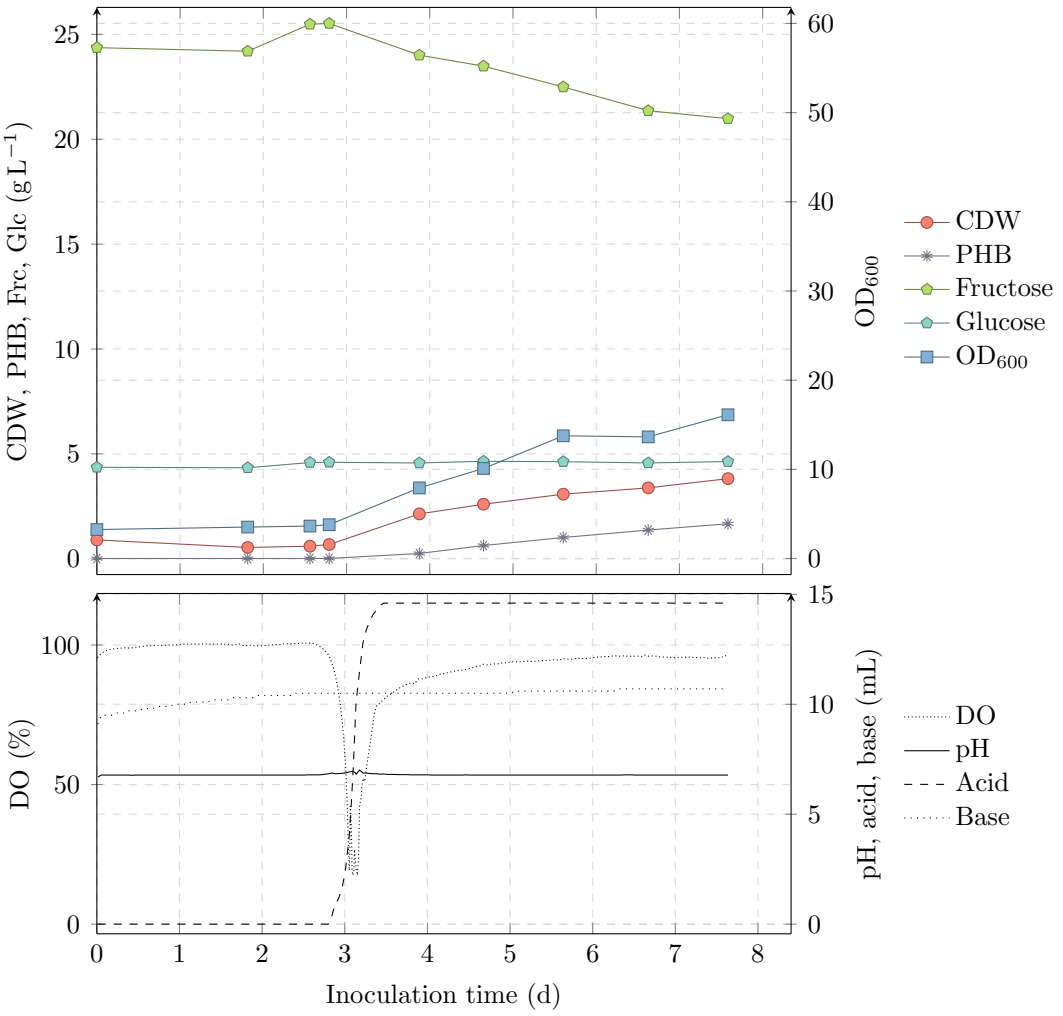

Figure 4.3: Fermentation course PHB-VII-428-1

4.3.1.2 PHB-VII-531-1 / Standard protocol

Fermentation 531-1, shown in Figure 4.4, finished basically after four days. At this time, a CDW of 3.1, and a PHB concentration of 1.6 g L^{-1} (53 %) were reached. The remaining fructose concentration was 16.0 g L^{-1}, and glucose content seemed to be unchanged.

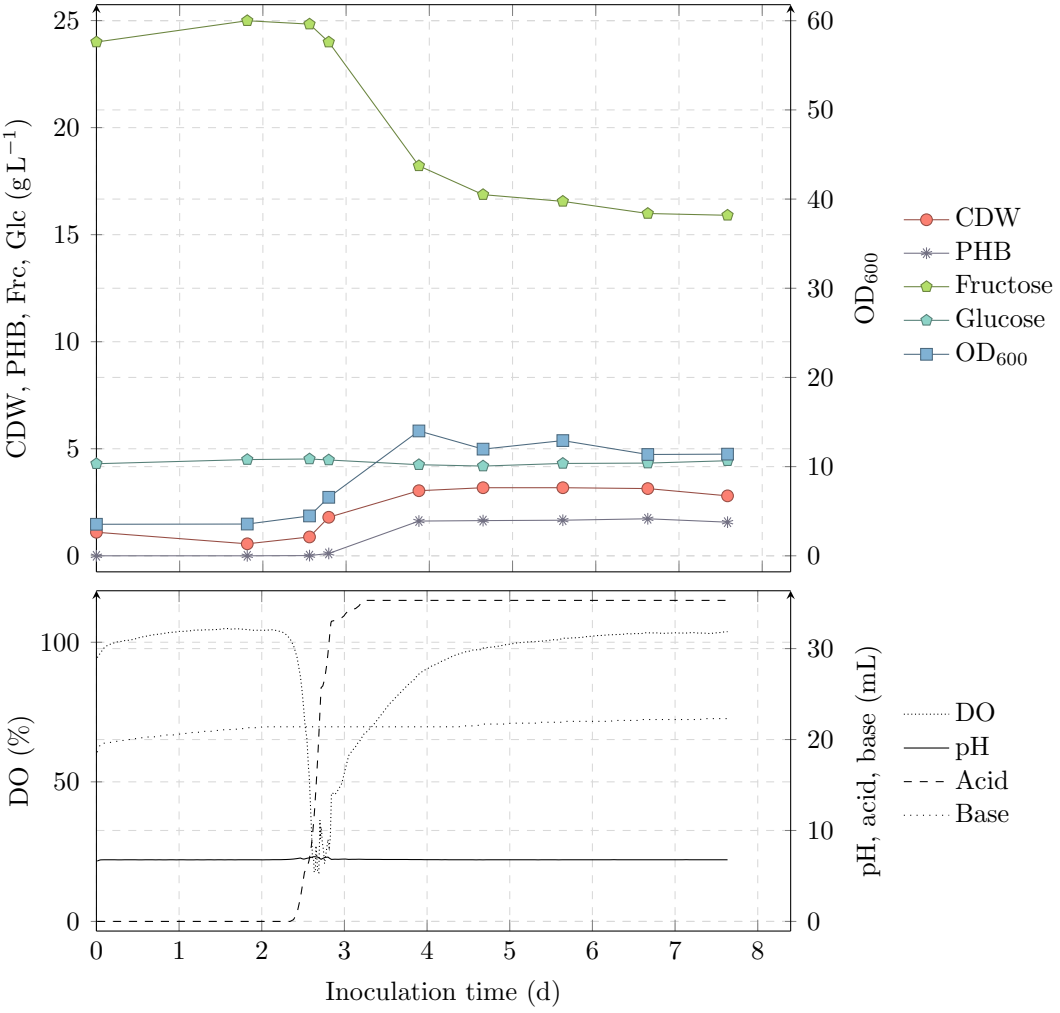

Figure 4.4: Fermentation course PHB-VII-531-1

4.3.1.3 PHB-VII-531-2 / Standard protocol

Fermentation 531-2 (Figure 4.5) from the first cycle performed better than the other ones. A PHB concentration of nearly $3\,\mathrm{g\,L^{-1}}$ was reached after a week. However, the lag phase was quite long also here, and the substrate was not consumed completely; while glucose was used entirely, about 50 % of the fructose was still in the medium after 8 days. When looking at the stepwise increase of cell dry weight as well as PHB concentration and regarding the DO concentration trend, two phases of bacterial activity can be observed.

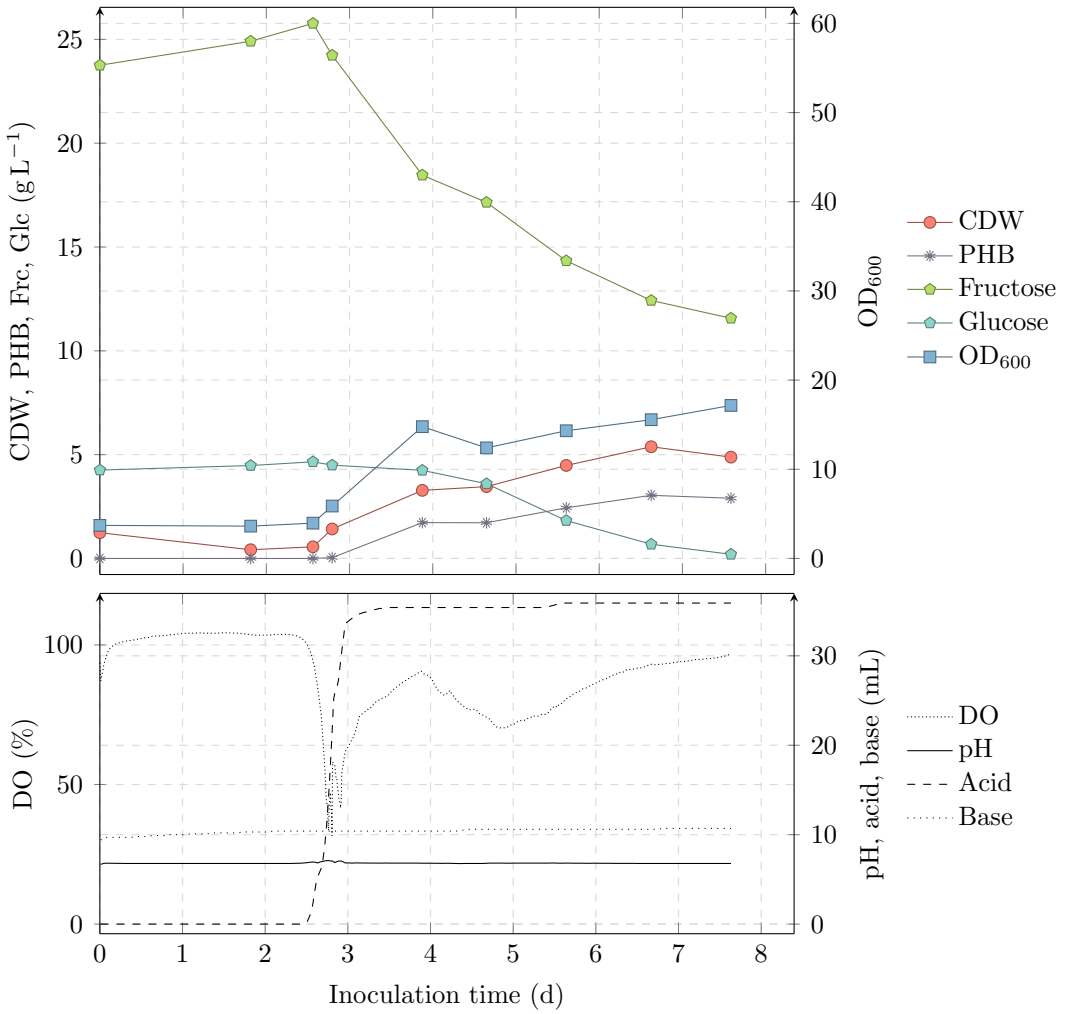

Figure 4.5: Fermentation course PHB-VII-531-2

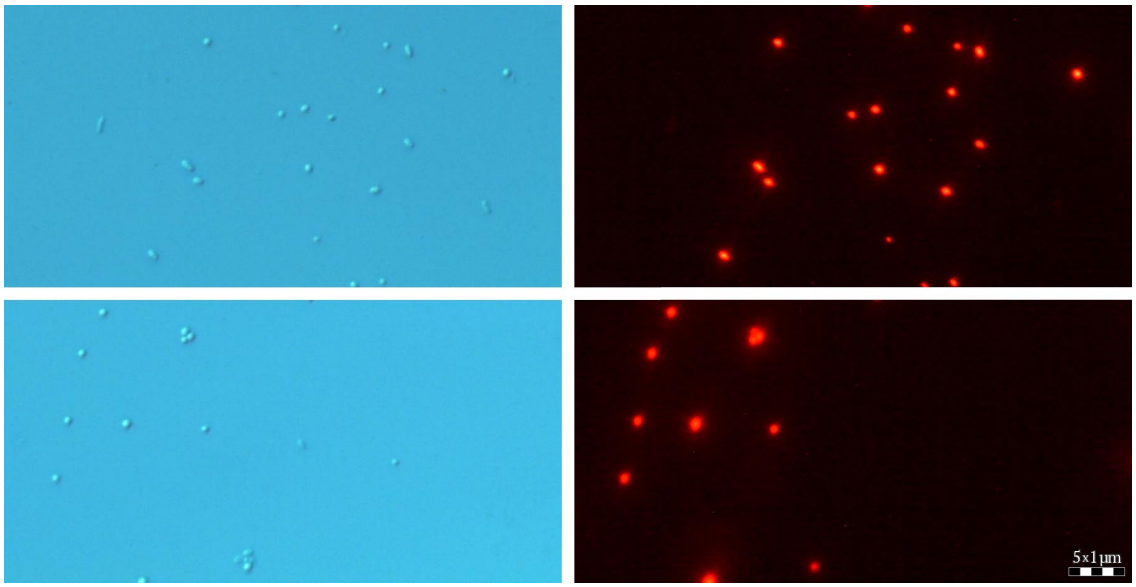

Figure 4.6: *C. necator* DSM 428 (above) and DSM 531 (below) after six days of fermentation in phase contrast and fluorescence microscopy

Under the microscope, bacteria of both strains looked small and had a globular to slightly rod-like shape. Figure 4.6 shows a phase contrast and a fluorescence microscopy image from the same specimen section of both strains. Most bacterial cells have diameter of approximately 1 µm or less. Only PHB producing bacteria have red fluorescence.

4.3.1.4 Discussion

Principally, three of four fermentations worked, in the sense that the desired organism grew and PHB accumulation occurred. However, one reactor (428-2, not shown here) was contaminated by an unknown organism. The other three reactors showed low PHB concentrations and incomplete substrate consumption (glucose and fructose) after 8 days.

Figures 4.3, 4.4, and 4.5 show nicely that after a cell growth phase (CDW increase) following the lag phase, the PHB accumulation phase began (PHB increase). This is especially visible between the third and fourth day in Figures 4.4 and 4.5. Residual cell mass then remained nearly constant over time until the end of fermentation. PHB accumulation seems to be not growth-associated for this strains of *C. necator*, which accords to the information in literature (Lee & Park 2002).

It can also be seen, that the cell dry weight decreased during the first two days, indicating cell death after inoculation. Resuspending the cell pellet with unmodified

hydrolysate during the preparation of the inoculum (Section 3.3.4.2) could explain that, as a hydrolysate pH of 4.5 might decrease the viability of the cells. Henceforward, the inoculum cell pellet was resuspended in previously to pH 7 adjusted hydrolysate.

Remarkable are the two growth phases observable in fermentation PHB-VII-531-2 (Section 4.3.1.3). It would be possible that these two growth and accumulation stages are resulting from the fact that the inoculum was prepared by combining two shaking flasks which were inoculated with two different clones or colonies. One clone may have started earlier, the second one later. During the second stage also the glucose content in the medium was consumed. DSM 428 (H16) is known to be glucose deficient (Orita et al. 2012). No information is available about strain 531 in the literature. However, even the glucose deficient described H16 is able to use glucose under certain conditions (Franz et al. 2010).

PHB-VII-531-1 and PHB-VII-531-2 show big differences in their general fermentation course. While the bacteria in PHB-VII-531-2 consumed glucose, PHB-VII-531-1 did not. As the inoculum was based on two single clones per reactor, this could indicate an inhomogeneous starting material (WCB). It was decided to no longer use single clones for further fermentations.

Although strain DSM 531 performed better than DSM 428 in terms of PHB productivity during this first fermentation cycle, further fermentations were mostly carried out with DSM 428. Preliminary trials showed that DSM 428 was able to totally consume all available fructose, whereas DSM 531 always left behind at least half of the sugar and produced less PHB.

4.3.2 NH_3 as NaOH replacement

During this fermentation cycle, the effect of nitrogen supplementation through the replacement of NaOH by NH_3 was investigated. The pH value of processes 428-1 and 428-2 (Figure 4.7 and 4.8) were regulated with NaOH, whereas the experiments 428-3 (Figure 4.9) and 428-4 used an aqueous NH_3 solution instead. Fermentation 428-4 is not shown here as the experiment did not work due to a contamination. Downwards regulation of the pH with hydrochloric acid was switched on after the growth phase finished to test whether the bacteria prefer a basic pH for growth.

4.3.2.1 PHB-XI-428-1 / no N supplementation

Fermentation 428-1 (Figure 4.7) produced a final PHB concentration of 1.6 g L^{-1} and a CDW of 4.1 g L^{-1} after 10 days. This corresponds to a PHB content in dry weight of 39 %. Most part of fructose was not consumed. Glucose concentration remained nearly constant over time.

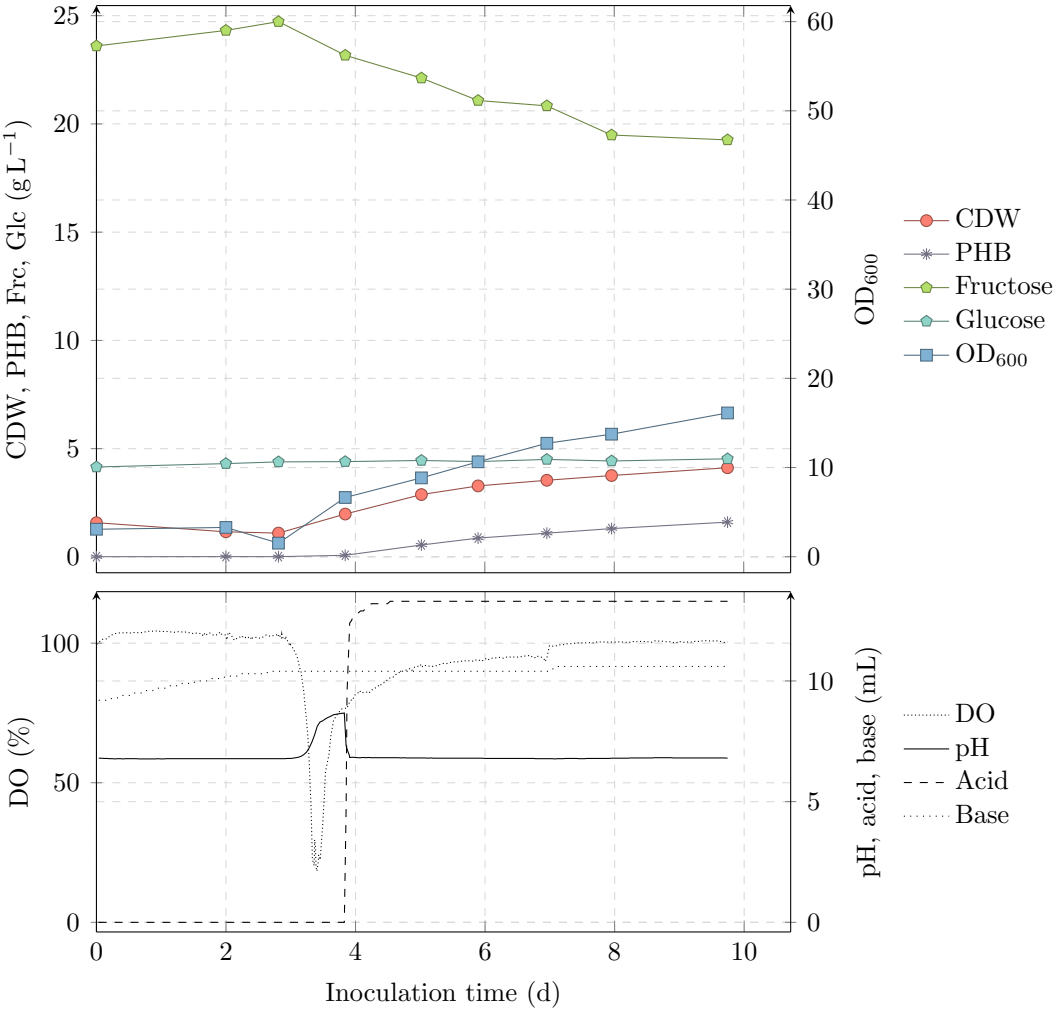

Figure 4.7: Fermentation course PHB-XI-428-1

4.3.2.2 PHB-XI-428-2 / no N supplementation

Fermentation 428-2 (Figure 4.8) gave almost the same results as 428-1. A final CDW of 4.5 g L^{-1} and PHB content of 1.7 g L^{-1} are comparable with previous fermentations of this strain with the same parameters. Also here, the biggest part of substrate was still in the medium after 10 days of ongoing process.

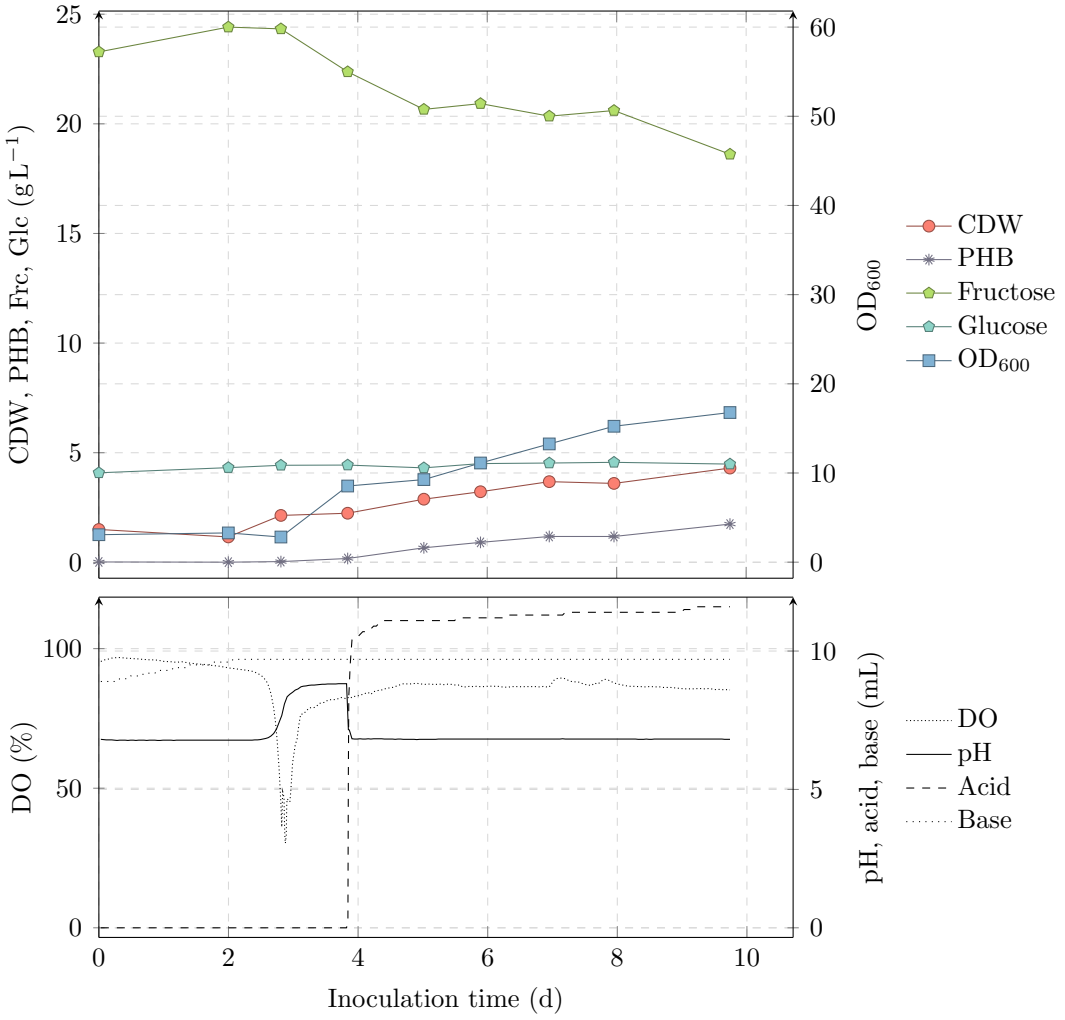

Figure 4.8: Fermentation course PHB-XI-428-2

4.3.2.3 PHB-XI-428-3 / N supplemented

The nitrogen supplemented fermentation 428-3 (Figure 4.9) differed strongly from the non supplemented ones. Optical density, as well as cell dry weight (10.6 g L^{-1}), and PHB concentration (4.1 g L^{-1}), were much higher than those of previous fermentations. Fructose as well as glucose were consumed almost completely after 10 days. However, the PHB content (38 %) was comparable with that of non supplemented fermentations of this cycle.

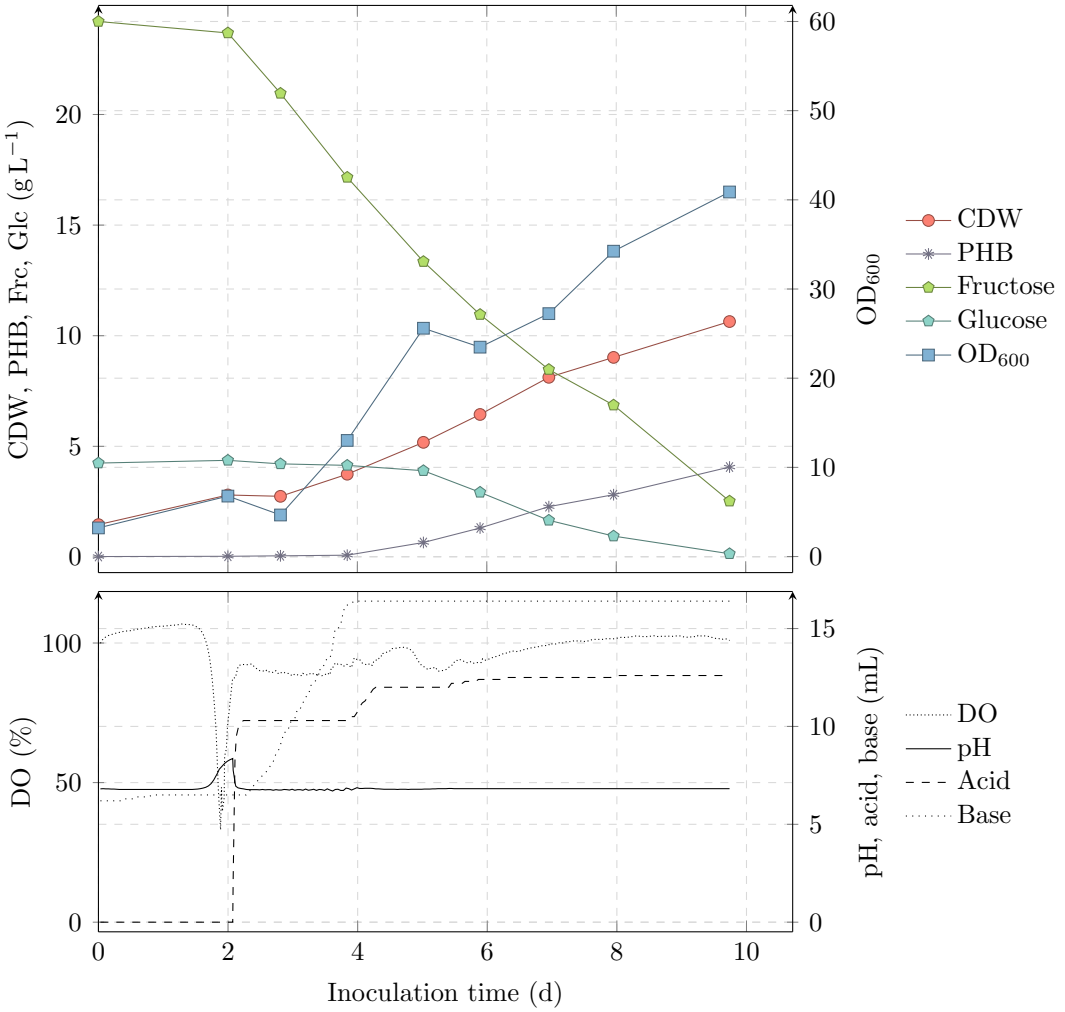

Figure 4.9: Fermentation course PHB-XI-428-3

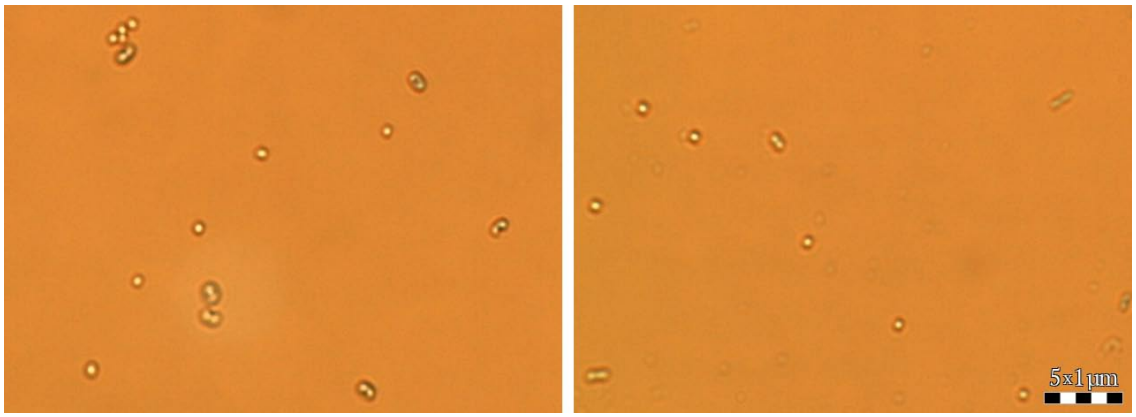

Figure 4.10: Bright field image of *C. necator* DSM 428 without (left) and with (right) nitrogen supplementation

Nitrogen supplemented and not supplemented bacteria were compared under the microscope in bright field. No obvious differences in cell size and shape could be determined (Figure 4.10).

4.3.2.4 Discussion

Unsupplemented (PHB-XI-428-1 and PHB-XI-428-2) and supplemented (PHB-XI-428-3) fermentations show clear differences in terms of PHB amount, CDW, and substrate consumption. However, there are no differences concerning PHB content and cell morphology. A PHB content of less than 40 % is very low compared to values known from the literature. Chanprateep et al. (2010) reached a PHA content of 78 % using the same strain and fructose as main substrate.

As the cell sizes show no difference (Figure 4.10) and the PHB content is the same for the fermentations with and without nitrogen, the increase in CDW and PHB concentration are due to a higher cell number in the fermentation medium. The cell proliferation was enabled by the higher nitrogen content.

The lag phase of the supplemented fermentation looks slightly shorter. The second nitrogen supplemented fermentation was not successful due to a contamination. After two days of lag phase, within one day, the whole sugar was consumed. No PHB was accumulated. Agar plate smears show different colony forms than those known from *C. necator* DSM 428.

The pH control was disabled at the begin of the fermentation, studying if the variable pH has any impact on the lag phase. After the pH climbed over 8, pH control was restarted. The data shown here suggest, that this variable pH has no

substantial effect on the lag phase.

4.3.3 $(NH_4)_2SO_4$ addition Minimal medium pre-culture I

As the replacement of NaOH by NH_3 resulted in higher PHB yields, the following experiment was the evaluation of different nitrogen concentrations. Because of handling and dosing advantages, $(NH_4)_2SO_4$ was used as nitrogen additive instead of ammonia.

Reactor PHB-XII-428-1 was used as blank, thus, without ammonium sulfate addition. PHB-XII-428-2 was supplemented with 0.5, PHB-XII-428-3 with 1.0, and PHB-XII-428-4 with $3.0\,\mathrm{g\,L^{-1}}$ $(NH_4)_2SO_4$. Looking at the base consumption of the reactor, it can be seen when $(NH_4)_2SO_4$ consumption began and ended.

Furthermore, bacteria were subcultured once on minimal medium plates, and inoculum shaking flasks were minimal medium based too. The procedure was changed due to the recommendations of a well-renowned PHB researcher, Martin Koller from the TU Graz. The exact minimal medium composition was already shown in Table 3.5 on page 33. DSM 428 cells came from the working cell bank at $-80\,^{\circ}\mathrm{C}$.

4.3.3.1 PHB-XII-428-1 / Without $(NH_4)_2SO_4$ addition

The unsupplemented reactor worked like expected from previous fermentations. It resulted in 2.4 g L^{-1} PHB and 5.6 g L^{-1} CDW, corresponding to a PHB content in dry weight of 43 %.

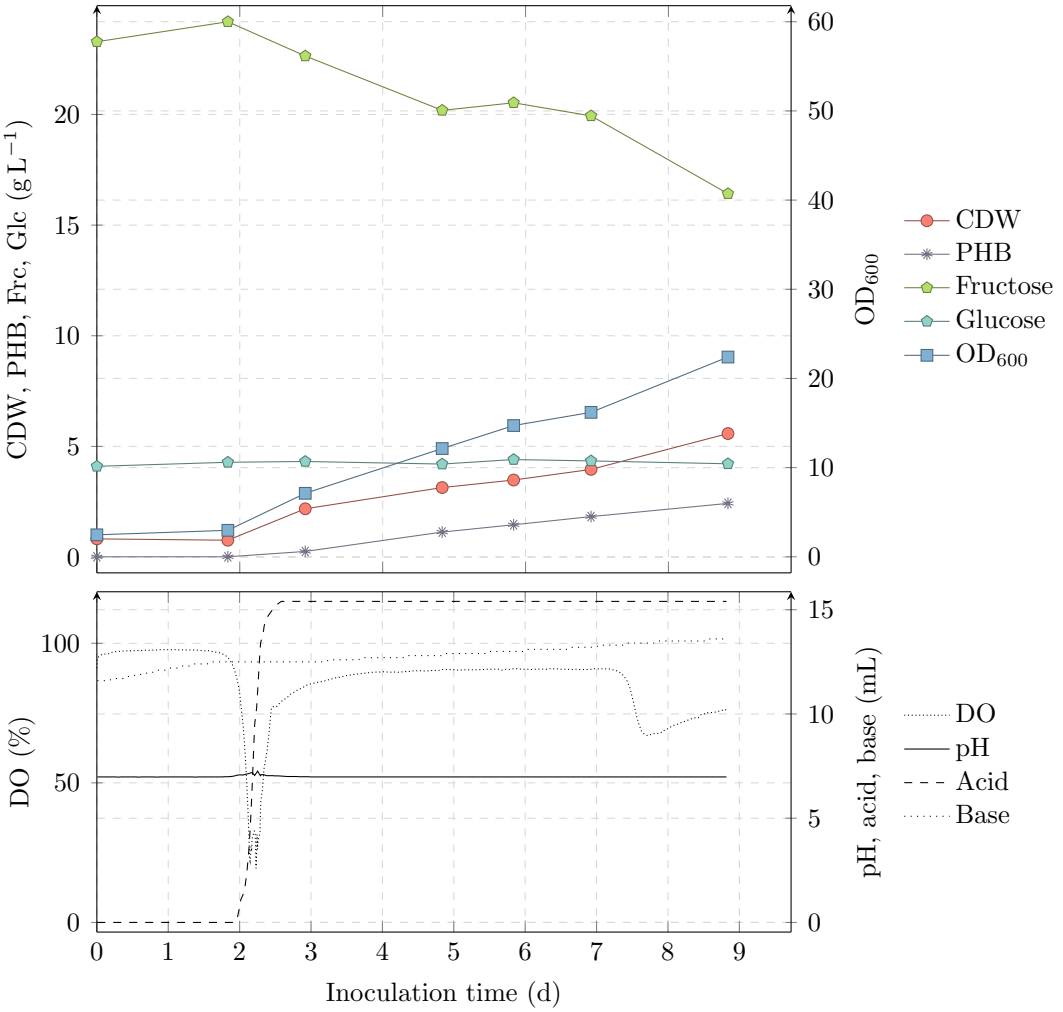

Figure 4.11: Fermentation course PHB-XII-428-1

4.3.3.2 PHB-XII-428-2 / 0.5 g L^{-1} $(NH_4)_2SO_4$

The reduced nitrogen supplementation does not seem to have any major effect on growth and PHB accumulation. 2.2 g L^{-1} PHB and a CDW of 4.9 g L^{-1} lead to a PHB content in dry weight of 45 %.

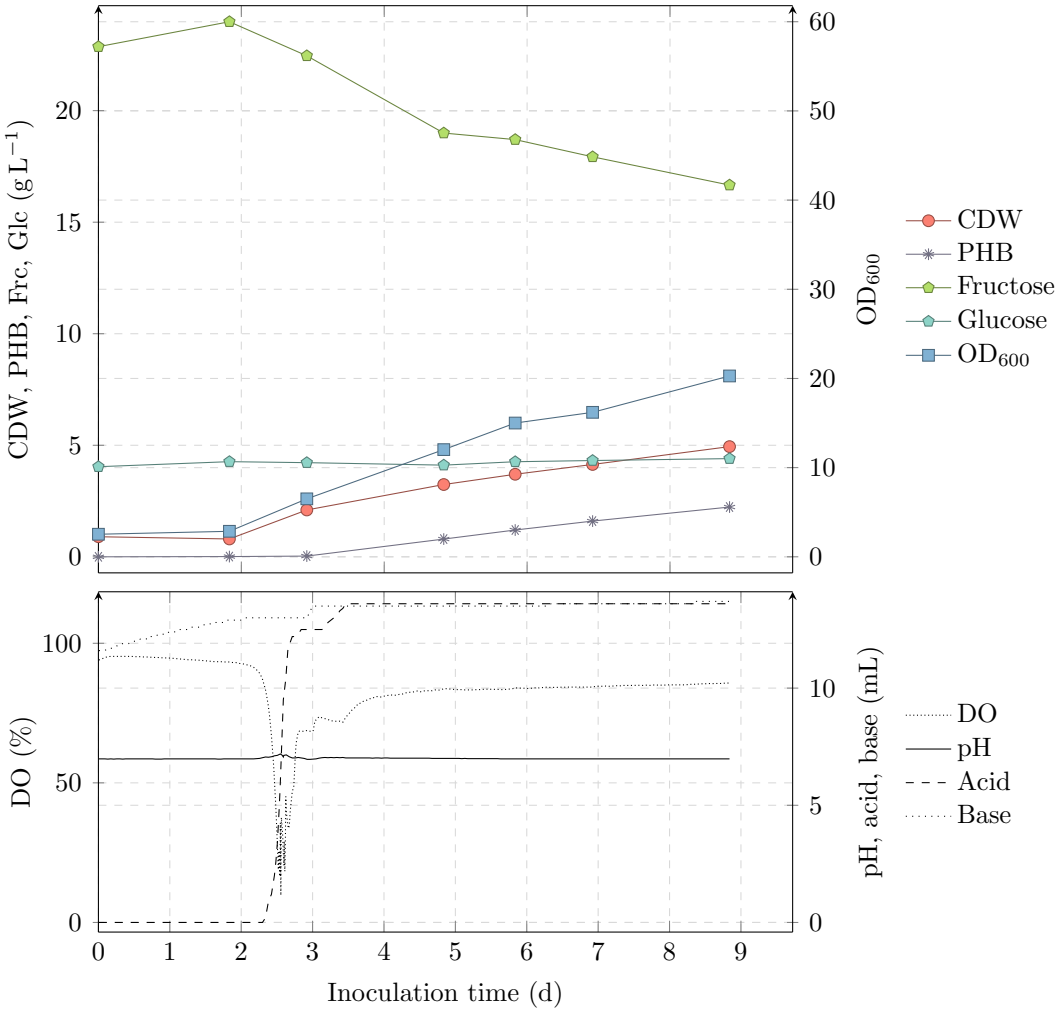

Figure 4.12: Fermentation course PHB-XII-428-2

4.3.3.3 PHB-XII-428-3 / 1.0 g L^{-1} $(NH_4)_2SO_4$

The base consumption was slightly higher due to the use of $(NH_4)_2SO_4$ because the consumption of ammonium leads to an acidification of the medium. No obvious positive effect of the nitrogen supplementation can be observed. 1.7 g L^{-1} PHB and a CDW of 4.7 g L^{-1} lead to a PHB content in dry weight of 36 %.

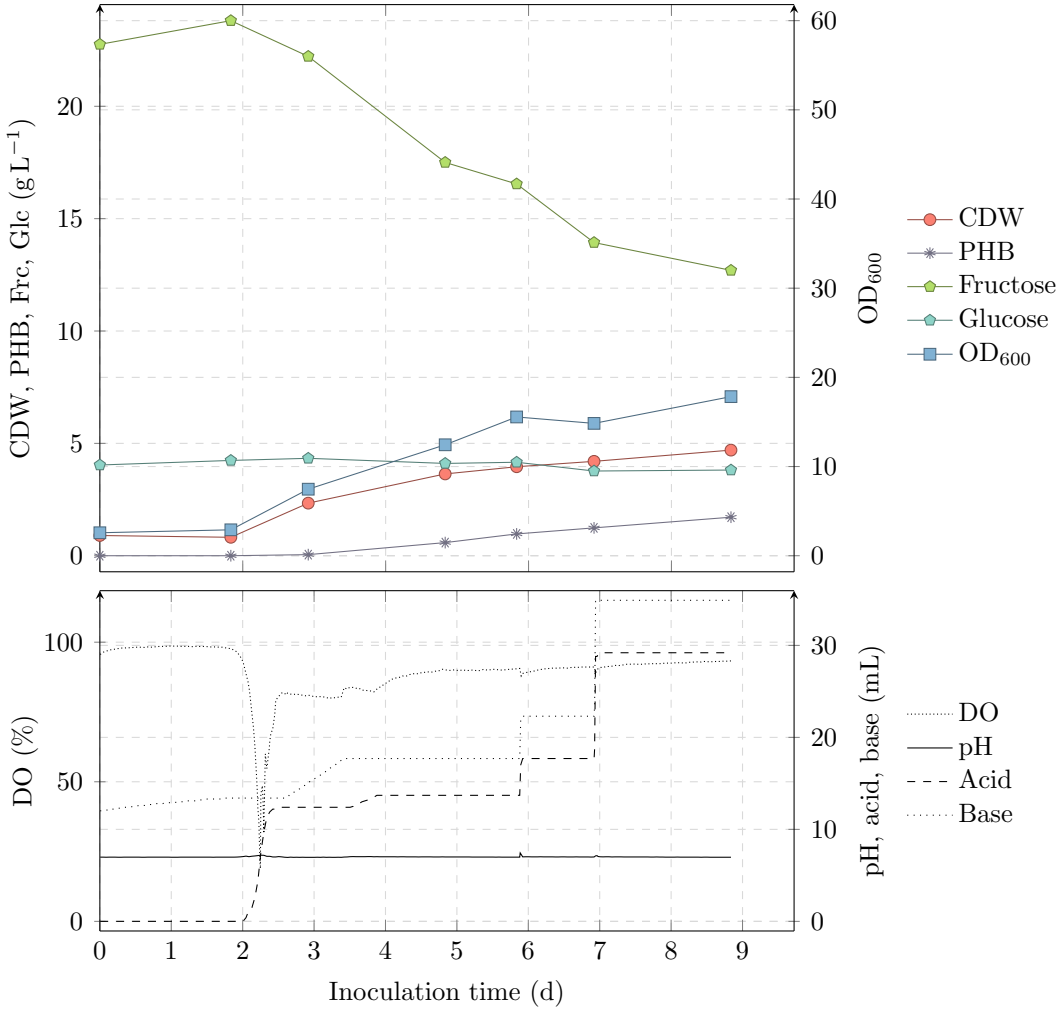

Figure 4.13: Fermentation course PHB-XII-428-3

4.3.3.4 PHB-XII-428-4 / 3.0 g L^{-1} $(NH_4)_2SO_4$

The initial course of fermentation 428-4 (Figure 4.14) is comparable with previous fermentations. The base consumption is quite high due the use of 3 g L^{-1} $(NH_4)_2SO_4$.

The process started with a normal high-activity growth phase, followed by a long cell growth phase during which no PHB accumulation occurred. The constant consumption of NaOH indicates a constant consumption of ammonium. After 5 days, the DO concentration decreased rapidly again, and the base consumption increased exponentially. After 7 days, fructose was totally consumed, and PHB had a maximal concentration of 4.5 g L^{-1}, and a CDW of 10.5 g L^{-1}, corresponding to a PHB content of 43 %.

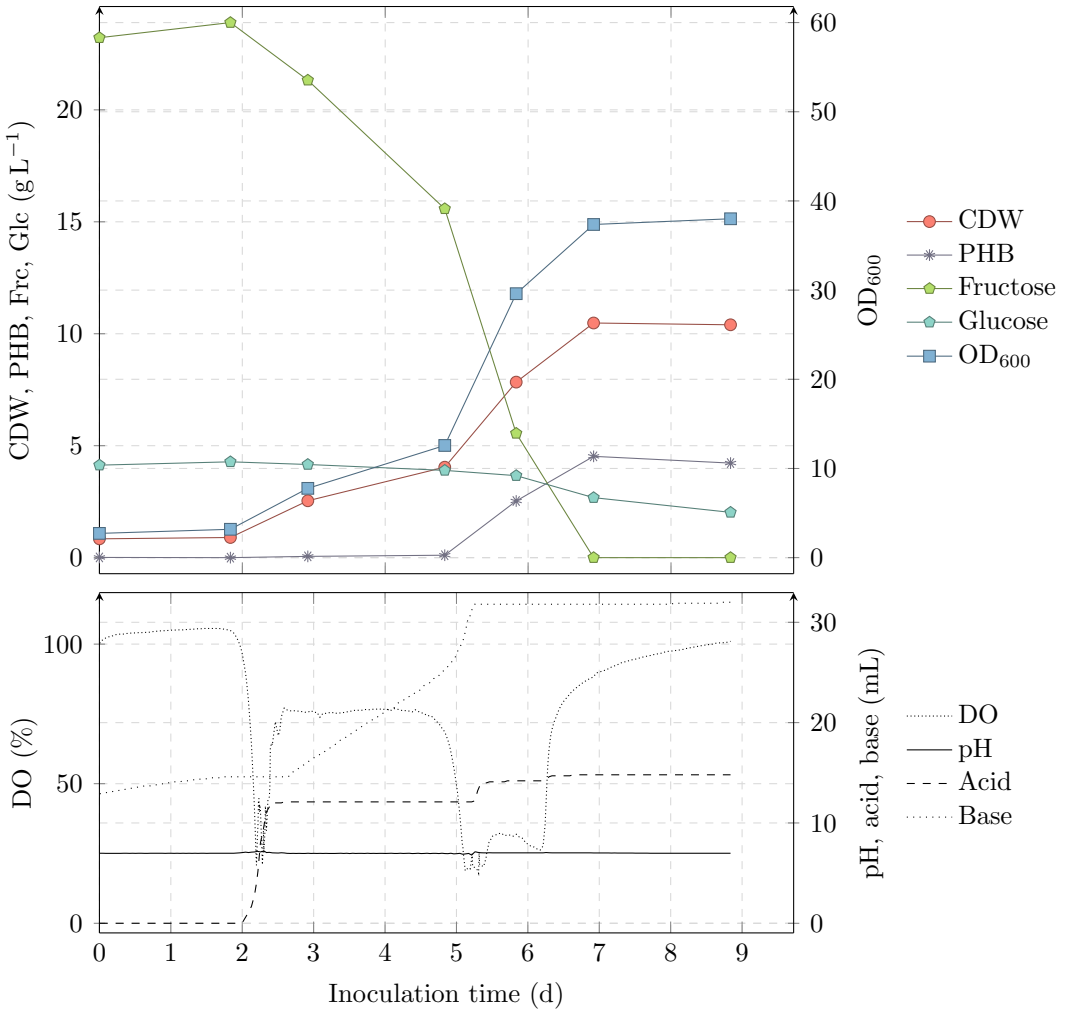

Figure 4.14: Fermentation course PHB-XII-428-4

4.3.3.5 Discussion

The first three fermentations of this cycle, which were supplemented with 0, 0.5, and 1.0 g L^{-1} $(NH_4)_2SO_4$, respectively, show neither a major difference in their general fermentation course nor in their PHB or CDW yield. Until day 5, also fermentation 428-4 (supplemented with 3.0 g L^{-1} $(NH_4)_2SO_4$) behaved similar. However, after 5 days, an unexpected increase in cellular activity could be observed during latter fermentation. It is implausible that the high nitrogen supplementation is solely responsible for the unexpected process course. Fermentation PHB-XI-428-3 from the previous cycle (Section 4.9) showed that a higher nitrogen content increases the cell proliferation, but neither increases dramatically the rate of PHB accumulation nor the activity during PHB accumulation phase. A spontaneous mutation could be possible but is unlikely. The most probable reason for the rapid "second start"-like increase in activity at day 5 is a metabolic adaptation to the substrate, promoted through the cultivation on minimal medium combined with the higher cell proliferation which was enabled by the high nitrogen supplementation. A streak plate, as well as microscopy images (not shown here), and the rapid PHB increase after day 5 suggest that no unwanted contamination is responsible for the rapid increase in activity during fermentation PHB-XII-428-4.

The LB medium streak plate created with cells taken at day 6 from fermentation PHB-XII-428-4 served as inoculum for the creation of minimal medium based streak plates.

4.3.4 Different nitrogen sources Minimal medium pre-culture I

The following four fermentations of cycle PHB-XIII were supplemented with four different nitrogen sources; urea, $(NH_4)_2SO_4$, soy peptone, and casein peptone. All four reactors got nitrogen supplementation in an amount equivalent to 3 g L^{-1} $(NH_4)_2SO_4$.

Like in the previous fermentation cycle (Section 4.3.3), bacteria were subcultured once on minimal medium plates, and inoculum shaking flasks were minimal medium based too.

4.3.4.1 PHB-XIII-428-1 / Urea

1.4 g L^{-1} urea were added to reactor 428-1, shown in Figure 4.15. A big part oft the fructose in the medium was consumed after 9 days. A cell dry weight of 7.6 g L^{-1} was produced. However, 2.0 g L^{-1} PHB lead to a PHB content of only 27 %.

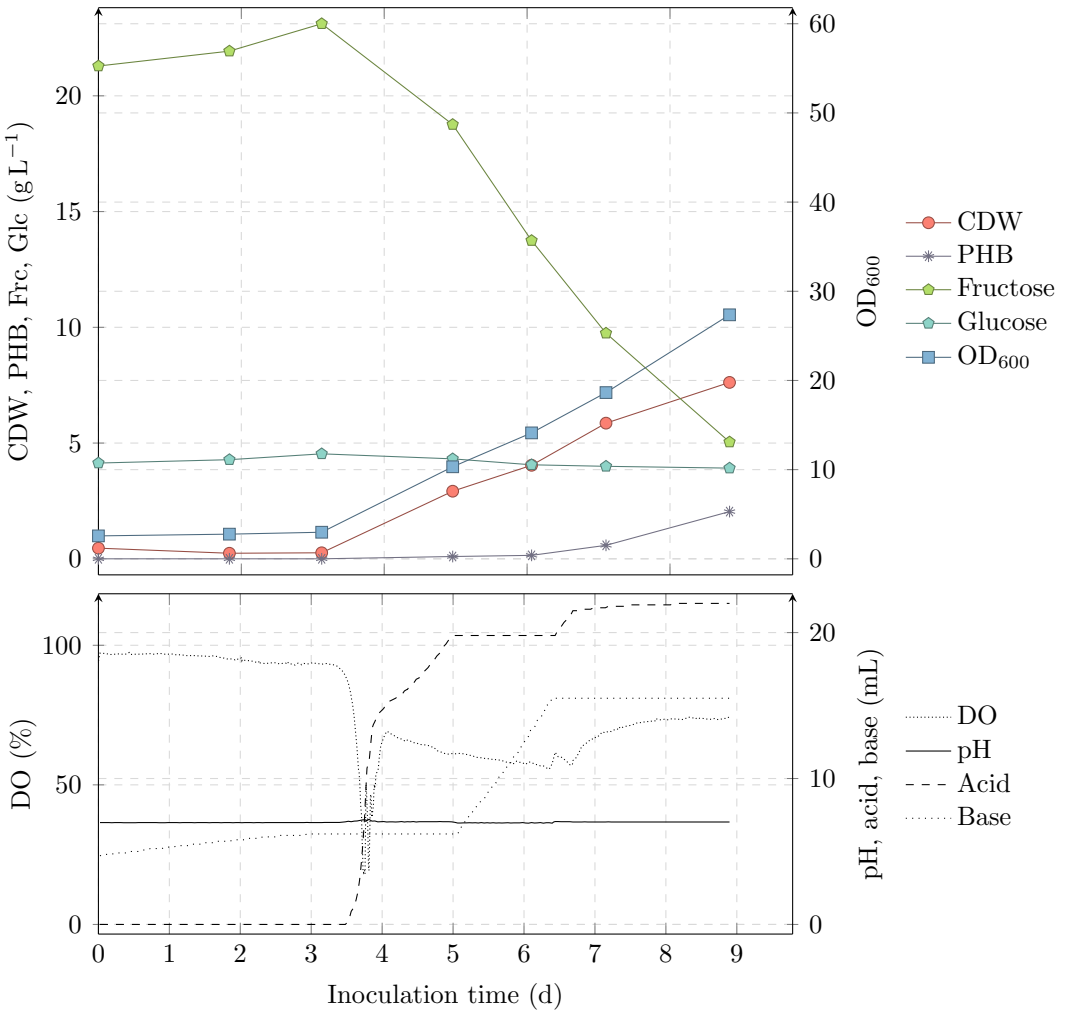

Figure 4.15: Fermentation course PHB-XIII-428-1

4.3.4.2 PHB-XIII-428-2 / $(NH_4)_2SO_4$

Reactor 428-2 (Figure 4.16) was supplemented with $3.0\,g\,L^{-1}$ $(NH_4)_2SO_4$. The general course of fermentation, like lag phase or DO course, was comparable to that of the urea supplemented one. Also here, the reached PHB content (33 %) was relatively low. $3.0\,g\,L^{-1}$ PHB and a CDW of $8.8\,g\,L^{-1}$ were produced.

It can be seen that the consumption of $(NH_4)_2SO_4$ would lead to a acidification of the medium. As the pH of the reactor was regulated, base was pumped in the reactor, starting at inoculation time 3.5 days, and ending after 6 days.

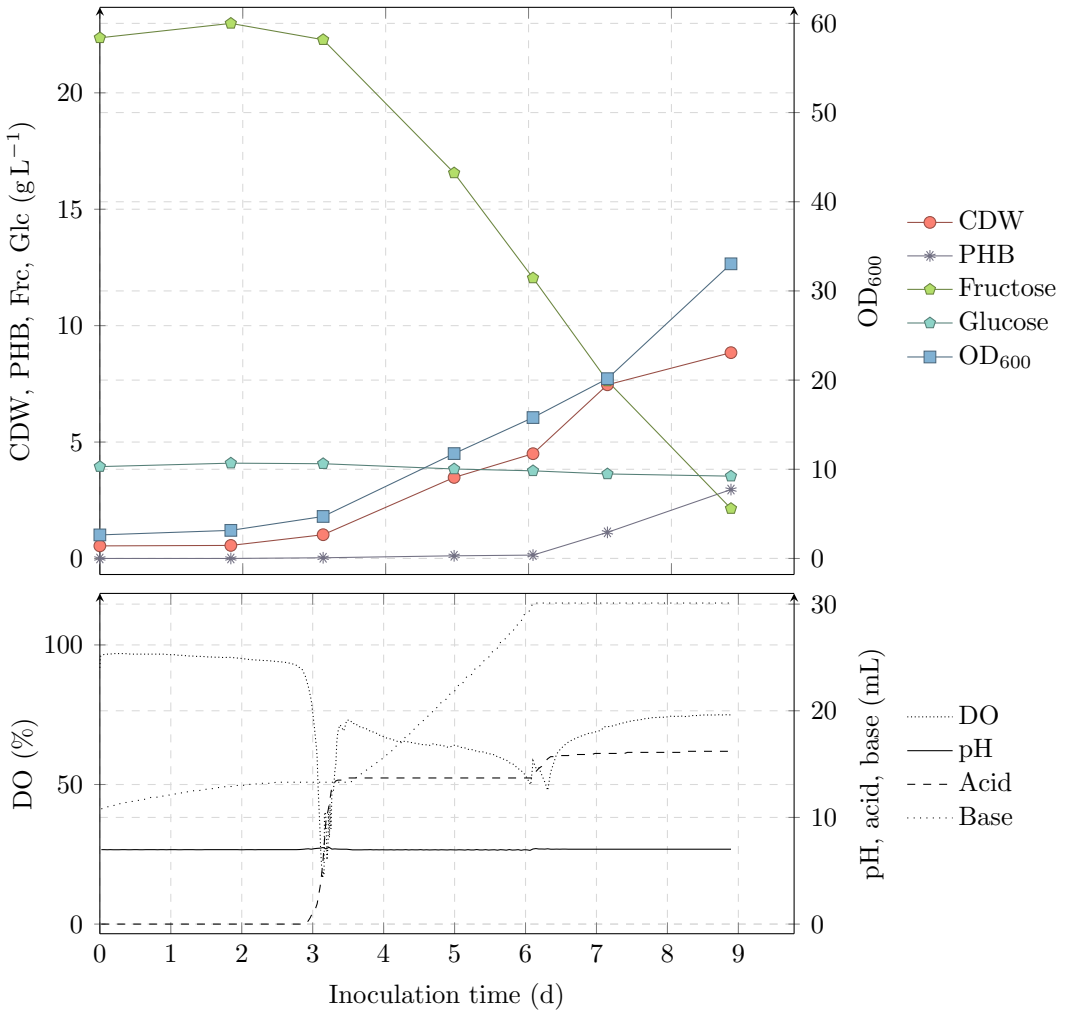

Figure 4.16: Fermentation course PHB-XIII-428-2

4.3.4.3 PHB-XIII-428-3 / Soy peptone

Reactor 428-3 (Figure 4.17) was supplemented with 6.6 g L^{-1} soy peptone. After 9 days, a final CDW of 8.1 and PHB content of 3.8 g L^{-1} was reached. This corresponds to a PHB content in dry weight of 47 %. The two days lasting lag phase was a little shorter than those of the urea and $(NH_4)_2SO_4$ supplemented fermentations.

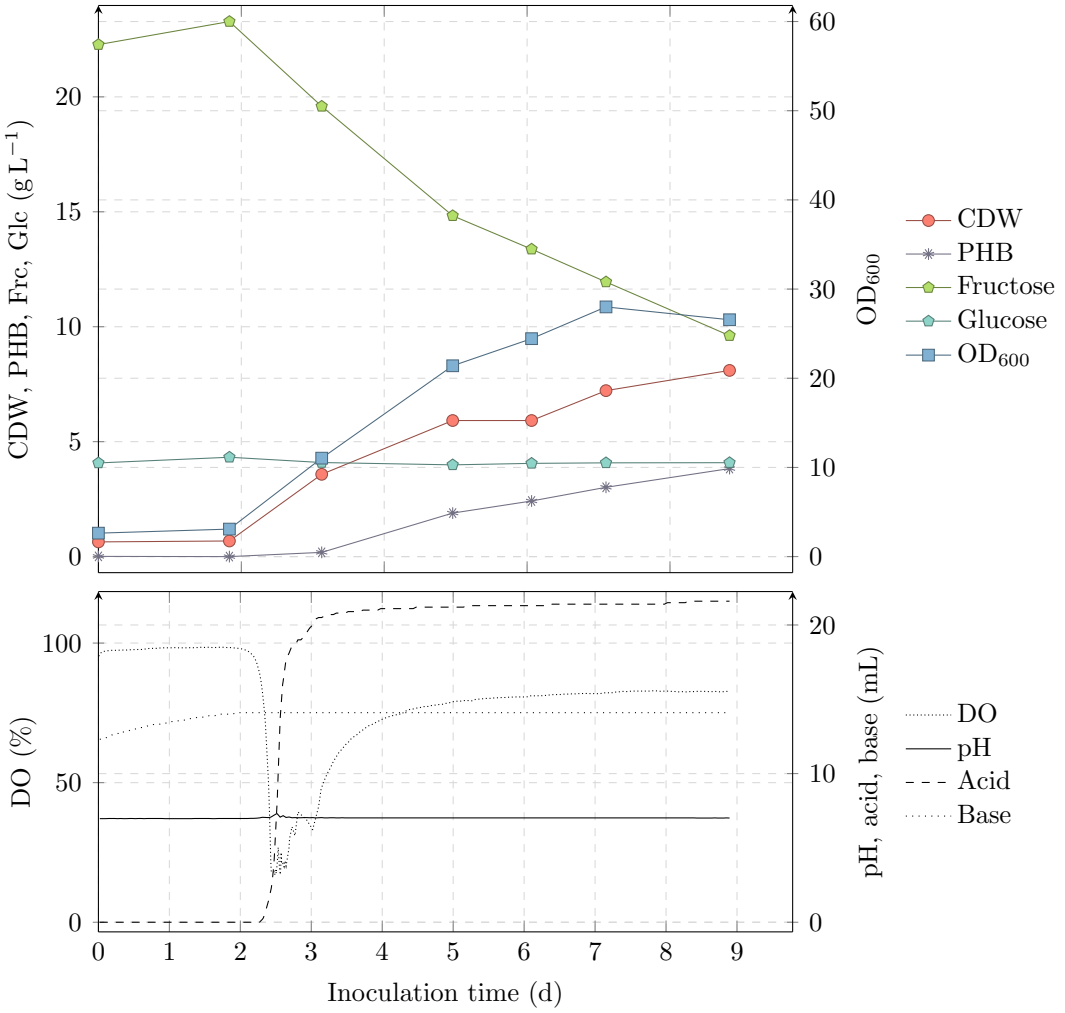

Figure 4.17: Fermentation course PHB-XIII-428-3

4.3.4.4 PHB-XIII-428-4 / Casein peptone

Reactor 428-4 (Figure 4.18) was supplemented with 5.3 g L^{-1} casein peptone and worked similar to the soy peptone supplemented one. The lag phase also for this peptone supplemented fermentation was pretty much two days long. Final PHB and CDW concentrations were 2.5 (35 %) and 7.1 g L^{-1}, respectively.

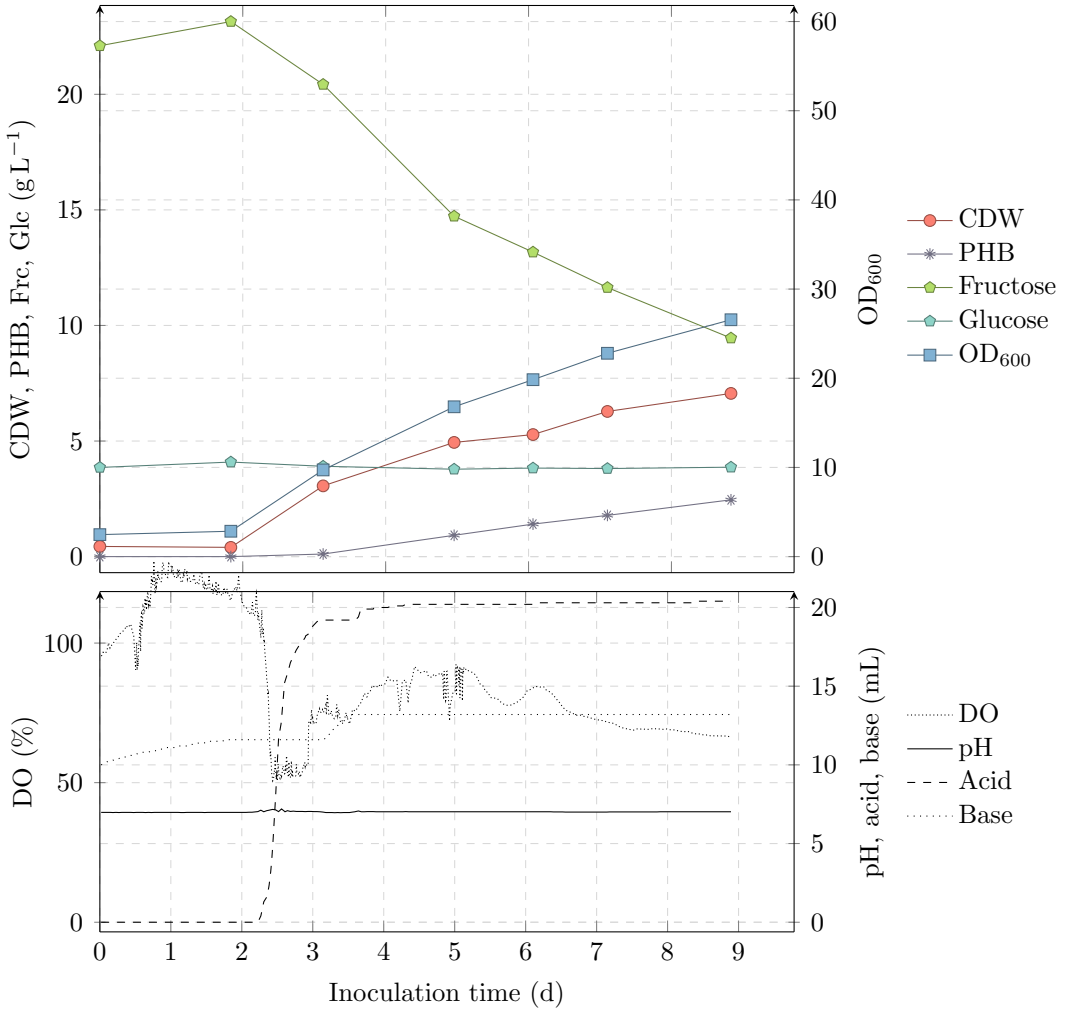

Figure 4.18: Fermentation course PHB-XIII-428-4

4.3.4.5 Discussion

Experiment cycle PHB-XIII showed that fermentations supplemented with complex nitrogen sources (peptones) show slightly shorter lag phase (about two days) compared to $(NH_4)_2SO_4$ and urea supplemented ones (three days). This corresponds to reports from the literature (Mahishi & Rawal 2002).

As peptones are hydrolysates of protein rich substrates, shorter lag phases on peptone supplemented fermentations make sense. Peptones contain free amino acids and short peptides which can easily be utilized by the bacteria for the synthesis of proteins. On the other hand, $(NH_4)_2SO_4$ or urea have to be converted first to amino acids which requires many different biosynthetic conversions.

Urea and $(NH_4)_2SO_4$ had a nearly complete substrate consumption after nine days. Residual cell mass was higher for urea and $(NH_4)_2SO_4$ supplemented fermentations. The two peptone supplemented fermentations had a residual fructose content of about 40 %.

Fermentation PHB-XIII-428-2, which was a duplicate of fermentation PHB-XII-428-4 (Section 4.3.3.4), could not reproduce previous results. Although both fermentations were supplemented with $3\,g\,L^{-1}$ $(NH_4)_2SO_4$, they differ strongly in their fermentation course. No second high-activity growth and PHB accumulation phase can be observed in fermentation PHB-XIII-428-2.

4.3.5 Different nitrogen sources Minimal medium pre-culture II

The four experiments of fermentation cycle PHB-XIIIb were carried out to get duplicates of the PHB-XIII fermentations. The same four nitrogen supplements and the same additive concentrations were used.

The only difference in the setup compared to PHB-XIII was the pre-culture. Cells were not taken directly from the WCB, but from a LB medium streak plate of cells taken from fermenter PHB-XII-428-4 at day 6 (Section 4.3.3.4, page 54). Subsequently, cells were subcultured twice on minimal medium plates before they grew in minimal medium based shaking flasks for 48 h.

As the cell density in the inoculum shaking flask was comparably high ($OD_{600} = 20 * 0.7$), the inoculum volume was halved from 500 to 250 mL.

4.3.5.1 PHB-XIIIb-428-1 / Urea

1.4 g L^{-1} urea were added to reactor 428-1 (Figure 4.19). The lag phase was clearly shorter than those of the previous fermentation, as it took only four to five hours (note the x-axis scale). After one day, fructose was totally consumed. A maximal CDW of 12.1 g L^{-1} and PHB content of 5.9 g L^{-1} were reached, corresponding to a PHB content in dry weight of 49 %. The DO concentration stayed at the minimal DO set point of 20 % as long as fructose was available in the medium.

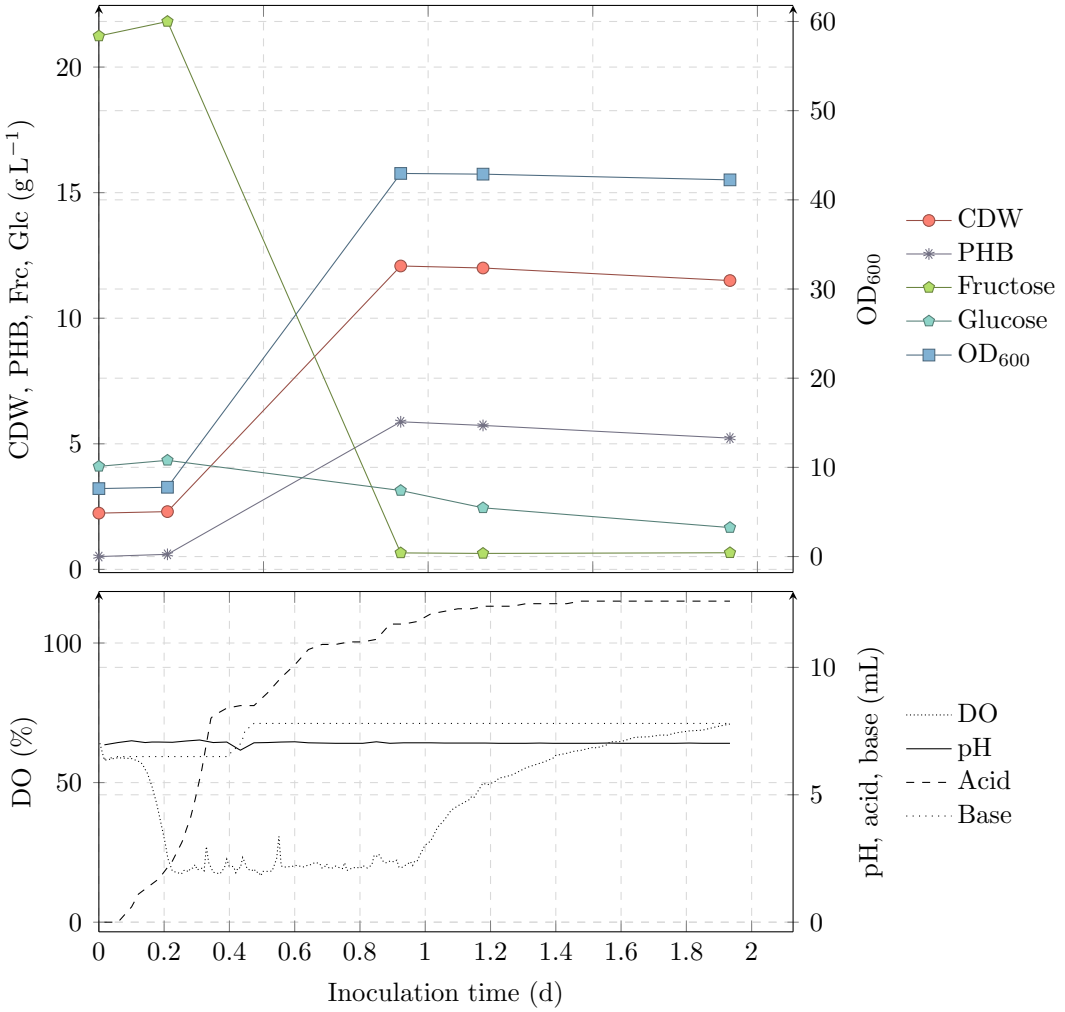

Figure 4.19: Fermentation course PHB-XIIIb-428-1

4.3.5.2 PHB-XIIIb-428-2 / $(NH_4)_2SO_4$

Reactor 428-2 (Figure 4.20) was supplemented with 3.0 g L^{-1} $(NH_4)_2SO_4$. It reached a maximal CDW of 11.0, and a PHB concentration of 5.1 g L^{-1} (47 %). The fermentation had a very short lag time (eight to ten hours) and the bacteria fully consumed the fructose after only 1.2 days.

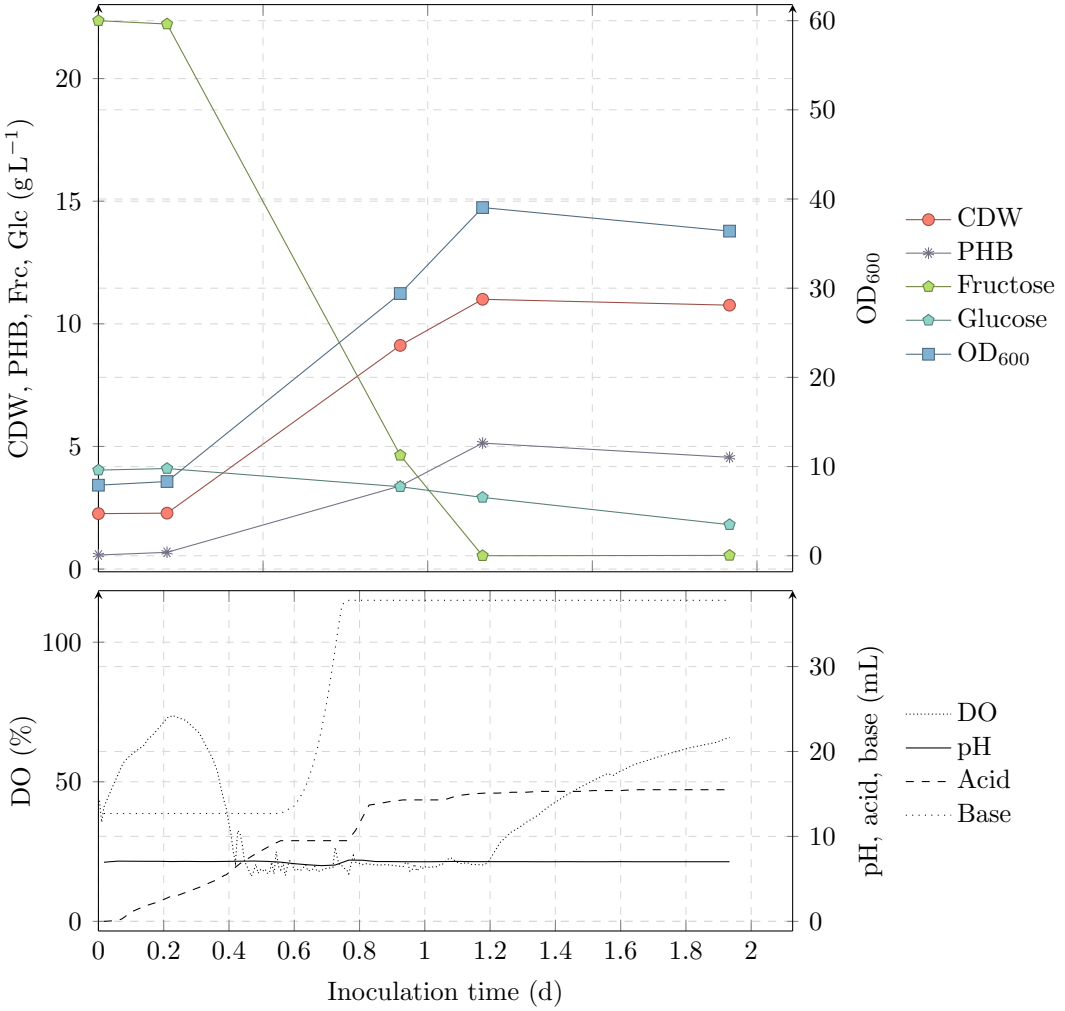

Figure 4.20: Fermentation course PHB-XIIIb-428-2

4.3.5.3 PHB-XIIIb-428-3 / Soy peptone

6.6 g L^{-1} soy peptone were supplemented to reactor 428-3 (Figure 4.21). After 1.5 to 2 days, fructose was totally consumed. 9.2 g L^{-1} PHB and 14.6 g L^{-1} CDW were produced. This corresponds to a PHB content in dry weight of 63 %.

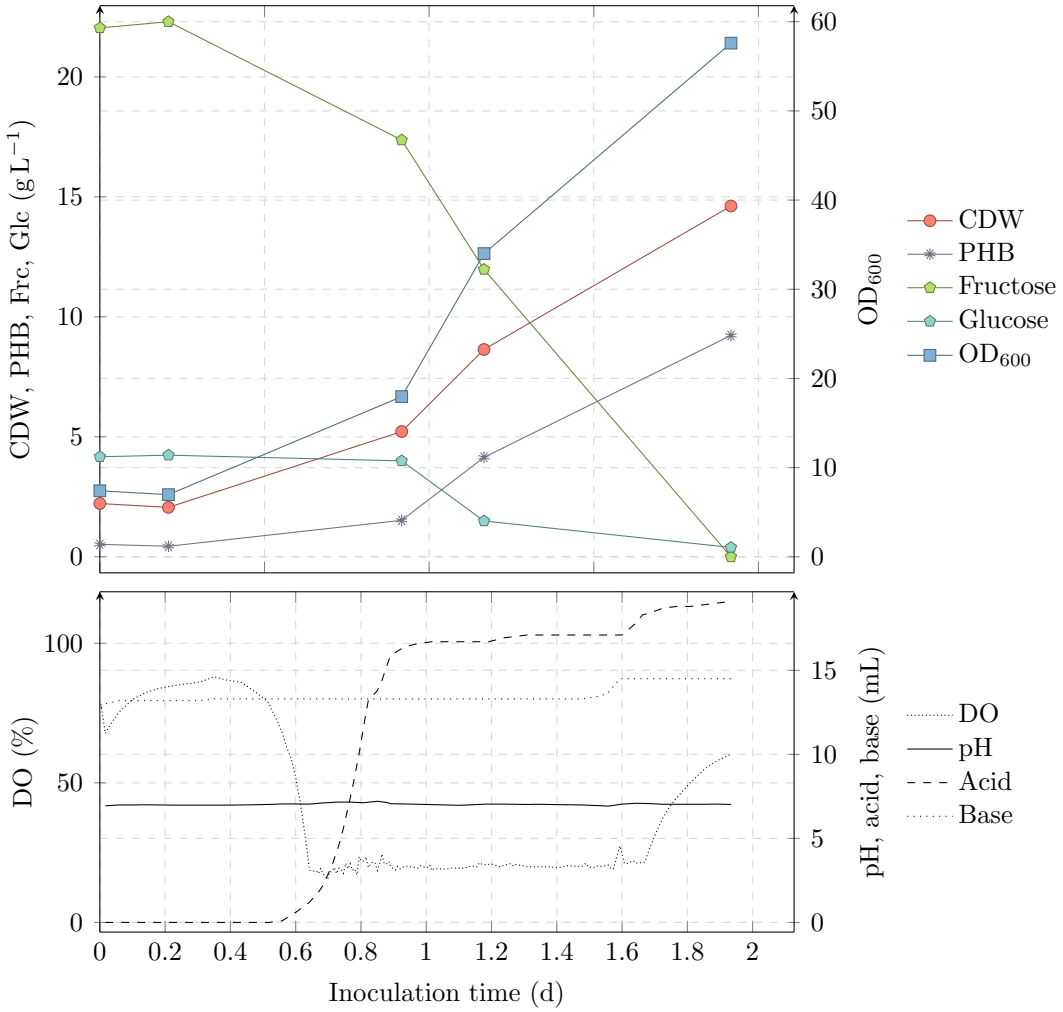

Figure 4.21: Fermentation course PHB-XIIIb-428-3

4.3.5.4 PHB-XIIIb-428-4 / Casein peptone

Reactor 428-4 (Figure 4.22) was supplemented with 5.3 g L^{-1} casein peptone. After slightly more than one day, 8.3 g L^{-1} PHB and 13.0 g L^{-1} CDW were produced, corresponding to a PHB content of 63 %.

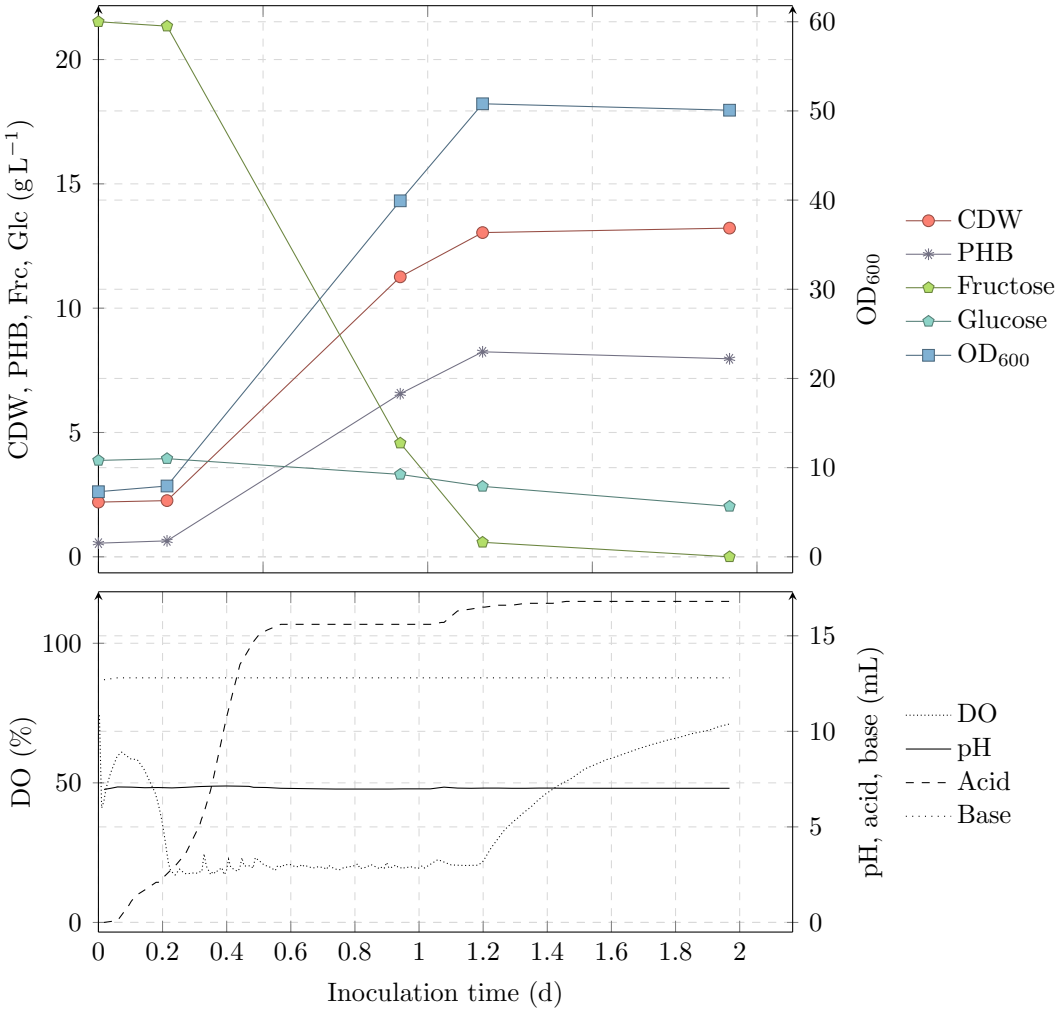

Figure 4.22: Fermentation course PHB-XIIIb-428-4

4.3.5.5 Discussion

All four experiments showed, by comparing them with older fermentations, an impressively shortened lag phase. Fructose was consumed within one to two days. It can further be seen that already in the inoculum flask small amounts of PHB were produced.

The two peptone supplemented fermentations showed slightly higher cell dry weights and considerably higher PHB contents, comparing them with the urea or ammonium sulfate supplemented ones. As already observed in PHB-XIII, the residual CDW increased and the PHB content decreased with nitrogen supplementation.

Due to the unexpectedly fast process, the sampling regime was not adequately timed for a proper display of the results.

4.3.6 Strain comparison

For fermentation cycles PHB-XIV and PHB-XVI, the well-working pre-culturing protocol of the last experiments was used. Beside strain DSM 428, also two other strains, DSM 531 and DSM 545 were tried out.

Minimal medium pre-culturing seemed to shorten lag phase dramatically and improve PHB accumulation capabilities. However, nitrogen supplemented fermentations resulted in quite low PHB contents in dry weight and high residual cell masses. As an attempt to channel carbon and energy flux towards PHB accumulation, the following experiments were done without nitrogen supplementation.

4.3.6.1 PHB-XIV-428-1 / DSM 428

428-1 reached the highest PHB concentration after three days ($4.4\,\mathrm{g\,L^{-1}}$), where the most part of fructose was already consumed. A CDW of $6.5\,\mathrm{g\,L^{-1}}$ led to a PHB content of 68 %. The lag phase was longer, compared to previous, nitrogen supplemented processes.

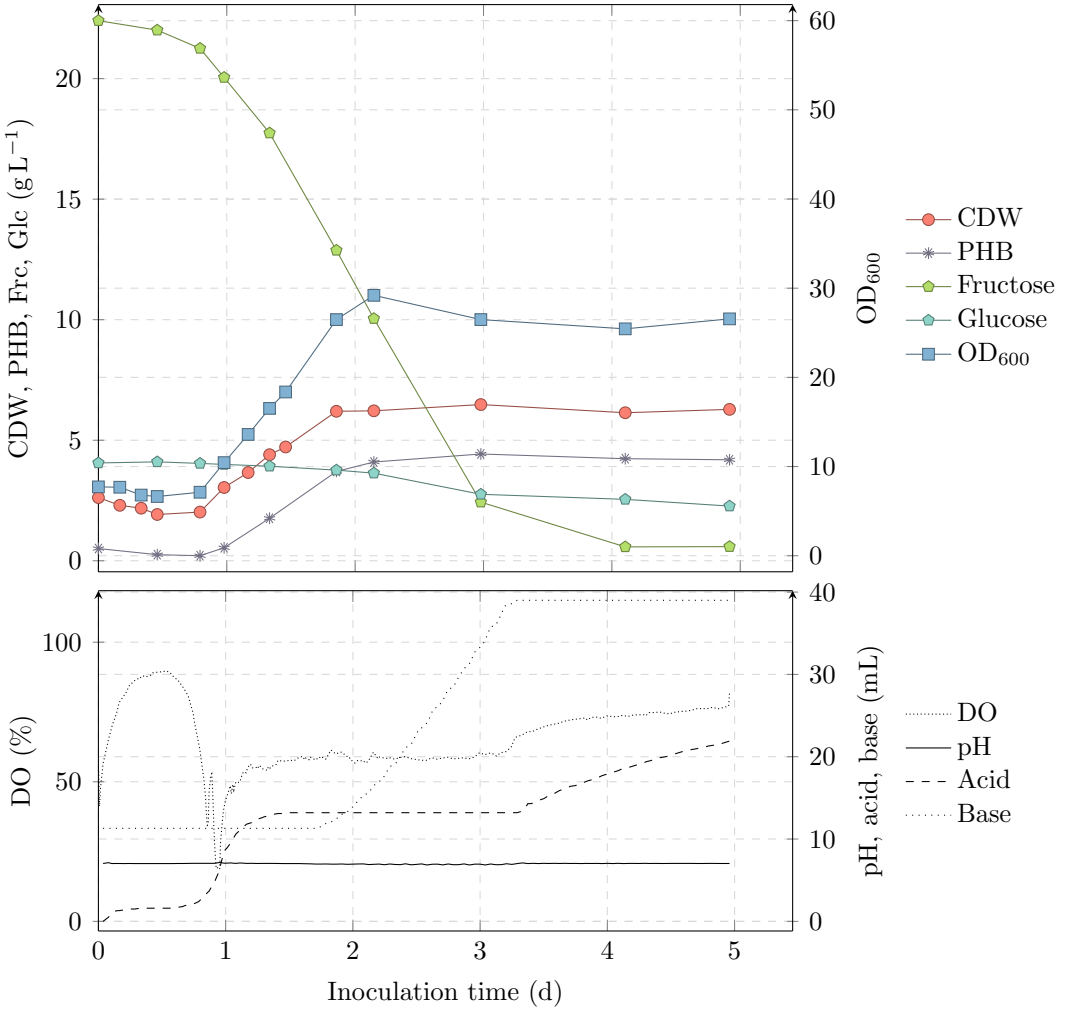

Figure 4.23: Fermentation course PHB-XIV-428-1

4.3.6.2 PHB-XIV-428-2 / DSM 428

Also for this experiment, 428-2, strain DSM 428 was used. After five days, fructose was consumed completely. A PHB concentration of 7.3 and a cell dry weight of 11.0 g L^{-1} led to a PHB content in dry weight of 66 %. Also here, the lag phase was longer than those of nitrogen supplemented fermentations.

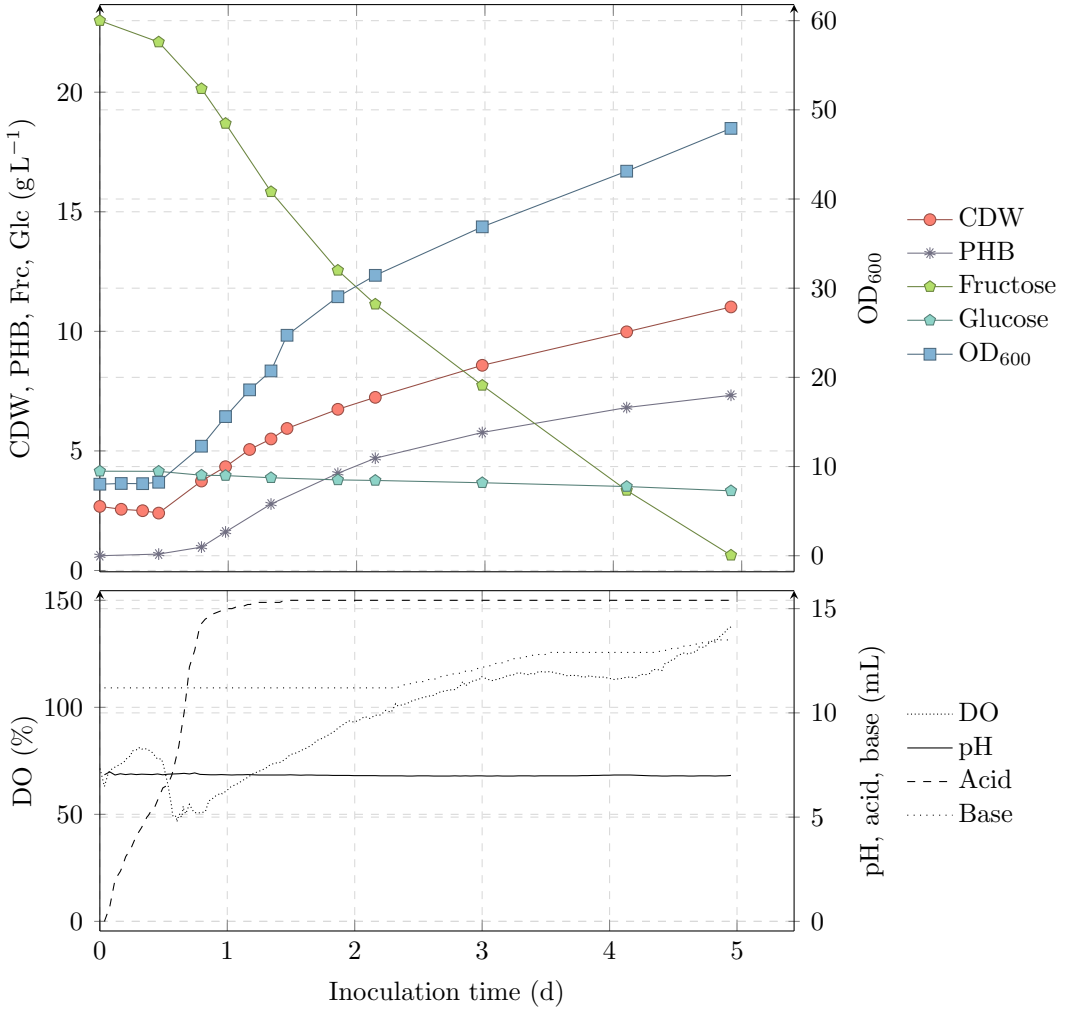

Figure 4.24: Fermentation course PHB-XIV-428-2

4.3.6.3 PHB-XVI-531-1 / DSM 531

After four days of fermentation, 1.4 g L^{-1} PHB and 3.0 g L^{-1} CDW were produced, corresponding to a PHB content in dry weight of 47 %.

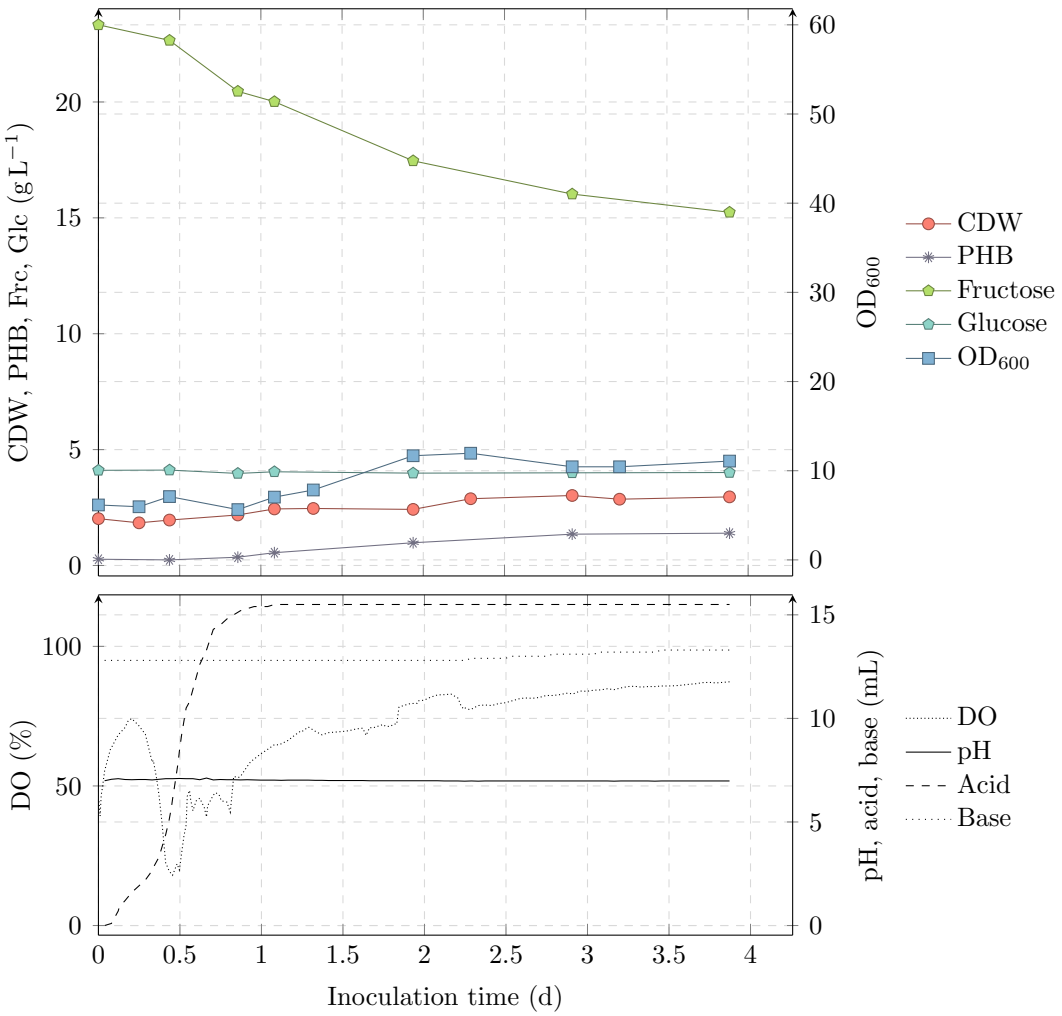

Figure 4.25: Fermentation course PHB-XVI-531-1

4.3.6.4 PHB-XVI-531-2 / DSM 531

Experiment 531-2 (Figure 4.26), in sense of process course, is very similar to 531-1. After four days, 1.5 g L^{-1} PHB and 3.1 g L^{-1} CDW were produced, corresponding to a PHB content in dry weight of 46 %.

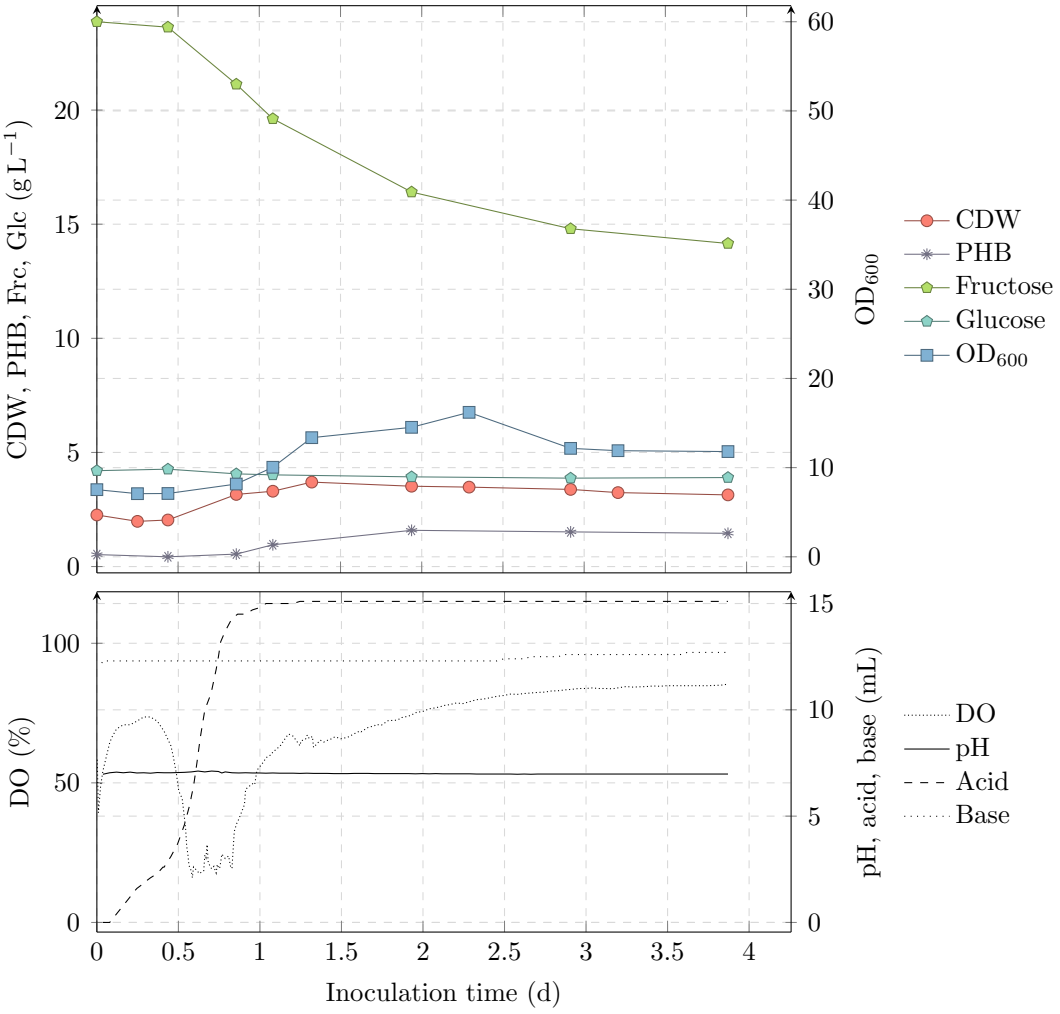

Figure 4.26: Fermentation course PHB-XVI-531-2

4.3.6.5 PHB-XIV-545-1 / DSM 545

Fermentation 545-1 was finished after three days. PHB accumulation starts with the beginning of the fermentation. No lag phase can be observed. 11.0 g L^{-1} PHB and 14.0 g L^{-1} CDW led to a PHB content in dry weight of 78 %.

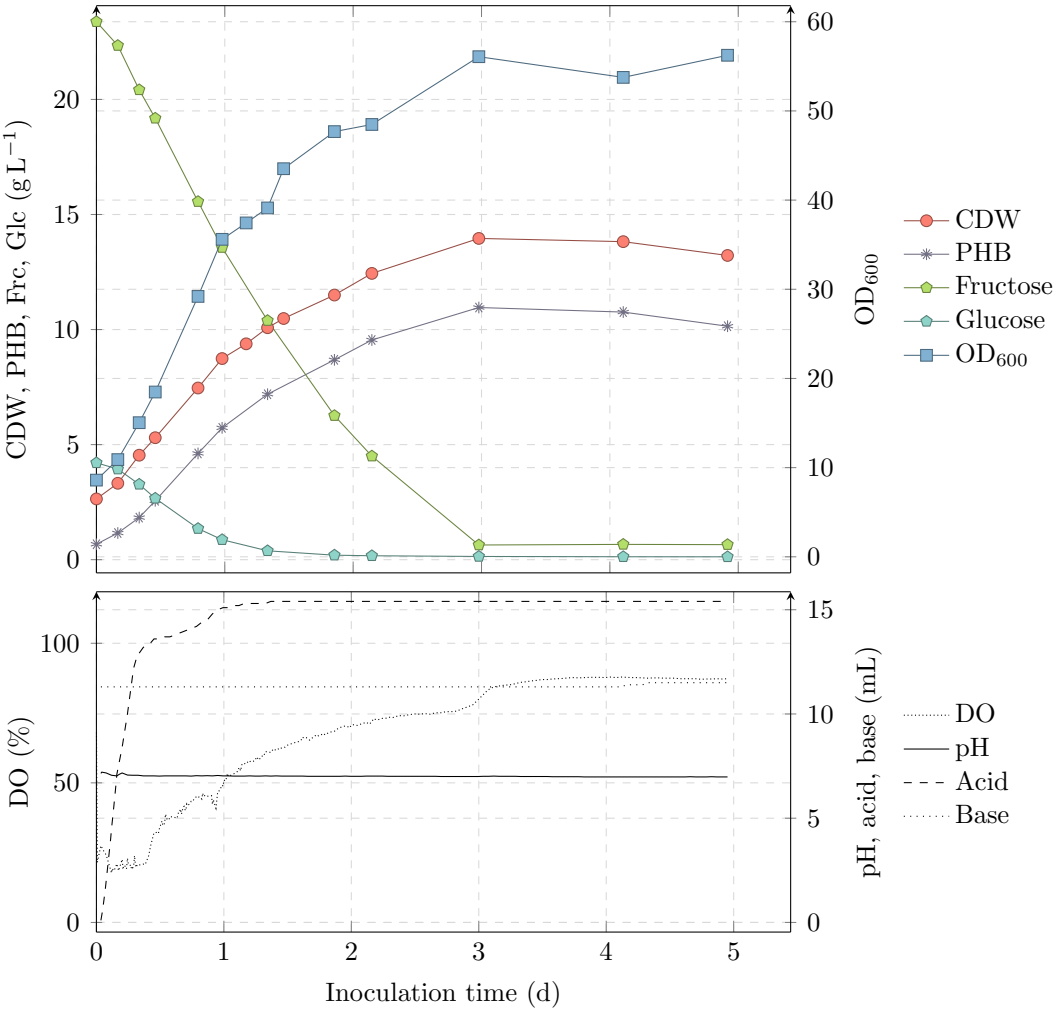

Figure 4.27: Fermentation course PHB-XIV-545-1

4.3.6.6 PHB-XIV-545-2 / DSM 545

Fermentation 545-2 was even faster than 545-1. After two days, 14.6 g L^{-1} CDW and 11.0 g L^{-1} PHB were produced, corresponding to a PHB content in dry weight of 78 %. There is no observable lag phase and also here, PHB accumulation starts already with the begin of the fermentation.

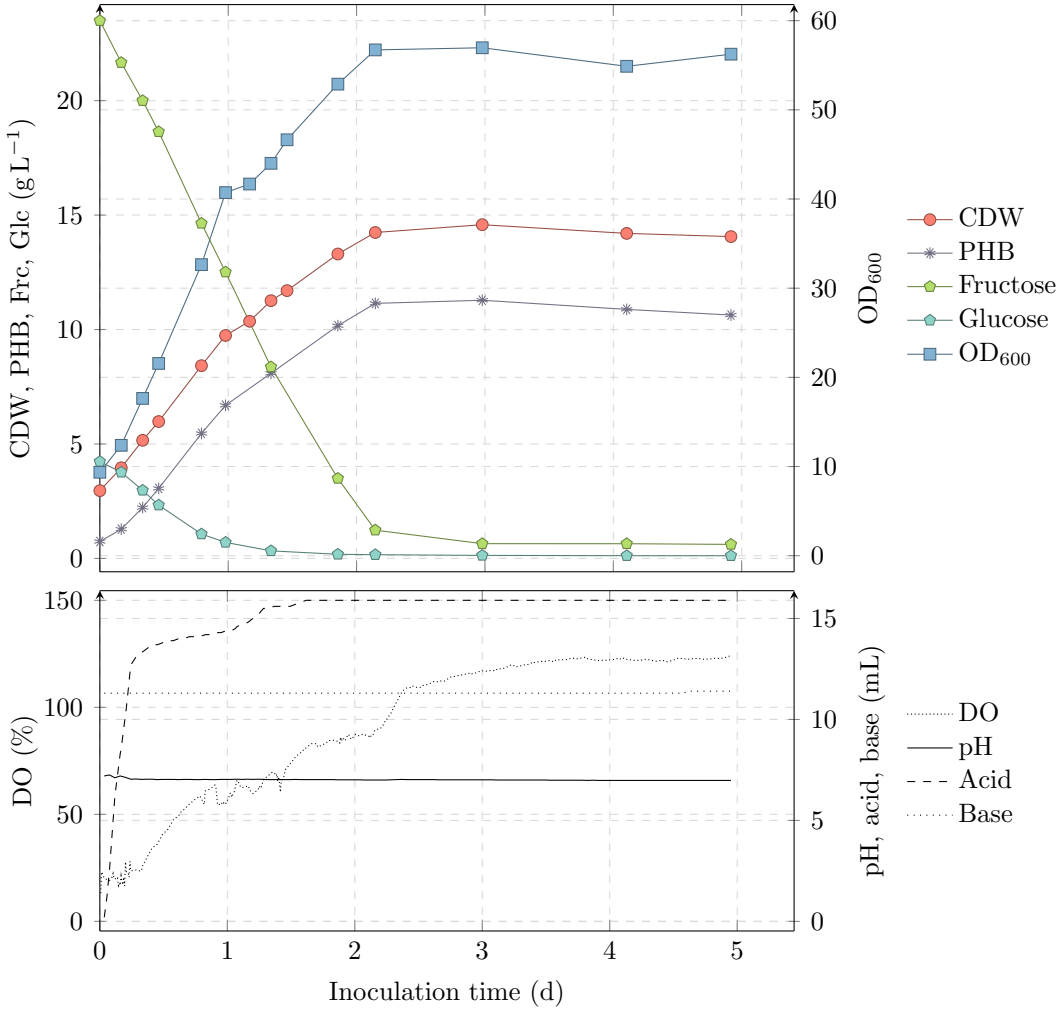

Figure 4.28: Fermentation course PHB-XIV-545-2

Compared to previous fermentations, the bacterial cells were more voluminous. DSM 428 looked more spherical (Figure 4.29), while DSM 545 had a clear rod shape (Figure 4.30).

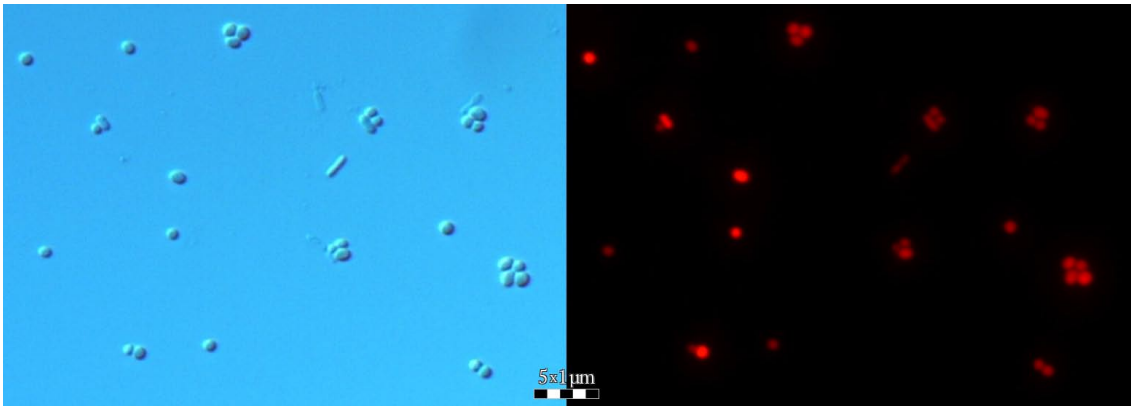

Figure 4.29: *C. necator* DSM 428 in phase contrast and fluorescence microscopy after two days of fermentation

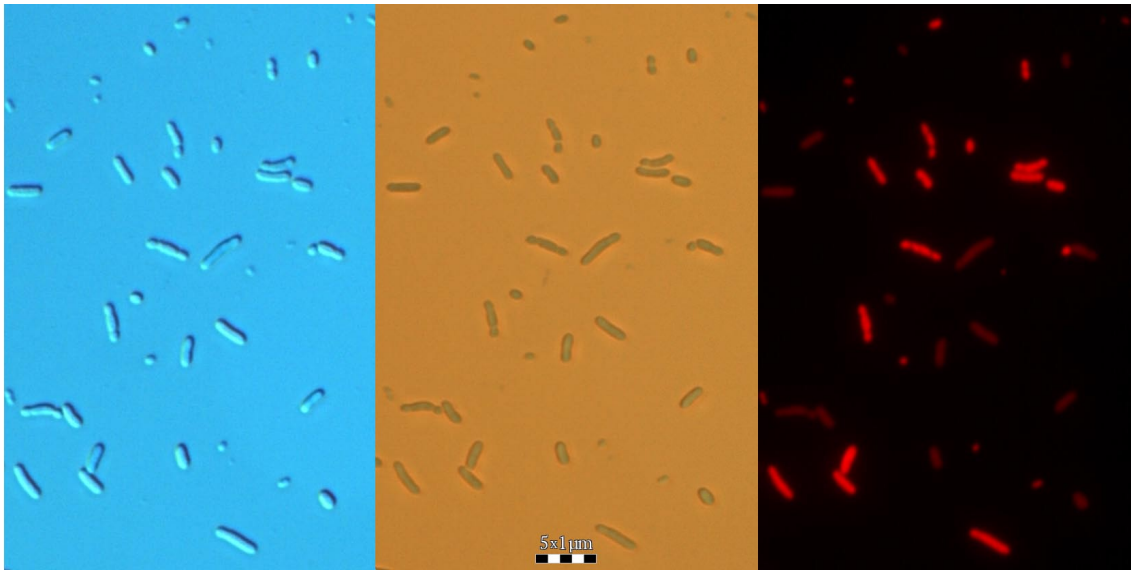

Figure 4.30: *C. necator* DSM 545 in phase contrast, bright field and fluorescence microscopy after two days of fermentation

4.3.6.7 Discussion

Fermentations 531-1 and 531-2 (Figures 4.25 and 4.26) showed incomplete substrate consumption and low PHB yields. The duration of the lag phase is comparable with those of strain DSM 428 (Section 4.3.6.1) but longer than 545 (Section 4.3.6.5). The amount of residual sugars at the end of fermentation (4 days) was quite high. In summary, strain DSM 531 showed far the lowest PHB productivity compared to 428 and 545 (all yields and productivities are shown in Table 4.2).

C. necator DSM 428 was used in experiments 428-1 and 428-2 (Figures 4.23 and 4.24). After four to five days, the available sugars were completely consumed. The lag phase was comparable with those of strain DSM 531 but longer than those of DSM 545.

Fermentations 545-1 and 545-2 (Figures 4.27 and 4.28) showed no visible lag phase as well as fast and high PHB accumulation. All available sugars were consumed within two to three days. PHB productivity was much higher than those of the other two strains. DSM 545 is far the most promising strain. As known from the literature, DSM 545 can utilize glucose as energy and carbon source and appears to produce PHB also with sufficient nutrient supply (Berezina 2013).

4.3.7 Detoxification

All detoxification experiments were carried out with *C. necator* DSM 545, as this was the most promising strain.

4.3.7.1 PHB-XVI-545-1 / Activated charcoal

Fermentation 545-1 (Figure 4.31), whose substrate was detoxified with activated charcoal, reached its PHB maximum after three days. A cell dry weight of 13.7 g L^{-1} and a PHB concentration of 10.3 g L^{-1} correspond to a PHB content of 75 %.

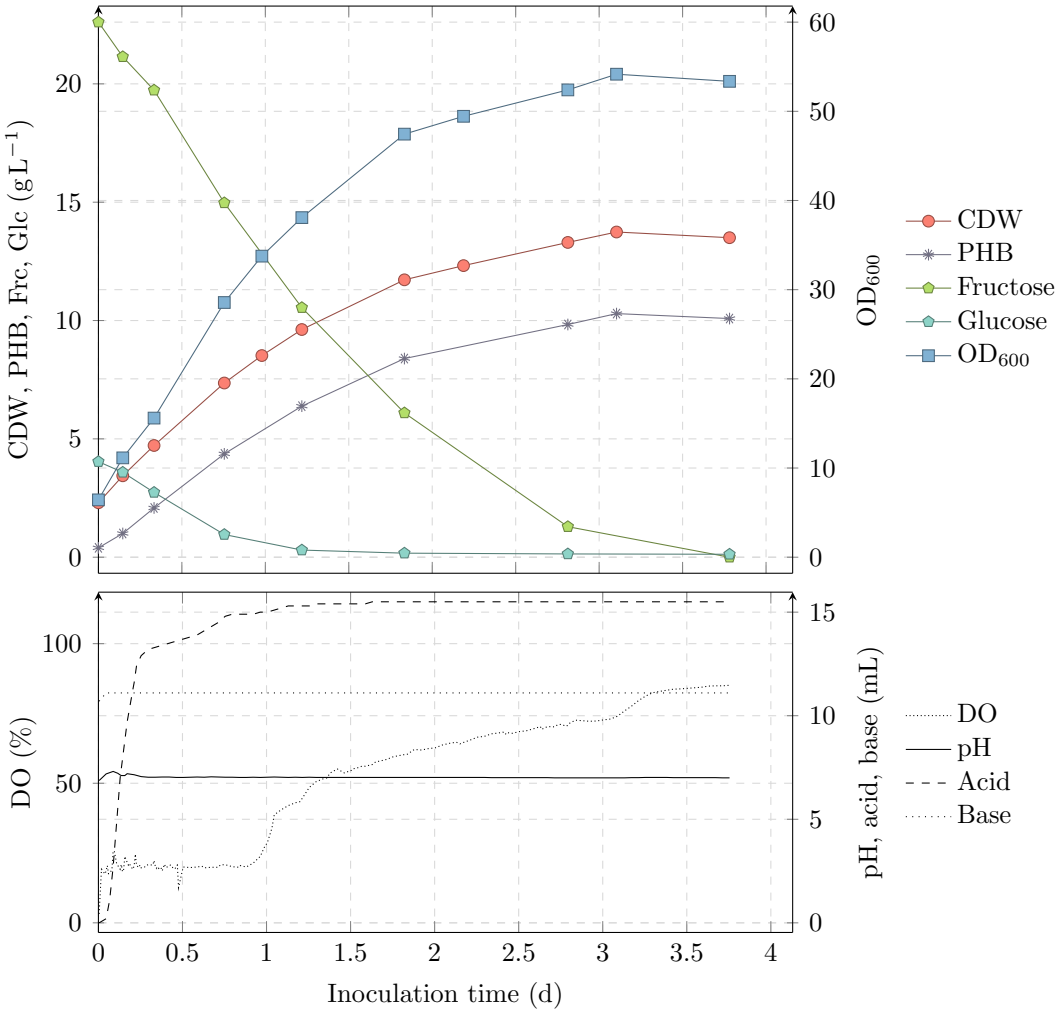

Figure 4.31: Fermentation course PHB-XVI-545-1

4.3.7.2 PHB-XVI-545-2 / Laccase

The substrate of fermentation 545 (Figure 4.32) was treated with laccase. A maximal PHB concentration of (10.2 g L^{-1}, 71 %) and a CDW of 14.2 g L^{-1} were reached after four days.

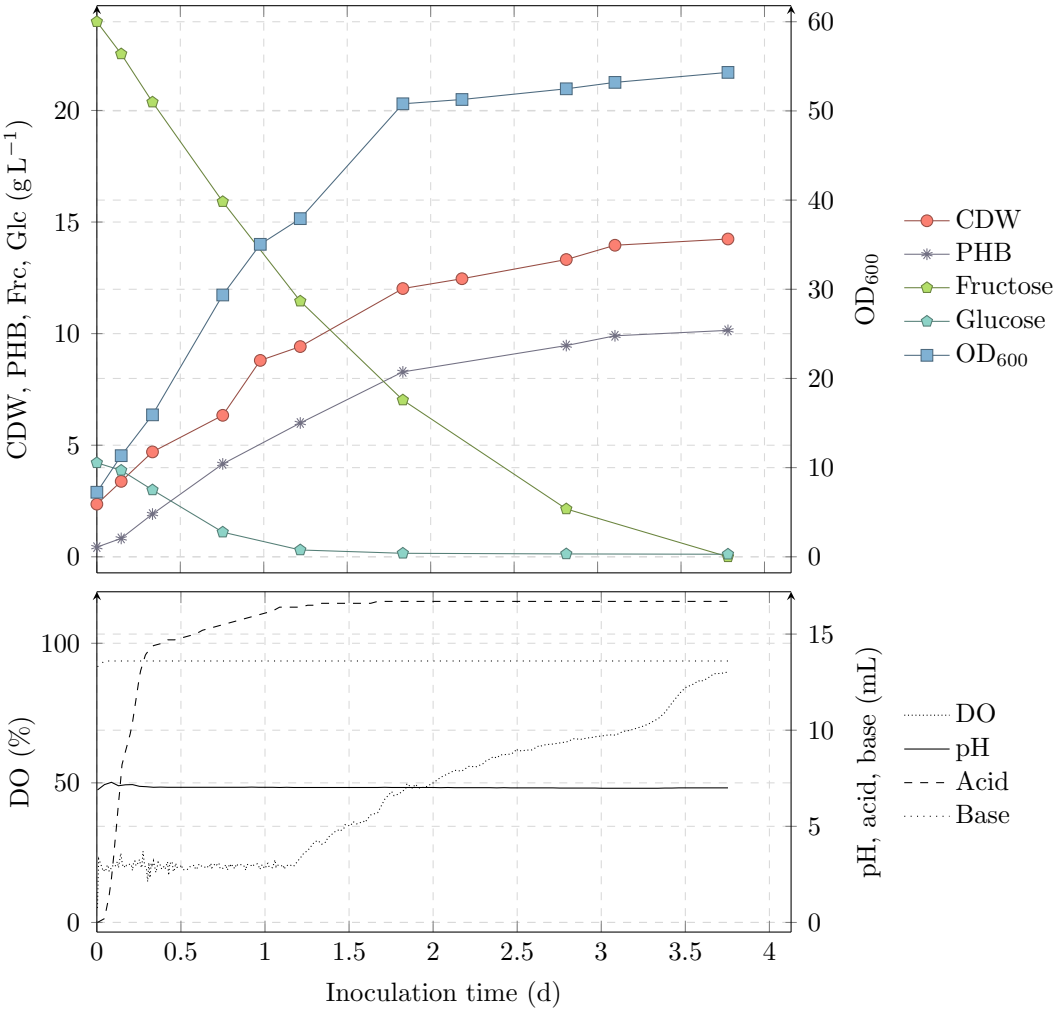

Figure 4.32: Fermentation course PHB-XVI-545-2

4.3.7.3 Discussion

Comparing fermentations without detoxifications and the two fermentations where charcoal or laccase were used, it can be said that no clear difference can be seen. Because the used substrate pretreatment processes ran at mild conditions, this results are not unexpected.

However, it must be said that these single experiments have a limited significance which makes it difficult to definitively discover any either positive or negative effect. Additional experiments will be required to obtain definite answers.

4.3.8 General results and discussion

4.3.8.1 Nitrogen consumption

Figure 4.33 shows the amount of total Kjeldahl nitrogen from non supplemented fermentations in the fermentation supernatant. Despite the fact that nitrogen consumption rates differed, it can be said that the TKN course of all fermentation experiments look very similar, as the amount of absorbed nitrogen does not really depend on the used *C. necator* strains for not supplemented fermentations. About

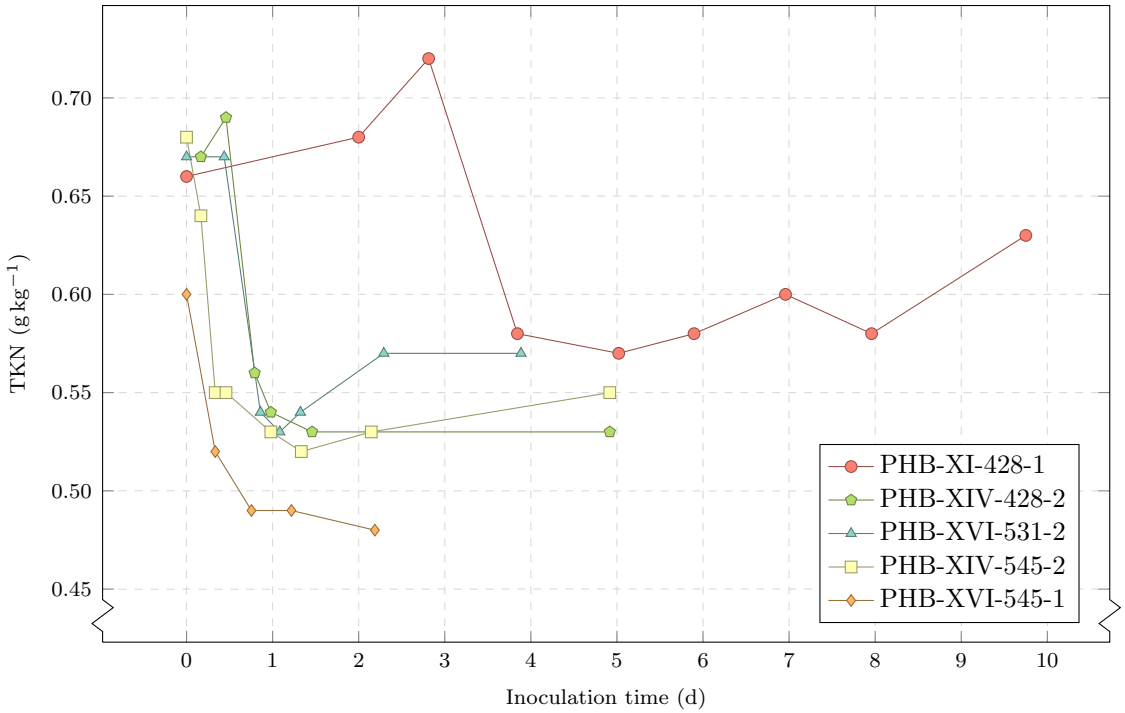

Figure 4.33: Nitrogen consumption - comparison between different strains and fermentations

20 to 25 % of the total Kjeldahl nitrogen were used by the organisms.

However, the speed of nitrogen consumption seems to be strain and pre-culturing dependent. Strain DSM 545 (PHB-XIV-545-2, PHB-XVI-545-1) consumed the available nitrogen very fast (as it grew also fast), DSM 428 (PHB-XIV-428-2) and 531 (PHB-XVI-531-2) are slower when using the same pre-culturing protocol. When using DSM 428 with standard protocol pre-culturing (non minimal medium pre-culture, PHB-XI-428-1), growth as well as nitrogen consumption rate is very low.

Table 4.1 shows the measured nitrogen values from the supernatant at start and end of fermentation cycle PHB-XIIIb. These experiments were supplemented with different nitrogen sources. A correlation between nitrogen uptake and amount of produced residual cell mass can be observed. The two peptone supplemented fermentations show lower amounts of consumed nitrogen compared to those of fermentations where urea or $(NH_4)_2SO_4$ was added. The nitrogen consumption of the fermentation supplemented with $(NH_4)_2SO_4$ was not determined through the difference in TKN, but through the difference in ammonium only. Therefore, the actual nitrogen consumption, including other nitrogen sources from the fermentation medium, is expected to be slightly higher.

4.3.8.2 Yields and productivities

Table 4.2 shows an overview of produced PHB amounts and productivities from all discussed fermentation experiments. The highest PHB contents and concentrations show fermentations carried out with *C. necator* DSM 545 (especially PHB-XIV-545-1). However, the highest volumetric productivities came from DSM 428 with peptone supplementation (e g.: PHB-XIIIb-428-4). Although the reached PHB concentrations and contents of latter fermentations were somewhat lower, the needed time was much shorter. Most of these fermentations were finished after one day. The highest

Table 4.1: Ammonium/TKN content in the supernatant of nitrogen supplemented fermentations (PHB-XIIIb) at start and end of the experiment

Additive	Method	N_{start} ($g\,kg^{-1}$)	N_{end} ($g\,kg^{-1}$)	N_{diff} ($g\,kg^{-1}$)	Residual cell weight ($g\,L^{-1}$)
Urea	TKN	1.35	0.72	0.63	6.2
$(NH_4)_2SO_4$	NH_4^+	0.74	0.10	0.64	5.9
Soy peptone	TKN	1.27	0.80	0.47	5.4
Casein peptone	TKN	1.26	0.86	0.40	4.7

Table 4.2: Summary: Product (PHB) productivities and yields

Fermentation ID	PHB_{start} ($g\,L^{-1}$)	PHB_{end} ($g\,L^{-1}$)	CDW_{end} ($g\,L^{-1}$)	PHB (%)	Time (d)	Productivity ($g\,L^{-1}\,h^{-1}$)	$Yield_{P/S}$ ($g\,g^{-1}$)
Starting protocol							
PHB-VII-428-1	0.0	1.7	3.8	45%	7.7	0.009	0.38
PHB-VII-531-1	0.0	1.6	3.1	52%	3.9	0.017	0.23
PHB-VII-531-2	0.0	3.0	4.9	61%	6.7	0.019	0.16
NH_3 vs. NaOH							
PHB-XI-428-1	0.0	1.6	4.1	39%	9.8	0.007	0.30
PHB-XI-428-2	0.0	1.7	4.5	38%	9.8	0.007	0.30
PHB-XI-428-3	0.0	4.1	10.6	39%	9.8	0.017	0.16
$(NH_4)_2SO_4$ supplementation + minimal medium pre-culture I							
PHB-XII-428-1	0.0	2.4	5.6	43%	8.9	0.011	0.21
PHB-XII-428-2	0.0	2.2	4.9	45%	8.9	0.010	0.31
PHB-XII-428-3	0.0	1.7	4.7	36%	8.9	0.008	0.15
PHB-XII-428-4	0.0	4.5	10.5	43%	8.9	0.021	0.17
N sources + minimal medium pre-culture I							
PHB-XIII-428-1	0.0	2.0	7.6	26%	8.9	0.009	0.11
PHB-XIII-428-2	0.0	3.0	8.8	34%	8.9	0.014	0.14
PHB-XIII-428-3	0.0	3.8	8.1	47%	8.9	0.018	0.27
PHB-XIII-428-4	0.0	2.5	7.1	35%	8.9	0.012	0.18
N sources + minimal medium pre-culture II							
PHB-XIIIb-428-1	0.5	5.9	12.1	49%	0.9	0.250	0.25
PHB-XIIIb-428-2	0.6	5.1	11.0	46%	1.2	0.157	0.21
PHB-XIIIb-428-3	0.5	9.2	14.6	63%	1.9	0.190	0.35
PHB-XIIIb-428-4	0.6	8.3	13.0	64%	1.2	0.269	0.36
Strain comparison							
PHB-XIV-428-1	0.5	4.4	6.5	68%	2.5	0.065	0.19
PHB-XIV-428-2	0.6	7.3	11.0	66%	4.9	0.057	0.31
PHB-XVI-531-1	0.3	1.4	3.0	47%	3.8	0.012	0.17
PHB-XVI-531-2	0.5	1.5	3.1	48%	3.8	0.011	0.15
PHB-XIV-545-1	0.7	11.0	14.0	79%	2.2	0.196	0.41
PHB-XIV-545-2	0.7	11.0	14.6	75%	3.0	0.143	0.41
Detoxification							
PHB-XVI-545-1	0.4	10.3	13.7	75%	3.5	0.118	0.39
PHB-XVI-545-2	0.4	10.2	14.2	72%	3.5	0.117	0.36

yields in turn were reached with non supplemented fermentations and DSM 545. During fermentations PHB-XIV-545-1 and PHB-XIV-545-2 0.41 gram PHB per gram consumed sugar were produced.

4.3.8.3 Discussion

Nitrogen, pre-culturing and productivities: Because the amount of used nitrogen was relatively constant for all three strains at different non supplemented fermentations (Figure 4.33), the consumed nitrogen seems to be the bioavailable nitrogen content of the chicory root hydrolysate for the used microorganisms. Bacterial growth was promoted when additional nitrogen sources were added. However, the increased cell proliferation consumed a lot of sugar which then could no longer be used as substrate for PHB accumulation. The assumption that higher nitrogen uptake promotes cell proliferation can be confirmed by the values of Table 4.1. These also show that bacteria consume less nitrogen when peptone additives were used compared to urea and $(NH_4)_2SO_4$ supplemented fermentations. This could be due to a lower bioavailability of the nitrogen from peptone or a slower uptake rate of peptone derived nitrogen.

It has been shown that the pre-culturing conditions have a strong effect on the lag phase duration as well as PHB accumulation capabilities. By prolonging the pre-culturing from one to two days and switching from LB to minimal medium during pre-culturing, the productivity could be increased more than an order of magnitude when using strain DSM 428.

Strain comparison experiments with the final protocol (minimal medium, no supplementation) showed that after changing the pre-culture conditions, DSM 428 was able to completely consume all available sugar within an acceptable time frame and without supplementation. However, also in this case the supplementation of nitrogen strongly increased the productivity.

DSM 531 clearly was the strain which achieved the lowest PHB amounts and productivities.

As DSM 545 was able to completely consume the available sugars from the chicory root hydrolysate within a short time frame, it can be assumed that additional nitrogen supplementation would reduce the final PHB concentrations. However, the supplementation of a low amount of nitrogen could accelerate the PHB accumulation by promoting cell proliferation at the fermentation start and therefore increase the volumetric productivity.

Contaminations: The first fermentations carried out with the standard protocol were highly susceptible to contaminations. This is not surprising since these experiments took extremely long and bacteria were not able to use the available sugar within a reasonable period of time. During this work, several fermentations failed because of this reason and due to the contaminations of the automatic propipette and/or the shaking incubator (fermentation plots of these failed experiments are not shown in the sections above). However, after cleaning the needed laboratory tools as well as optimizing the protocol and enabling faster growth and nutrient consumption, all contamination problems were gone.

Further remarks: As PHB acts as energy and carbon storage, bacteria use it when other sources of carbon and energy have been used up. The use of PHB reserves can be seen when looking at the decreasing PHB concentrations after the depletion of free sugars. This can be observed in fermentation PHB-XII-428-4, PHB-XIIIb-428-1, PHB-XIV-545-1, or PHB-XIV-545-2. However, PHB consumption was relatively slow over a longer period of time. The decrease in PHB concentration usually was accompanied by a decrease in optical density.

5. Summary and conclusion

It has been shown that the chicory root hydrolysate has viable properties for the production of PHAs through bacterial fermentation. Problematic is the low sugar content, limiting maximal PHA and CDW concentrations.

The three investigated strains showed different PHA accumulation properties. The most promising strain, *Cupriavidus necator* DSM 545, was able to consume the full sugar content within two days, producing $11.1\,\mathrm{g\,L^{-1}}$ P(3HB) (78 %) and a cell dry weight of $14.2\,\mathrm{g\,L^{-1}}$. This result was reached without any nutrient supplementation or detoxification process. While DSM 428 and 531 started PHB accumulation only after depletion of nitrogen, DSM 545 accumulated PHB also while some nitrogen was still present.

The highest volumetric productivities were reached by using nitrogen supplementation, as higher cell numbers are able to accelerate the overall process of substrate consumption and PHA accumulation. Nitrogen clearly enhances bacterial growth and proliferation. However, too high nitrogen addition leads to a redirection of the carbon and energy fluxes towards residual cell mass, decreasing PHA concentration and impairing productivity.

The pre-culture conditions are crucial, as minimal medium inoculum enhanced the subsequent fermentation step tremendously. The inoculum volume was halved, the inoculum incubation time was doubled, the lag phase was shortened by 80 to 90 %, and the sugar content was consumed within a few days, depending on the used strain.

The cell morphology of *C. necator* strongly depends upon environmental conditions, physiological state, and pre-culturing. The cell shape can vary widely, and strains which grew at the same conditions can look rather different.

The next steps for the establishment of the process should be a scale-up to pilot scale. Furthermore, precursors for the production of PHA copolymers should be examined, in order to improve the mechanical properties of the polymers.

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