# UTILIZATION OF WASTE STREAMS FROM THE BIODIESEL INDUSTRY FOR THE PRODUCTION OF SHORT CHAIN LENGTH POLYHYDROXYALKANOATES

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# KURZFASSUNG

Polyhydroxyalkanoate (PHAs) sind Polymere, die biologisch abbaubar und biokompatibel sind und von vielen Prokaryoten als Teil ihres natürlichen Metabolismus produziert werden. Abhängig vom verwendeten Bakterienstamm und der Fütterungstaktik, können viele verschiedene Arten von PHAs hergestellt werden, die eine große Bandbreite an Eigenschaften abdecken. Aufgrunddessen stellen diese Biopolymere eine vielversprechende Alternative zu den herkömmlichen Polymeren auf Erdölbasis dar. Allerdings besitzen sie einen schwerwiegenden Nachteil, nämlich ihre vergleichsweise hohen Produktionskosten.
Eine Möglichkeit dieses Manko zu überwinden, ist die Verwendung von neuen, billigeren Substraten, an Stelle von reinem Zucker. Genau das ist auch das Ziel des FP7 Projekts ANIMPOL, das den Einsatz von Abfallströmen der Biodieselindustrie und der Fleisch verarbeitenden Industrie als Substrate für die Produktion von PHA vorschlägt. Auf diese Weise würden gleichzeitig auch einige Abfallprobleme dieser Branchen gelöst werden.

Als Teil dieses Projekts wurde der Bakterienstamm *Cupriavidus necator DSM 545* hinsichtlich seiner Verwertung von zwei verschiedenen Abfallströmen der Biodieselindustrie untersucht. Der Einfluss der rohen Glycerolphase (CGP) und der Biodieselfraktion, die aus gesättigten Fettsäuren besteht, auf das Wachstum und die Speicherphase der Bakterien wurde überprüft. Darüberhinaus wurde vor allem auf die Anwendung des neuartigen 3HV Precursors Margarinsäure Wert gelegt, die sowohl in Biodiesel, als auch in tierischen Fetten enthalten ist und somit ebenfalls eine günstige Alternative im Vergleich zu herkömmlichen Precursoren wie Propionsäure darstellt.

Zuerst wurden mehrere Schüttelkolbenversuche durchgeführt, um den optimalen Emulgator für den verwendeten Biodiesel zu finden und um einen ersten Eindruck hinsichtlich der Anwendbarkeit von CGP, Biodiesel und Margarinsäure als Substrate zu gewinnen.
Die Ergebnisse dieser ersten Untersuchungen wurden genutzt, um sie auf drei verschiedene Fermentationen in Bioreaktoren anzuwenden.
Bei der ersten Fermentation wurden 10 g/L Glucose und der 4HB Precursors γ-Butyrolacton verwendet, um das Copolymer P(3HB-co-4HB) herzustellen. Die Ergebnisse, die bei der Anwendung dieser herkömmlichen Substrate erzielt wurden, wurden als Referenzwerte herangezogen, um die anderen beiden Experimente zu evaluieren.
Bei der zweiten Fermentation wurden 10 g/L reines Glycerol als Kohlenstoffquelle eingesetzt und PHB mit geringerem Molekulargewicht erzeugt.
Für die letzte Fermentation wurden 5 g/L Biodiesel und 2 g/L Margarinsäure als Substrat verwendet, um P(3HB-co-3HV) herzustellen.

# ABSTRACT

Polyhydroxyalkanoates (PHAs) are completely biodegradable and biocompatible polymers, produced by many prokaryotes as part of their natural metabolism. Depending on the utilized strain and the feeding strategies many different types of PHAs, showing a wide range of properties, are known to be synthesized. Thus they constitute a very promising alternative to the conventional petrol-based polymers, possessing one major drawback: Their comparatively high production costs.
One way to overcome this obstacle is the utilization of novel cheaper substrates than the commonly used purified sugars. This constitutes also the objective of the FP7 project ANIMPOL which suggests to use waste streams from biodiesel plants and from the meat converting industry as substrates for PHA production, thus solving disposal problems of these branches at the same time.

As part of this project the utilization of two different biodiesel waste streams by the strain *Cupriavidus necator DSM 545* was investigated. The impact of the crude glycerol phase (CGP) as well as that of the biodiesel fraction which consists of saturated fatty acid methyl esters on microbial growth and accumulation phase was evaluated. Additionally special attention was paid to the application of the novel 3HV precursor margaric acid which is contained in biodiesel as well as in animal lipids, thus presenting a cheap alternative to common precursors like propionic acid.

First several shaking flask experiments were conducted in order to find the optimal emulsifier for the utilized biodiesel and to gain a first insight concerning the applicability of CGP, biodiesel and margaric acid as substrates.
The results of these preliminary experiments were used to conduct three different bioreactor fermentations.
The first fermentation was utilizing 10 g/L glucose in combination with the 4HB precursor γ-butyrolactone to produce the copolymer P(3HB-co-4HB). The results obtained from these well-established substrates were used as references to evaluate the other two experiments.
The second fermentation was conducted applying 10 g/L of pure glycerol as carbon source, thus producing PHB with low molecular masses.
For the last fermentation 5 g/L of biodiesel were used as substrate together with 2 g/L of margaric acid as precursor for P(3HB-co-3HV) production.

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# ABBREVIATIONS

$\mu_{max}$ ..... maximum specific growth rate [1/h]
3HB ..... 3-hydroxybutyrate
3HD ..... 3-hydroxydecanoate
3HDD ..... 3-hydroxydodecanoate
3HHx ..... 3-hydroxyhexanoate
3HO ..... 3-hydroxyoctanoate
3HV ..... 3-hydroxyvalerate
4HB ..... 4-hydroxybutyrate
AFM ..... atomic force microscopy
*C. necator* ..... *Cupriavidus necator*
CDM ..... cell dry mass [g/L]
CGP ..... crude glycerol phase
CoA ..... coenzyme A
FAME ..... fatty acid methyl ester
FFA ..... free fatty acid
GC ..... gas chromatography
HPLC ..... high pressure liquid chromatography
HX ..... caproic acid
KFU ..... Karl Franzens University, University of Graz
lcl ..... long chain length
MA ..... margaric acid
MBM ..... meat and bone meal
mcl ..... medium chain length
OD ..... optical density
P(3HB-co-3HV) ..... poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
P(3HB-co-4HB) ..... poly(3-hydroxybutyrate-co-4-hydroxybutyrate)
PHA ..... polyhydroxyalkanoate
PHB ..... poly(3-hydroxybutyrate)
$pO_2$ ..... partial pressure of oxygen, dissolved oxygen [%]
q ..... specific production rate [1/h]
r ..... production rate [g/Lh]
scl ..... short chain length
spec. $V_{prod}$ ..... specific production rate of PHA during the fermentation [1/h]
spec. $V_{prod,acc}$ ..... specific production rate of PHA during accumulation phase [1/h]
TCA ..... tricarboxylic acid cycle
$T_g$ ..... glass transition temperature [°C]
$T_m$ ..... melting temperature [°C]
$V_{prod}$ ..... volumetric production rate of PHA during the fermentation [g/Lh]
$V_{prod,acc}$ ..... volumetric production rate of PHA during accumulation phase [g/Lh]
$X_r$ ..... residual biomass [g/L]
Y ..... yield factor [g/g]

# 1 INTRODUCTION

The research done for this master thesis is part of the project ANIMPOL which is funded by the European Union under the Seventh Framework Programme (FP7). Its full title is "Biotechnological conversion of carbon containing wastes for eco-efficient production of high added value products". Its aim is to develop an industrial process which combines the cost-efficient production of bio-based polyhydroxyalkanoate (PHA) biopolymers and biodiesel with the solution of existing waste problems of slaughterhouses, the rendering industry and the biodiesel industry.
This master thesis has the objective of developing and optimizing a fermentation process which allows the production of the copolymer P(3HB-co-3HV) starting from different waste streams from the biodiesel industry. Special attention will be paid to the application and the effects of the novel 3HV precursor margaric acid which is the most common odd-numbered fatty acid contained in animal lipids.

## 1.1 PROJECT AIMS OF ANIMPOL

During the slaughtering of animals huge amounts of waste streams accrue. For example in Austria only about a third of a cattle is sold on the food market ***(1)***. Although a certain quantity of the produced animal wastes can be processed to meat and bone meal (MBM) and tallow by rendering sites, still a considerable amount of surplus material (e.g. hides, inwards) remains, which is of no economical value and has to be disposed of ***(2)***. In addition the rendering industry has to fight with accruing waste streams for which no considerable market value exists.
One of the basic ideas of ANIMPOL is to convert these materials, which are inconvenient for the respective branches, to comparatively cheap substrates for the bio mediated production of PHAs. This way this sector gains an enormous benefit, since the major drawback of PHAs compared to conventional polymers are their considerable production costs which are partly due to rather expensive feedstocks like sugar cane or molasses ***(3)***. Once they can compete with fossil-based polymers in this context, they constitute an attractive alternative because of their biodegradability and biocompatibility ***(4)***.

The biodiesel industry fits perfectly well into this scenario. On the one hand, same as PHA production, it is in need of new substrates which, in contrast to the commonly used vegetable oils, are relatively cheap and do not threat the food chain ***(5)***. On the other hand it has to solve the problem of its by-products, for example the crude glycerol phase (CGP) which accrues in considerable amounts during biodiesel production. Another waste stream consists of saturated fatty acid methyl esters (FAMEs) which in fact are low quality biodiesel, since they have a negative impact on the fuel's cold weather behavior and therefore should be removed ***(6)***.
For these reasons another objective of the project ANIMPOL is to assess the use of CGP and different lipid waste streams from slaughterhouses and rendering industry (e.g. tallow) as feedstocks for biodiesel production and the use of the unwanted unsaturated FAMEs fraction as substrate for PHA-producing bacteria.

The main cost drivers of PHA production are the raw materials for providing adequate carbon and nitrogen sources. Also the precursors for special building blocks which lead to polymers with improved properties are an important cost factor. These are for example odd numbered fatty acids (e.g. margaric acid) whose addition during PHA accumulation phase causes the generation of 3HV-building blocks which lead to the formation of the copolymer P(3HB-co-3HV). This material is advantageous in terms of melting point, crystallinity, ductility and toughness when compared with PHB homopolyester ***(7,8)***.
As carbon sources for the final industrial process, which will be designed as part of ANIMPOL, CGP and the saturated FAMEs generated during the biodiesel production are taken into account. Also the hydrolysis of

the lipid part of animal wastes is a good possibility to obtain fatty acids which are applicable as carbon source during PHA production.
Some of them have an odd-numbered carbon chain, thus constituting a perfect 3HV precursor. In addition also biodiesel itself contains a certain amount of different odd-numbered fatty acids, above all margaric acid, which leads to a decreased need of additional precursors during P(3HB-co-3HV) production.
Even MBM could be directly used as carbon and nitrogen source, if it lost its market value as during the crisis over bovine spongiform encephalopathy (BSE, mad-cow disease) ***(9)***.
Animal wastes consist, not only of lipid material, but also of proteinaceous material which can be hydrolyzed to generate the needed nitrogen source for PHA-producing bacteria.
Apart from the cost-intensive raw materials the PHA production process can also be improved by using renewable energies for fermentation or for the isolation of PHAs. This could be achieved by using the biomass which was separated from the biopolymer as a feedstock for a biogas plant.
The proposed connection of meat processing industry, biodiesel industry and PHA production is shown in Figure 1-1.

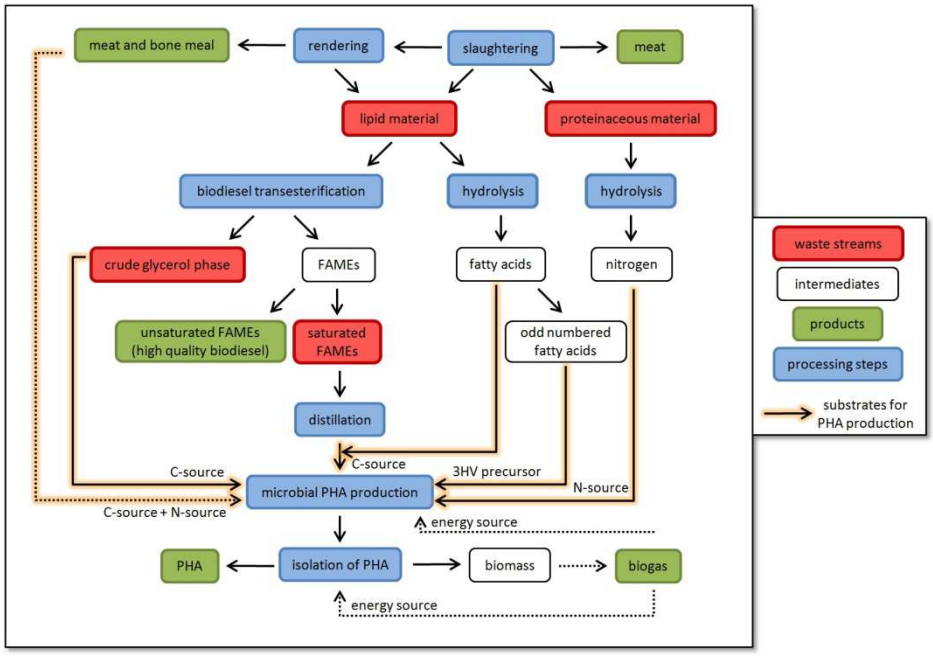


***Figure 1-1*** *Scheme of the project ANIMPOL: Use of different waste streams for the production of PHAs and biodiesel.*

# 1.2 CONSORTIUM OF ANIMPOL

In order to achieve the discussed goals several different companies and research facilities from all over Europe are working together on this project.

- **Graz University of Technology; Institute of Biotechnology and Biochemical Engineering (Austria)**
  The group of Professor Braunegg is responsible for the coordination of the project. Additional tasks are the biotechnological conversion of the processed waste streams *via* different PHA producing strains together with the production of raw biopolymers and biomass and with the generation of relevant fermentation data, also including the experiments conducted for this thesis.

- **Graz University of Technology; Institute for Process and Particle Engineering (Austria)**
  Professor Narodoslawsky's and Professor Schnitzer's working groups are responsible for creating a process design for large scale PHA production and to assess its ecological and economical impacts by means of life cycle analysis (LCA). As part of the additionally conducted cleaner production studies also the application of renewable energies, such as solar thermal energy or biogas are investigated.

- **University of Padua; Department of Agricultural Biotechnology (Italy)**
  The Microbiology Group is responsible for the evaluation and the genetic improvement of the used PHA-producing strains. In addition it is conducting ecocompatibility tests for produced polymer formulations.

- **University of Zagreb; Faculty of Food Technology and Biotechnology (Croatia)**
  They are responsible for the mathematical modeling of a possible large scale process, using the obtained experimental data.

- **University of Graz; Institute of Chemistry (Austria)**
  The working group Chemistry and Technology of Renewable Resources is responsible for the conversion of lipid wastes to FAMEs, the distillation of the produced biodiesel to remove unwanted impurities and the hydrolysis of animal waste streams to fatty acids, respectively nitrogen compounds. Moreover it controls the quality of the obtained biodiesel.

- **Argent Energy Limited (United Kingdom)**
  Argent Energy is a waste-to-energy biodiesel producer and operates a multi feedstock biodiesel plant, which processes mainly tallow and used cooking oils. It was built in 2005 for a capacity of 50 000 tons per year.
  They are responsible for processing animal wastes to biodiesel and for providing saturated FAMEs and CGP.

- **Termoplast (Italy)**
  Termoplast works in the field of flexible plastic packaging production, mainly for food and medical applications.
  They are responsible for the assessment of the market potentials and the production of blends with other biopolymers. In addition they characterize the produced polymers in terms of biocompatibility, biodegradability and processing properties.

- **University of Pisa; Department of Chemistry and Industrial Chemistry (Italy)**
  The Laboratory of Bioactive Polymeric Materials for Biomedical and Environmental Applications (BIOlab) is responsible for the production of polymer blends and the assessment of the biocompatibility of different formulations. It also has the task of characterizing the produced polymers.

- **Polish Academy of Sciences; Centre of Polymer and Carbon Materials in Zabrze (Poland)**
  They are responsible for the preliminary characterization of raw polymers and the final characterization of produced formulations applying for example elemental analysis (EA), gel permeation chromatography (GPC), nuclear magnetic resonance ($^{1}H$ and $^{13}C$; NMR), thermogravimetric analysis (TGA) and multistage mass spectrometry (ESI-MS).

- **National Institute of Chemistry (Ljubljana, Slovenia)**
  Their tasks are comparable to those of the Polish Academy of Sciences, but they are using supplemental methods, for example size exclusion chromatography with multi angle light scattering detection (SEC-MALS), differential scanning calorimetry (DSC) and dynamic mechanical analysis (DMA).

- **ARGUS Umweltbiotechnologie GmbH (Germany)**
  ARGUS Umweltbiotechnologie operates in the field of organic pollutants treatment in soil and water and also in the field of food production.
  This company is responsible for the development of the downstream processing, starting from the drying and degreasing of the produced biomass to the isolation of pure PHA.

- **Ulrike Reistenhofer GmbH (Austria)**
  They are a small meat processing company and are responsible for the delivery of animal wastes.

# 2 POLYHYDROXYALKANOATES

Polyhydroxyalkanoates (PHAs) are completely biodegradable and biocompatible polymers of hydroxyalkanoic acids. They are produced by many prokaryotes (archaea, bacteria) as part of their natural metabolism. Generally PHAs are accumulated intracellularly when there is enough carbon present in the substrate, but one of the other nutrients needed for cell division is lacking ***(10,11,12)***.
PHAs represent the only kind of bacterial plastics which are solely biologically synthesized: the production of their monomers as well as their polymerization occurs *in vivo* ***(13)***. Thus many different monomer compositions can be achieved by varying strains, substrates and/or cosubstrates, consequently providing polymers with a wide range of properties ***(10)***.
Due to all these facts PHAs constitute a very promising alternative to conventional petrol-based polymers. Of course, first their major drawback has to be overcome: Their comparatively high production costs ***(14,15,16)***.

## 2.1 TYPES OF PHAS

Depending on the number of carbon atoms of the incorporated monomer units, three different types of PHAs can be distinguished:

- **short chain length (scl) PHAs:**
  The monomers consist only of 3 to 5 carbon atoms. Scl PHAs are thermoplastic and some of them have very similar properties as polypropylene ***(4)***.
  Examples for typical scl PHA building blocks are: 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV), 4-hydroxybutyrate (4HB) (Figure 2-1).

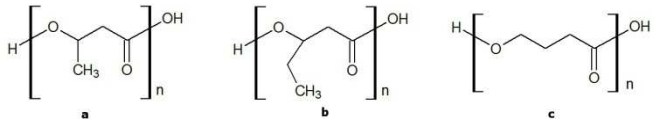


***Figure 2-1*** *Building blocks of scl PHAs: a 3HB; b 3HV; c 4HB.*

- **medium chain length (mcl) PHAs:**
  At least some of the incorporated monomers are C6 to C14 atoms. They are elastomeric with lower melting points, lower glass transition temperatures and lower crystallinity than scl PHAs ***(4)***.
  Examples for typical mcl building blocks are: 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD), 3-hydroxydodecanoate (3HDD) (Figure 2-2).

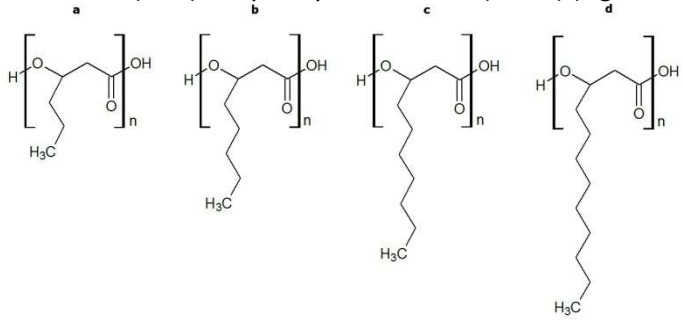


***Figure 2-2*** *Building blocks of mcl PHAs: a 3HHx; b 3HO; c 3HD; d 3HDD.*

- **long chain length (lcl) PHAs:**
  The monomers consist of more than 14 carbon atoms and, unlike scl and mcl PHAs, can only be produced *in vitro* ***(15)***.

This thesis further only refers to scl PHAs.

# 2.2 HISTORICAL OVERVIEW OF SCL PHAS

## *2.2.1 DISCOVERY OF PHB*

According to Sudesh et al. ***(11)*** and Williamson and Wilkinson ***(17)***, the first references of PHA in literature can be found in 1901 (made by Martinus Beijerinck and by Arthur Meyer, who already was able to isolate intact granules). But back then nobody was aware of what they were really looking at and so the granules were spuriously referred to as "lipid inclusions".
In the 1920s the French bacteriologist Maurice Lemoigne was searching for the cause of the acidification of aqueous suspensions of *Bacillus megaterium*. Soon he discovered it to be due to the production of 3-hydroxybutyric acid ***(18)***, which he rightly suspected to originate from a reserve material accumulated within the cells of the bacterium ***(19)***. In 1925 he already was able to isolate an amorphous product with chloroform which he considered to be the parent substance of 3-hydroxybutyric acid that was formed during autolysis ***(20)***. Two years later he further characterized this substance: "The purified product is white...it forms a transparent film if it is thin, a white and opaque one if it is thicker...it's graph is the inverse of the graph of β-hydroxybutyric acid." He came to the conclusion that the intracellular reserve material "consists of a product of polymerization and dehydration of β-hydroxybutyric acid with the empirical formula $(C_4H_6O_2)^n$. During autolysis this product depolymerizes thus yielding a new substance of the same empirical formula, but with a lower melting point, and β-hydroxybutyric acid." ***(21)*** The product he was describing is known today as PHB ***(22)***.

## *2.2.2 REDISCOVERY OF PHB*

Lemoigne and his co-workers kept on studying the polymerized 3-hydroxybutyric acid until the 1950s, but without drawing too much attention to their results. In 1944 they suggested that the occurrence of the "lipid" granular material found in the cells of *B. megaterium* was correlated with the extracted polymer of 3-hydroxybutyric acid ***(23)***. Nine years later the Swedish microbiologist Weibull was able to confirm: The "lipid" inclusions actually consisted of a polymer ***(24)***. Around this time some scientists finally started to become interested in this topic.
After all, in the year 1958, PHB had its final breakthrough.
In the Bacteriology Department of the University of Edinburgh Wilkinson and co-workers had started their research on the polymer of poly-3-hydroxybutyric acid produced by *Bacillus megaterium* and *Bacillus cereus*. In 1958 they postulated that it may act as reserve carbon and energy source for the cells ***(25,26)***. In the same year they introduced the "hypochlorite method of estimating the poly-β-hydroxybutyrate contents" instead of the chloroform extraction which was used until then. Thus it was now possible to liberate intact granules of PHB ***(17)***.
Independently from these events Forsyth examined the metabolic products of a purple-pigmented pseudomonad at the Colonial Microbiological Research Institute in Trinidad and isolated a solid material which he identified as PHB. Being aware of the fact that Lemoigne had found an analogous substance in *Bacillus megaterium* and in *Azotobacter chroococcum*, he screened several aerobic gram-negative bacteria for PHB and was able to detect it among others in *Azotobacter vinelandii* and in some *Chromobacteria* and *Pseudomonades*. His results were published in the year 1958 as well ***(27)***.
Thereupon Doudoroff and his colleagues at the Department of Bacteriology of University of California, Berkeley, also became involved with the research on PHB. They started to conduct tracer experiments with the photosynthetic purple bacterium *Rhodospirillum rubrum* and 1959 they observed that the polymer was an endogenous carbon source for the synthesis of cell-constituents ***(28)***.
When Merrick, one of Doudoroff's team, left Berkeley to start working in New York at Syracuse University, four scientists there (Lundgren, Slepecky, Alper and Marchessault) were inspired to look into the subject of bacterial polymers ***(29,30)***. In 1965, they compared the properties of PHB which they had obtained from different investigators and came to the conclusion that the applied extraction method had a huge impact on the molecular mass of the polymer. The commonly used alkaline hypochlorite method only yielded PHB with rather low molecular masses, a fact which was attributed to its degradative nature ***(31)***.

Already in the 1960s and 70s many scientists got involved with the identification of the enzymes which play a role in the biosynthesis and degradation of PHB ***(32,33,34,35,36)***.

## *2.2.3 OTHER POLYMERS THAN PHB*

The first time it was realized that bacteria also were able to synthesize other polymers than PHB was in 1972, when Wallen and Davis examined activated sludge from the municipal sewage treatment plant in Peoria, Illinois. They found a polyester which, although it contained 3-hydroxybutyric acid, had a different structure than PHB ***(37)***. Two years later Wallen reported on a new heteropolymer with similar properties as PHB and which was composed of 3-hydroxyvaleric and 3-hydroxybutyric acids. He referred to it as "polyhydroxyalkanoate", today it is known as P(3HB-co-3HV) ***(38)***.
It took almost another ten years until other polymer building blocks than 3HB and 3HV were discovered: Five years after Braunegg and colleagues of the Graz University of Technology had developed a new method of PHB determination using gas chromatography (GC) in 1978 ***(39)***, two researchers at the Florida State University were able to detect at least eleven different units of 3-hydroxy fatty acids in polymers isolated from marine sediments, based on Braunegg's method. They also postulated that the bacterial storage polymer was actually a mixed polymer of short-chain 3-hydroxy fatty acids which they called "poly-β-hydroxyalkanoates (PHA)" and even went as far as doubting the existence of the homopolymer PHB ***(40)***.
In the same year the first mcl building block was reported by Dutch researchers: They found inclusions in *Pseudomonas oleovorans* which resembled PHB granules, but didn't contain poly-3-hydroxybutyric acid and had the empirical formula $C_8H_{14}O_2$ (poly-3-hydroxyoctanoate) ***(41)***.
In 1988 another new copolyester which had been produced by *Cupriavidus necator* and contained 3-hydroxybutyrate and 4-hydroxybutyrate units, P(3HB-co-4HB), was reported ***(42)***.

## *2.2.4 LARGE SCALE PRODUCTION OF SCL PHA*

PHA attracted commercial attention for the first time in the late 1950s when the US company W. R. Grace & Co started to produce small quantities of PHB for business evaluation ***(8)***. In 1962 Baptist and Werber, two of the company's researchers, filed two patents, one concerning the fermentation, the other concerning the extraction process of PHB ***(43,44)***. But not long afterwards the project was terminated due to the thermal instability of PHB ***(45)***.

During the oil crisis of 1973 the commercial production of biopolymers became more interesting ***(11)***. Thus Imperial Chemical Industries Ltd. (ICI, United Kingdom) got involved with PHAs in the 1970s and 80s. They set up the first pilot plant and introduced the copolymer P(3HB-co-3HV) to the market under the trademark Biopol ***(45,46,47,48,22)***.
In the 1990s Wella (Germany) tried to use Biopol as material for shampoo bottles, but this experiment turned out to be economically not feasible ***(11,16)***.
In 1996 ICI sold their Biopol patents to Monsanto (USA) ***(49)*** who resold them in 2001 to Metabolix ***(50)***, a US bioscience company who had already started working with PHA in the 1980s ***(16)***. Three years after their purchase of the Biopol assets, together with Archer Daniels Midland (ADM) they formed the joint venture Telles for the manufacturing and marketing of their new PHA product line Mirel ***(51)***.

Another company who started to produce PHA in the 1980s was Chemie Linz (Austria), but they concentrated on PHB and reached a capacity of 1000 kg/week ***(16)***. A few years later their polymer sector was incorporated by Petrochemie Danubia GmbH (PCD) ***(52)***, who sold their strains and their know-how of PHB production in 1993 to Biomer (Germany) ***(53)***.

Because of the 1973 oil crisis, the Brazilian government launched the program "Pró-Álcool" with the goal of replacing conventional fossil fuels for automobiles by ethanol produced from sugar cane. As a consequence many already existing sugar cane mills were combined with distilleries, thus providing a perfect starting point for additionally integrating a PHA production process.

As substrate for the bacteria the in-house product saccharose can be used, the needed energy is produced by burning of the waste product bagasse, the remaining waste streams could be recycled as fertilizers for the sugarcane fields. As solvent for the extraction of PHA from the bacterial cells fusel alcohols (e.g. iso-pentanol) from the ethanol production could be used (see Figure 2-3) ***(14,54,4)***.
Such a process was realized by PHB Industrial S.A. (Brazil) who started their laboratory studies on the production of PHB and P(3HB-co-3HV) in 1992. Already three years later they installed a pilot plant designed for a capacity of 5 tons/a which was integrated into a sugarcane mill in São Paulo. In 2000 they remodeled the plant and increased its capacity to 50 t/a ***(55)***.

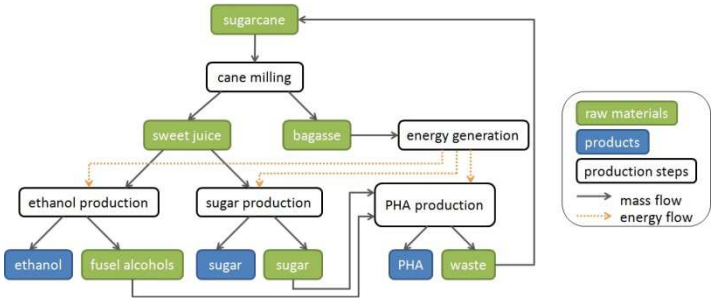


***Figure 2-3*** *Scheme of a sugarcane process with integrated PHA production. Adapted from* (**54**).

In the past few years several companies in China also started their work in the PHA sector, for example Tianjin Green Bio-Science Co. (TGBS). In 2008 they found a huge investor in DSM Venturing which enabled them to build a P(3HB-co-4HB) manufacturing plant with the capacity of producing 10000 t/a ***(56,49)***.

An exemplary process flow sheet for industrial-scale PHB production was suggested by Choi and Lee ***(57)*** and is shown in Figure 2-4.

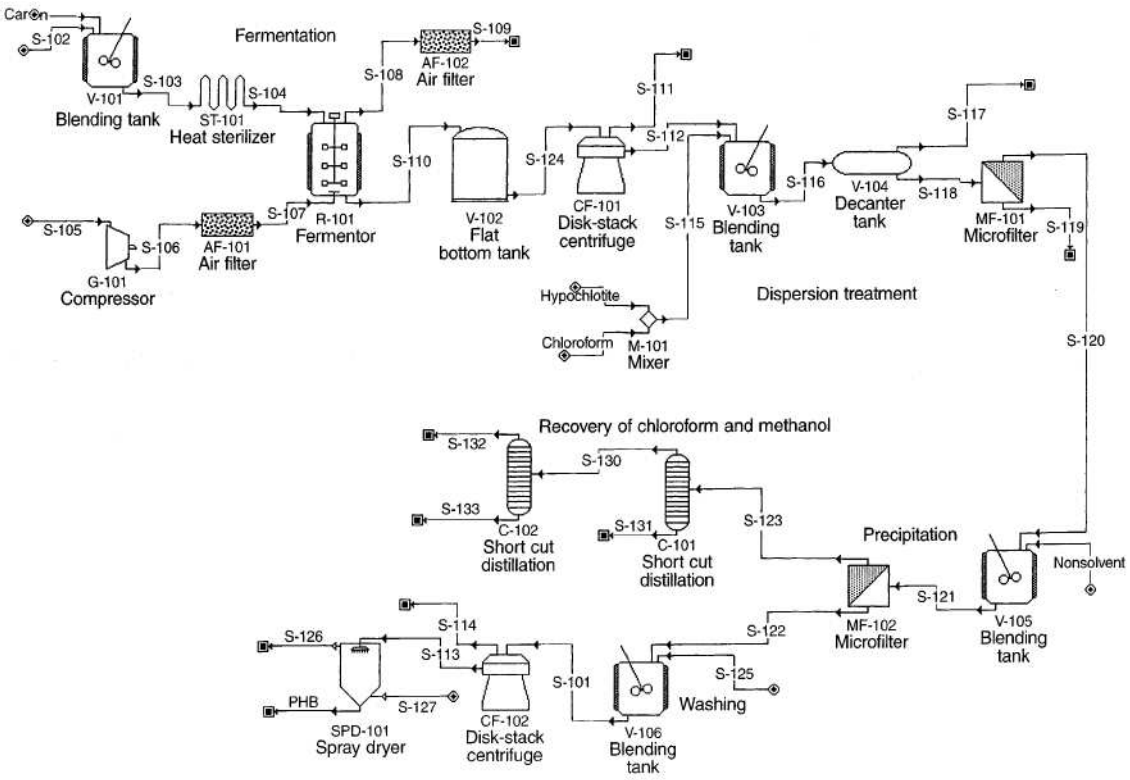


***Figure 2-4*** *Process flow sheet for PHB production with PHB recovery via dispersion of chloroform and hypochlorite. Source:* (**57**).

# 2.3 MICROBIOLOGICAL ASPECTS OF PHA PRODUCTION

## 2.3.1 CUPRIAVIDUS NECATOR

One of the best examined bacterial species regarding scl PHA production is *Cupriavidus necator,* a fact which is not too obvious at the first glance, because its name was subject to several changes during the last decades.
After its first isolation by Bovell in 1957 it was designated as *Hydrogenomonas eutropha*, but was not formally described and eleven years later it was renamed for the first time to *Alcaligenes eutrophus*. In 1987 it was discovered that this species was not only highly resistant to copper, but it was even stimulated in its growth. It was described as being a nonobligate bacterial predator. Thus it was reassigned to a completely new genus and its name was changed again, this time to *Cupriavidus necator*, which means "copper loving slayer" ***(58)***. Less than ten years later, in 1995, it was renamed *Ralstonia eutropha* ***(59)***, only to get its new name *Wautersia eutropha* assigned after nine years ***(60)***, which in turn was changed back again to *Cupriavidus necator* even in the same year ***(61)***, which is still in use today.

The cells of *Cupriavidus necator,* which are the shape of straight rods, have a size of about 1 µm in length, 0.7 µm in diameter and move through two to ten peritrichous flagella. After two days their colonies are off-white, glistening, smooth, convex, with an entire edge and with 2 to 4 mm in diameter. They can be found mainly in soil, but have also been isolated from clinical specimens of humans. PHA rich cells can be seen in Figure 2-5 ***(58,61)***.

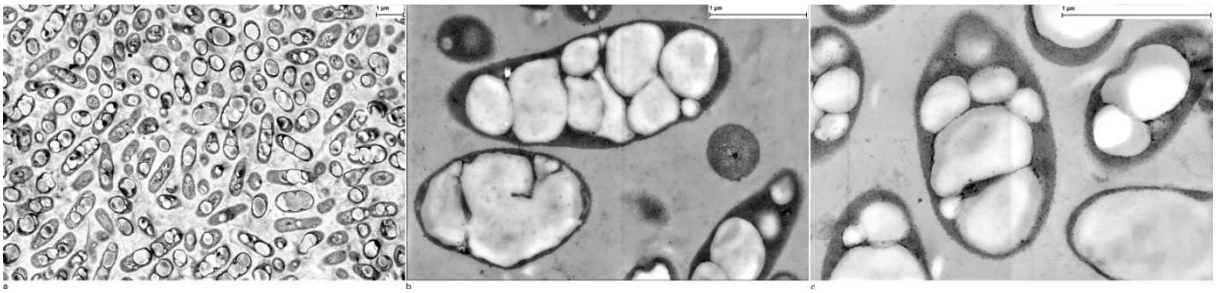

***Figure 2-5*** *PHA-rich C. necator cells. Magnification: a: 1:20.000; b: 1:72.000; c: 1:150.000. Pictures provided by Dr. Elisabeth Ingolić, FELMI-ZFE, Graz, Austria.*

For the experimental part of this thesis solely *Cupriavidus necator* was used, hence other PHA producing strains will not further be elaborated.

## 2.3.2 METABOLISM OF PHA SYNTHESIS

### 2.3.2.1 BASICS OF METABOLIC PROCESSES

The metabolism of a cell consists of the following steps:

1. Catabolism: The nutrients are broken down to smaller pieces, providing the energy and the components needed.
2. Anabolism: In a first step, building blocks (e.g. amino acids) are assembled from the obtained components. These are further synthesized to macromolecules (e.g. proteins, reserve materials, constituents of the cell wall).

For each of the occurring metabolic conversions a particular enzyme is responsible which acts as a biocatalyst, reducing the activation energy, thus allowing these processes. Enzymes are proteins and as such consist of amino acids and have a very high substrate specificity.
In contrast, coenzymes are low-molecular compounds which transfer broken down pieces of nutrients, constituting substrates for enzymes. One of the most common is coenzyme A (CoA-SH; Figure 2-6) which reacts only with its thiol-group to form thioesters (e.g. acetyl-CoA) ***(62,63)***.

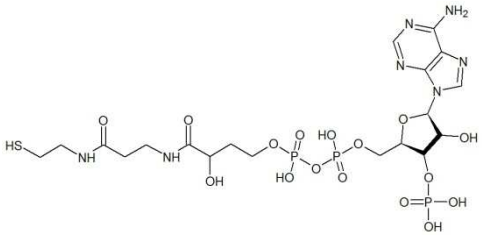

***Figure 2-6*** *Structure of Coenzyme A.*

If the surrounding substrate contains all the substances needed for bacterial growth (carbon and energy source, nitrogen, phosphorus, sulfur, water, oxygen, trace elements), the cell converts them *via* different metabolic pathways into biomass. One of these pathways is the tricarboxylic acid cycle (TCA, also known as citric acid cycle; Figure 2-7), where Acetyl-CoA is terminally oxidized to $CO_2$, generating energy and biosynthetic precursors which play an important role for the availability of nitrogen, phosphorus and other elements. For example, oxaloacetate, one of the products of the TCA cycle, is crucial for the synthesis of amino acids.

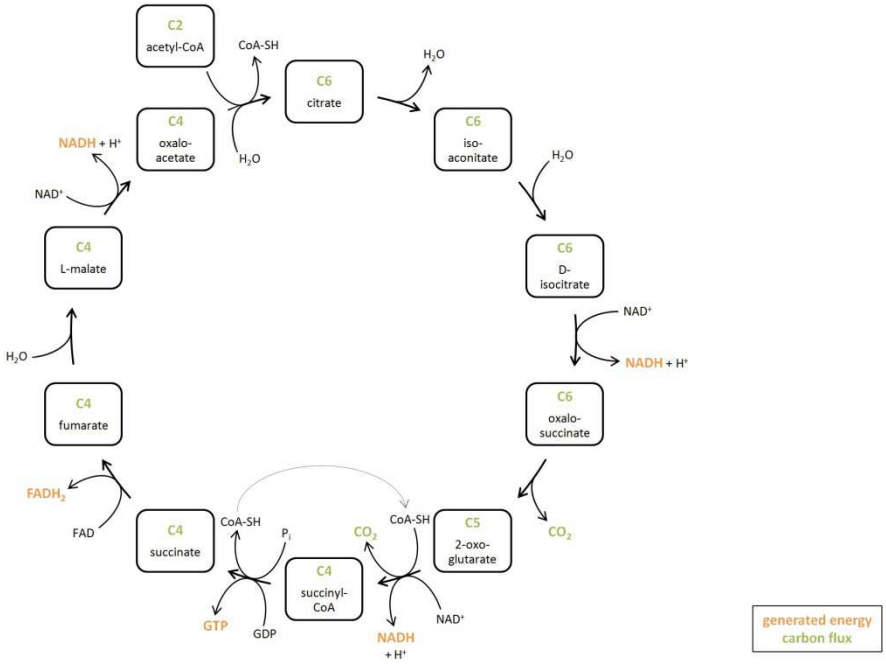


***Figure 2-7*** *Scheme of TCA cycle. Source:* (**62**).

If there is a lack of one nutrient in the substrate the cell is not able to synthesize proteins anymore and the reproductive growth stops. In this case PHA producing bacteria have the ability to store the surplus carbon in form of PHA granules if there is an excess of carbon available: Due to the nutrient imbalance the TCA cycle is inhibited and the subsequently accruing Acetyl-CoA is redirected towards PHA synthesis (Figure 2-8) ***(62,7,10,64,65)***.

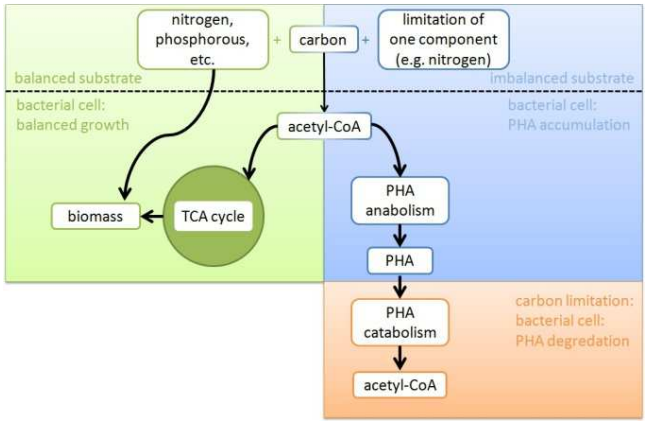


***Figure 2-8*** *Scheme of acetyl-CoA flux under balanced and imbalanced nutrient conditions. Adapted from* (**47**,**62**).

Once the carbon source is limited, the PHA producing bacteria are able to degrade the accumulated polymer again to carbon building blocks, thus gaining a huge advantage over other organisms in matters of survival. But PHA not only acts as carbon and energy source if the external carbon is restricted, it also allows enhanced survival under other environmental stress conditions like heat, cold, UV irradiation, desiccation, osmotic shock or the presence of organic solvents ***(66,67,68,69)***.

### *2.3.2.2 METABOLISM OF PHB IN CUPRIAVIDUS NECATOR*

The single steps of the metabolic pathway of PHB synthesis in *C. necator* are shown in Figure 2-10 and Figure 2-9.

At the beginning the carbon source is broken down to C2 molecules of acetyl-CoA. For glucose this happens *via* the Entner Doudoroff pathway, which is also known as 2-keto-3-desoxy-6-phosphogluconate (KDPG) pathway, where two molecules of the C3 intermediate pyruvate are generated per each sugar molecule. These pyruvates are then decarboxylated to form acetyl-CoA. Fatty acids are broken down *via* the β-oxidation pathway (see Figure 2-11) and yield, depending on their chain length, several acetyl-CoA units.

Two molecules of the formed acetyl-CoA are combined in a condensation reaction which is catalyzed by the enzyme β-ketothiolase and acetoacetyl-CoA is formed. This intermediate is reduced by acetoacetyl-CoA reductase to the (R)-isomer of 3-hydroxybutyryl-CoA. The latter is the monomer which, in a last step, is incorporated into the PHB-chain by the synthase enzyme ***(70,10,32,33,34)***.

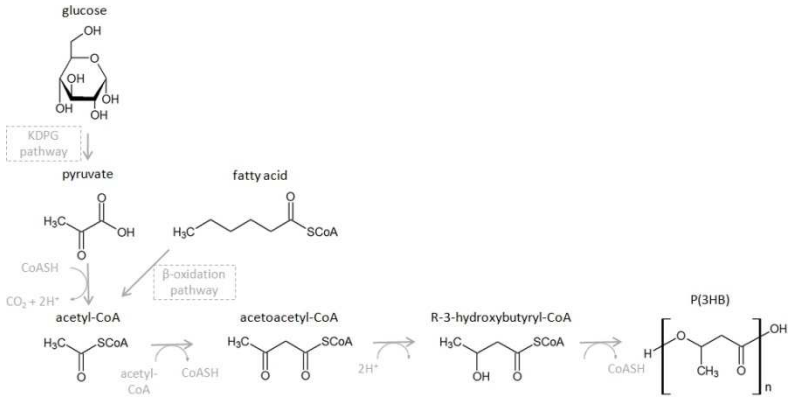


***Figure 2-9*** *Biosynthesis of PHB in C. necator. Adapted from:* (**71**,**72**).

Once there is a limitation of the carbon source, the anabolism of PHB is stopped in favor of the catabolism. The depolymerase enzyme converts the polymer to monomers of (R)-3-hydroxybutyric acid, based on which acetyl-CoA is generated *via* the intermediates acetoacetic acid and acetic acid. The produced acetyl-CoA molecules can enter the TCA cycle, thus supplying the cell with energy and components needed for its survival ***(33,35,36,70,11)***.

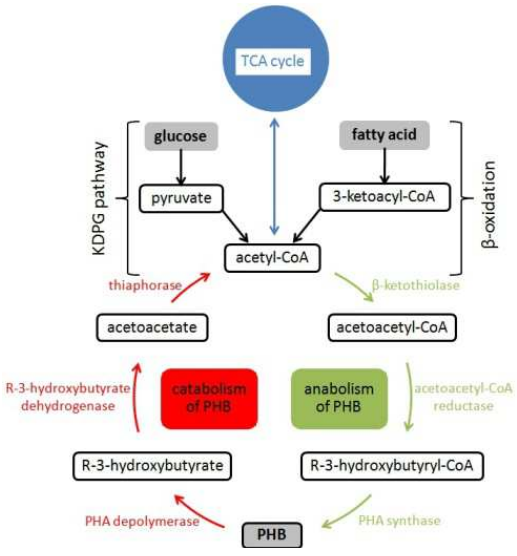


***Figure 2-10*** *Scheme of PHB metabolism in C. necator. Adapted from* (**11**).

### 2.3.2.3 METABOLISM OF P(3HB-CO-3HV) IN CUPRIAVIDUS NECATOR

To allow the biosynthesis of the copolymer P(3HB-co-3HV) in *Cupriavidus necator* a precursor for the building of the 3HV units is needed in addition to the main carbon source. This could be propionic acid or another odd-numbered fatty acid which is broken down *via* β-oxidation to one or more molecules of acetyl-CoA and one molecule of propionyl-CoA, which acts as substrate for the generation of the 3HV-building block (see Figure 2-11).

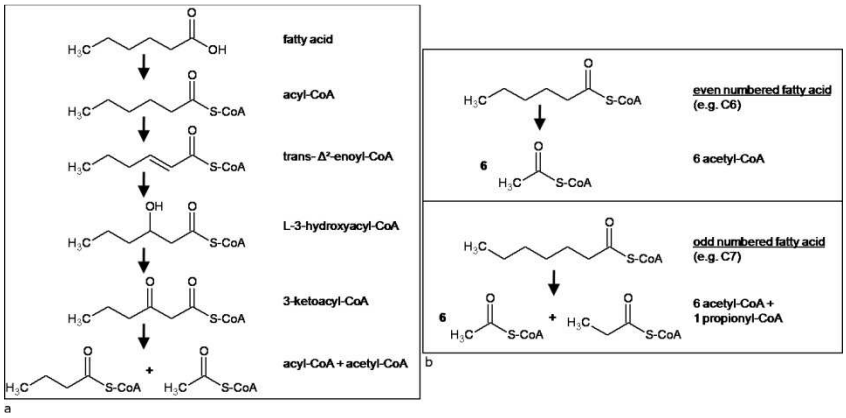


***Figure 2-11*** *a: Steps of the* β*-oxidation of a fatty acid; b: Different results of* β*-oxidation for even and odd numbered fatty acids. Adapted from* (**11**).

Subsequently, one propionyl-CoA reacts with one acetyl-CoA, enzymatically catalyzed by 3-ketothiolase, building 3-ketovaleryl-CoA. This is transformed to R-3-hydroxyvaleryl-CoA, the 3HV building block which can be incorporated into the copolymer by PHA synthase ***(71,72,10)***. The 3HB units are generated starting from acetyl-CoA *via* the same metabolic pathway as for the synthesis of the homopolymer PHB.

Associated with the biosynthesis of P(3HB-co-3HV) is also an undesired oxidative loss of $CO_2$: The propionyl-CoA can lose its carbonyl atom to build acetyl-CoA, which then only can be used for synthesis of 3HB units, lowering the yield of the expensive 3HV precursor and the 3HV production rates. This process can be suppressed through a reduction of the concentration of dissolved oxygen ***(72,15)***.

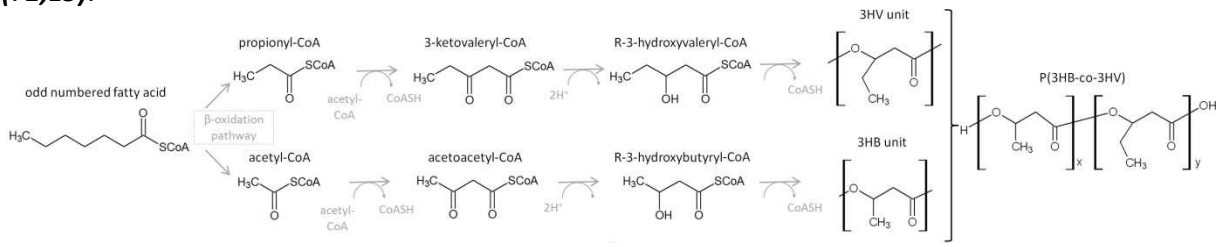


***Figure 2-12*** *Biosynthesis of P(3HB-co-3HV) in C. necator. Adapted from* (**71**,**72**).

### 2.3.2.4 METABOLISM OF P(3HB-CO-4HB) IN CUPRIAVIDUS NECATOR

Same as for P(3HB-co-3HV), the biosynthesis of the copolymer P(3HB-co-4HB) requires an appropriate precursor like 4-hydroxybutyric acid, γ-butyrolactone, 1,4-butanediol or 4-chlorobutyric acid. All of them can be converted to 4-hydroxybutyric acid, which acts as starting material for 4-hydroxybutyryl-CoA which in turn is polymerized, adding 4HB building blocks to the intracellular polymer (see Figure 2-13).

During this process parts of the carbon provided by the precursor get lost. On the one hand, starting from 4-hydroxybutyryl-CoA, 3HB units can be synthesized *via* several intermediates like butyryl-3-ene-CoA. On the other hand 4-hydroxybutyryl-CoA can also be converted to succinyl-CoA, thus entering the TCA cycle ***(73,74,75,10)***.

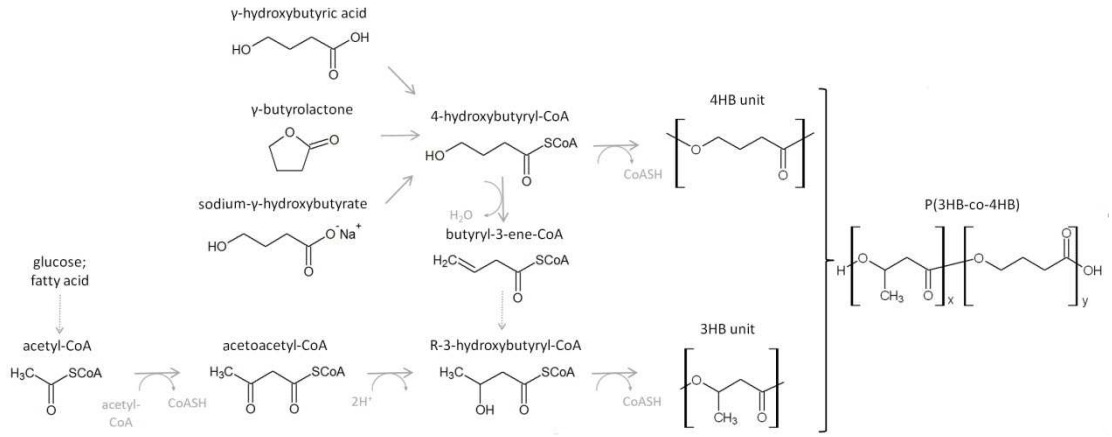


***Figure 2-13*** *Biosynthesis of P(3HB-co-4HB) in C. necator. Adapted from* (**74**,**73**,**75**,**10**).

# 2.4 PROPERTIES

## *2.4.1 PROPERTIES OF PHAS*

PHA producing bacteria are known to be able to polymerize more than 150 different monomers of (R)-hydroxyalkanoic acids ***(76)***. Depending on the R-substituent of the β-carbon (see Figure 2-14), which can range from a simple methyl group (in case of PHB) all the way to long chain units even containing rings, double bonds or branched chains, major differences in the properties of the produced PHA are obtained. For example the polymer becomes more elastomeric with an increasing number of side-chain carbons ***(45)***.

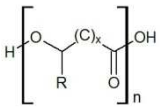

***Figure 2-14*** *General structure of PHAs.*

Generally PHAs are aliphatic polyesters which are biodegradable, biocompatible, piezoelectrical, optically active and hydrophobic ***(77)***.
Inside the bacterial cell, PHAs are located in the cell cytoplasm as a mobile amorphous polymer ***(78)*** and have the form of granules with an average diameter of 0.2 to 0.5 µm (cf.Figure 2-5) ***(11)***. These granules are surrounded by a protein monolayer membrane, which can be seen as globular particles in an atomic force microscopy (AFM) image (Figure 2-15) ***(79)***.

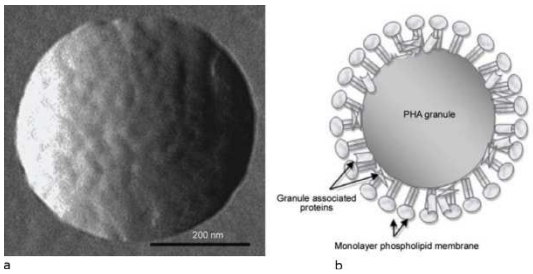


***Figure 2-15*** *Morphology of PHA granules: a: AFM image of a PHA granule, showing globular particles on its surface; b: scheme of the granule surface (not true to actual scale). Source:* (**73**).

## 2.4.2 PROPERTIES OF PHB

PHB is a highly crystalline polymer which crystallizes slowly, forming large spherulitic structures. It is susceptible to thermal degradation and displays a rather low elongation at break ***(22)***. As shown in Table 2-1 it has very similar properties as polypropylene (PP) in terms of melting point, glass-transition temperature and degree of crystallinity, but it is stiffer and more brittle. Its processability is also limited due to the relatively small difference between its melting point and its decomposition temperature. For these reasons PHA copolymers are often favored over PHB ***(10,8,80)***.
It was proposed that PHB molecules in their crystalline state form a right-handed helix with a double screw axis along the chain. Based on energy considerations a molecular PHB model was computed whose helical conformation is only stabilized by nonbonded energies and polar interactions (Figure 2-16) ***(81)***.

***Table 2-1*** *Properties of PHB.*

| [mol%] | $T_m$ [°C] | $T_g$ [°C] | tensile strength [MPa] | elongation to break [%] | sources |
|---|---|---|---|---|---|
| PP | 170 | -10 | 35 | 400 | ***(82,11)*** |
| PHB | 180 | 9 | 40 | 3 | ***(54,11)*** |

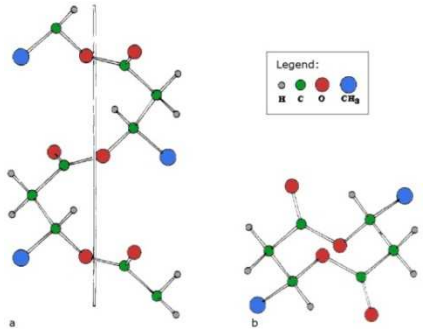


***Figure 2-16*** *Molecular model of PHB with the lowest energy. a: Viewed normal to the helix axis; b: viewed along the helix axis. Source:* (**81**).

## 2.4.3 PROPERTIES OF P(3HB-CO-3HV)

The comonomer units of P(3HB-co-3HV) are statistically randomly distributed ***(22)***. The incorporated 3HV building blocks cause lower melting points and crystallinity and an increase of ductility and toughness in comparison to PHB, providing a big advantage in terms of processability ***(7,8)***. The exact properties depend on the 3HV content, as shown in Table 2-2.
P(3HB-co-3HV) is isodimorphic: Because the 3HB and 3HV monomers are of similar size and crystalline conformation, the crystal lattice of one building block can accommodate the other one and *vice versa*. Below the pseudo-eutectic point of approximately 30 mol% 3HV, a coherent PHB phase crystallizes which incorporates 3HV units, above this point a PHV lattice accommodating 3HB units is formed ***(83)***.

***Table 2-2*** *Properties of P(3HB-co-3HV); n.d. = no data.*

| [mol%] | $T_m$ [°C] | $T_g$ [°C] | tensile strength [MPa] | elongation to break [%] | crystallinity [%] | sources |
|---|---|---|---|---|---|---|
| PHB | 179 | 10 | 40 | 7 | 80 | ***(54,7)*** |
| P(3HB-co-3% 3HV) | 170 | n.d. | 38 | n.d. | n.d. | ***(4)*** |
| P(3HB-co-9% 3HV) | 162 | 6 | 37 | 20 | 60 | ***(54,7)*** |
| P(3HB-co-20% 3HV) | 145 | 1 | 32 | 45 | 35 | ***(54,7)*** |
| P(3HB-co-34% 3HV) | 97 | -8 | 18 | 97 | n.d. | ***(54)*** |
| P(3HB-co-71% 3HV) | 83 | -13 | 11 | 5 | n.d. | ***(54)*** |

### 2.4.4 PROPERTIES OF P(3HB-CO-4HB)

P(3HB-co-4HB) is like P(3HB-co-3HV) a random copolymer whose properties are defined by its 4HB content, which enhances its properties compared to PHB ***(42,84)***. Above 40 mol% 4HB it shows a very similar behavior as elastic rubbers ***(67)***. Its properties depending on the 4HB content are shown in Table 2-3.

***Table 2-3*** *Properties of P(3HB-co-4HB); n.d. = no data.*

| [mol%] | $T_m$ [°C] | $T_g$ [°C] | tensile strength [MPa] | elongation to break [%] | crystallinity [%] | source |
|---|---|---|---|---|---|---|
| PHB | 177 | 4 | 40 | 6 | 59 | ***(85,4)*** |
| P(3HB-co-6% 4HB) | 162 | -1 | 28 | 45 | 56 | ***(85,4)*** |
| P(3HB-co-10% 4HB) | 159 | -3 | 26 | 440 | 46 | ***(85,4)*** |
| P(3HB-co-28% 4HB) | n.d. | -15 | n.d. | n.d. | 23 | ***(85)*** |
| P(3HB-co-85% 4HB) | 48 | -41 | 15 | 600 | 29 | ***(85,4)*** |
| P(3HB-co-90% 4HB) | 50 | -44 | n.d. | n.d. | n.d. | ***(85)*** |
| P(3HB-co-94% 4HB) | 51 | -46 | n.d. | n.d. | 42 | ***(85)*** |
| P(4HB) | 54 | -50 | 104 | 1000 | n.d. | ***(85,4)*** |

## 2.5 COMPARISON OF PHAS WITH CONVENTIONAL PLASTICS

Petrol-based plastics have the major disadvantage of causing huge problems once they are disposed of. Either they are placed on landfills which are already overflowing with refuse or they are incinerated, thus contributing to global warming and the greenhouse effect. Another possibility to deal with plastic waste is recycling, but this is also associated with problems like the requirement of a certain sorting accuracy and a relatively high purity of the disposed items. Moreover this process is connected to high collection costs and leads to an increase of brittleness of the recycled material ***(86)***.

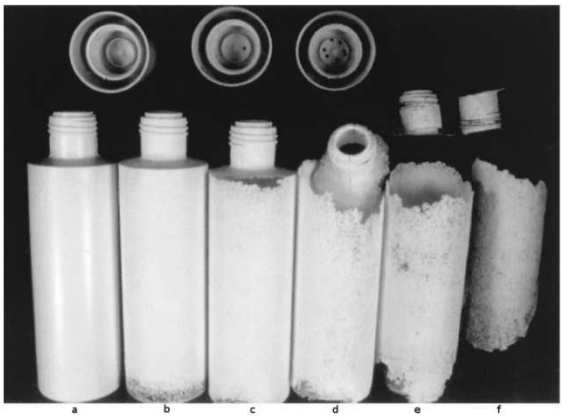


***Figure 2-17*** *Degradation of P(3HB-co-3HV) in aerobic sewage sludge after a: 0; b: 2; c: 4; d: 6; e: 8 weeks. Source:* (**87**).

Here PHAs clearly are advantageous, since they are completely biodegradable (Figure 2-17): Provided that there are microorganisms present, they are metabolized to carbon dioxide and water, the only compounds remaining of the former plastic item ***(54,7)***. In addition PHAs are part of a balanced carbon cycle, thus not affecting the climate. The carbon dioxide which is released during their breakdown will again be fixed by plants which indirectly serve as carbon source for the biosynthesis of PHAs, thus closing the cycle (Figure 2-18) ***(4)***. In addition to their biodegradability PHAs are also biocompatible: 3HB and its oligomers even occur in human blood and tissue ***(88)***.

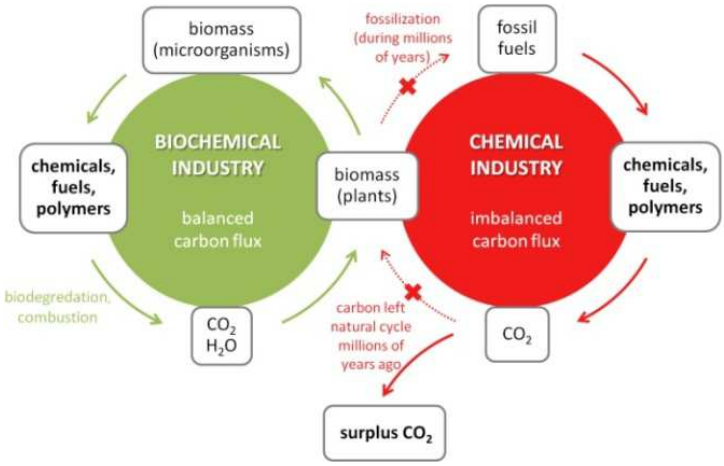


***Figure 2-18*** *Comparison of the carbon fluxes of the biochemical industry (biodiesel, PHAs) and of the chemical industry (petrol-based fuels and polymers). Adapted from* (**9**).

Another problem of conventional plastics constitutes the fact that they are dependent on fossil fuels, whose feedstocks will be depleted one day. In addition their prices are subject to political events as demonstrated during the oil crises of the 1970s and 2000s (Figure 2-19).
Here PHAs provide an attractive alternative as well, since they are completely independent from fossil fuels, provided that the energy required for their production is exclusively based on renewable resources ***(4)***.

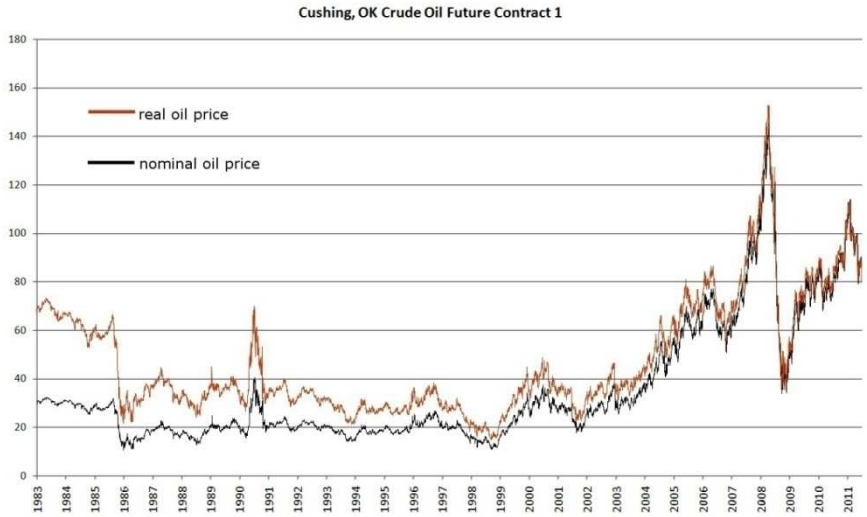


***Figure 2-19*** *Development of the crude oil price after the first oil crisis; data from Cushing, Oklahoma (Future Contract 1;* (**89**,**90**)*. Real oil price: inflation corrected on the basis of September-2011; Nominal oil price: not inflation corrected.*

But although the price of PHAs is not as instable as the price of crude oil, at the same time it represents its major drawback. In terms of costs bioplastics with a price of approximately 5 US$ per kg PHB cannot yet compete with conventional plastics, which cost less than 1 US$ per kg in the case of polyethylene and polypropylene ***(57)***. ICI sold its copolymer Biopol even at a price of approximately 16 US$ per kg ***(57)***. The decisive cost drivers of PHA production are the raw materials for the substrate (carbon and nitrogen source, precursors for special building blocks) and the isolation and purification steps needed to obtain the pure polymer ***(4)***. Also the achieved PHA content of bacterial cells, the yield and the productivity should not be disregarded in terms of economic feasibility ***(91)***.

# 2.6 APPLICATIONS OF PHAS

Due to the wide range of properties featured by PHAs they can be utilized in many different areas.
For example they can be used as disposable items of everyday life (e.g. razors, diapers, cosmetic containers, cups) as well as packaging materials (e.g. compostable shopping bags, containers, paper coatings) ***(77)***. But they are also used for agricultural applications (e.g. mulching films, plant pots), as biofuels and, because of their optically pure R-configuration, as synthons for fine chemicals, which often have higher market values than the polymers themselves (e.g. vitamins, antibiotics, pheromones, aromatics, perfumes) ***(13,4,15,92)***. They are also interesting for the printing industry, since they can be easily stained ***(93)***. Examples for the different applications of PHAs are shown in Figure 2-20.


***Figure 2-20*** *Different applications of Telles' product line Mirel. Source:* (**94**).

A very promising area for the utilization of PHAs is the field of medical applications, where biocompatibility is of the utmost importance. Among other things they can be used for wound management (e.g. sutures, skin substitutes, nerve cuffs), for the vascular system (e.g. heart valves, cardiovascular fabrics), for orthopedic applications (e.g. spinal fusion cages, bone graft substitutes, screws), as drug deliver carriers (e.g. micro- and nanospheres for anticancer therapy), for urological applications (e.g. urological stents), for tomography and for ultrasound (as contrast agents) ***(95,82)***.

# 3 BIODIESEL

Biodiesel consists of monoalkylesters of long chain fatty acids which are generally produced from oils (e.g. food-grade rapeseed oil) *via* an alkali catalyzed transesterification process (Figure 3-2) ***(96)***. Biodiesel is one of many different kinds of biofuels, which, in contrast to petrol-based fuels, are produced from renewable resources. Biofuels can be divided into four main groups:

- **First generation biofuels:**
  They are produced from feedstocks which are related to the food chain in some way. Either they also could be used as food or feed or they are based on food residues (e.g. sugar, starch, vegetable oil, animal fats). Examples of first generation biofuels are biodiesel, sugarcane ethanol and corn ethanol ***(97)***.
- **Second generation biofuels:**
  Their feedstocks are not connected to the food chain (e.g. cellulose, hemicellulose, lignin). Examples of second generation biofuels are cellulosic ethanol, substitute natural gas (SNG) and Fischer-Tropsch diesel ***(97)***.
- **Third generation biofuels:**
  For the production of these biofuels algae biomass is used as feedstock ("oilgae") ***(98)***.
- **Fourth generation biofuels:**
  These biofuels are based on genetically engineered feedstocks and are carbon negative. During the growth of the required biomass more $CO_2$ is consumed than will be set free by the combustion of the produced biofuel ***(98)***.

Due to their comparatively good exhaust emission characteristics and their independence from fossil feedstocks biofuels have experienced a boom in the last several years ***(4,99)***. In 2003 the European Commission issued the Directive 2003/30/EC which set the goal of reaching a biofuel penetration of 5.75% in the transport sector by 2010. In 2009 the Directive 2009/28/EC raised this share to 10% for each member state until 2020 ***(99)***. But also in non EU countries, the production of biofuel is on the rise (Figure 3-1).

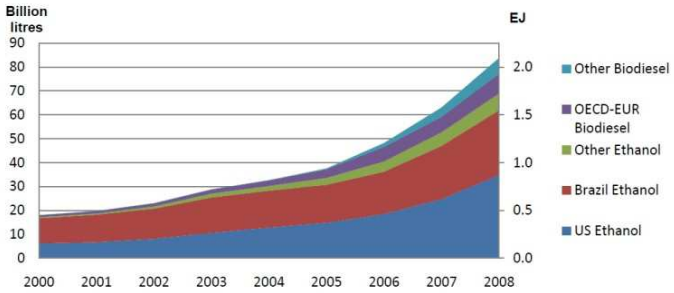


***Figure 3-1*** *Trend of global biofuel production. Source:* (**97**).

Like all first generation biofuels, biodiesel has to struggle with the major drawback of being limitated with regard to the available cultivation area and at the same time of coming into conflict with the food chain ***(100)***. One promising approach in solving this problem is the utilization of waste lipids like animal fats from the slaughtering and rendering industries, soap stocks, waste cooking oils and restaurant greases ***(4)***.

# 3.1 TRANSESTERIFICATION

During transesterification (also known as alcoholysis) oils or fats, which consist of 90 to 98% of triglycerides ***(6)***, react with an alcohol, normally in presence of a catalyst ***(96)***, building the respective fatty acid alkyl esters and generating glycerol as side product. This process consists of three steps: The triglyceride is converted to glycerol *via* the intermediates diglyceride and monoglyceride, after each step generating a molecule of fatty acid alkyl ester, the desired end product biodiesel (Figure 3-2) ***(5)***. Being an equilibrium reaction, an excess of alcohol shifts the equilibrium of a transesterification towards ester formation ***(96)***.

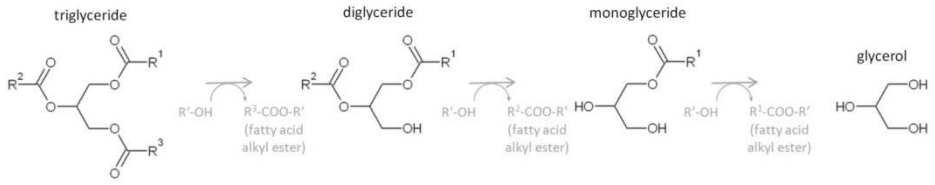


***Figure 3-2*** *General equations for the transesterification reactions of triglycerides. Adapted from:* (**5**).

In most cases the applied alcohol is methanol, thus generating fatty acid methyl esters (FAMEs; Figure 3-3). At the end of such a methanolysis process a two-phase system exists, consisting of a lower glycerol phase and an upper FAME phase ***(5)***.

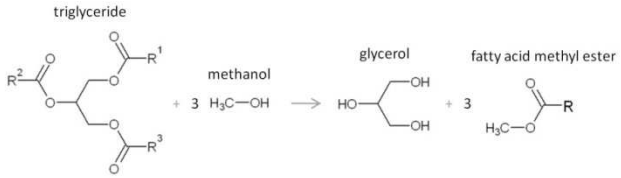


***Figure 3-3*** *General equation for the methanolysis of triglycerides. Adapted from:* (**5**).

# 3.2 TRANSESTERIFICATION METHODS

Regarding the applied catalyst four different transesterification methods can be distinguished.

- alkali catalyzed transesterification
- acid catalyzed transesterification
- enzyme catalyzed transesterification
- supercritical transesterification

## *3.2.1 ALKALI CATALYZED TRANSESTERIFICATION*

Alkaline catalysis is the most common method for biodiesel production and molar alcohol to oil ratios of up to 6 to 1 are used ***(5)***.

Examples of alkaline catalysts are sodium hydroxide (NaOH), potassium hydroxide (KOH), sodium methoxide ($NaOCH_3$) or potassium methoxide ($KOCH_3$) ***(96)***.

It has the advantage of allowing high conversion in a short time ***(96)***, but at the same time it is very sensitive to free fatty acids (FFAs) which are found in relatively huge amounts in low-quality feedstocks (e.g. animal fats). They react with the alkaline catalyst, forming soap and causing a severe decrease in yields and hindering the separation of the glycerol phase after the transesterification. For this reason a pretreatment step is needed if the FFA content exceeds 0.5 to 5%. This is usually achieved through an acid catalyzed esterification of the FFAs to esters prior to the actual transesterification (Figure 3-4) ***(5,6,96)***.

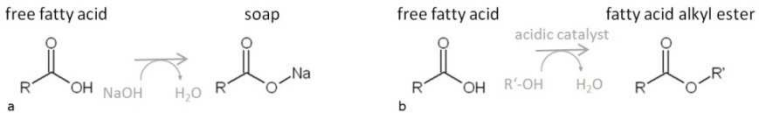


***Figure 3-4 a:*** *Equation for the soap formation reaction; b: Pretreatment of FFAs via esterification. Adapted from:* (**96**).

Also the presence of water during an alkali catalyzed transesterification causes poor yields ***(96)***.
An example of a typical biodiesel production process applying alkaline methanolysis is shown inFigure 3-5.

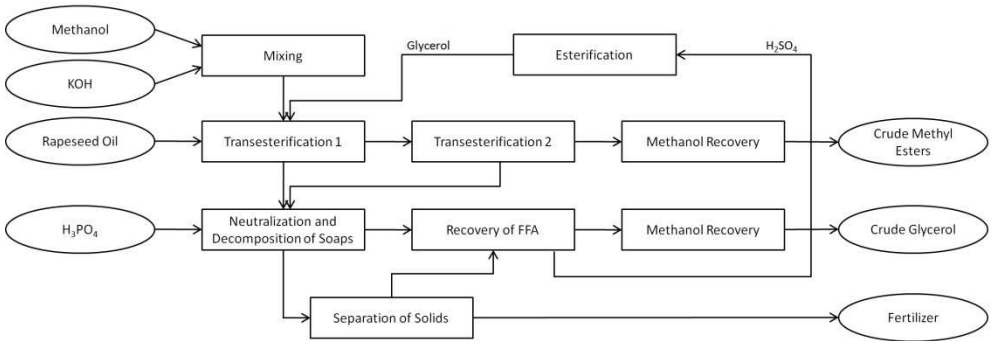


***Figure 3-5*** *Scheme of a typical biodiesel production process via alkaline methanolysis. Source:* (**5**).

## *3.2.2 ACID CATALYZED TRANSESTERIFICATION*

During acid catalyzed transesterification FFAs do not cause any problems in terms of soap formation, but for this method to be effective, also a bigger amount of alcohol is needed (molar ratios up to 30 to 1) and also the corrosive behavior of the acidic catalyst is disadvantageous ***(5,6)***.
Examples of acidic catalysts are sulfuric acid ($H_2SO_4$), phosphoric acid ($H_3PO_4$) and hydrochloric acid (HCl) ***(96)***.

## *3.2.3 ENZYME CATALYZED TRANSESTERIFICATION*

Transesterifications can also be enzymatically catalyzed, utilizing lipases. Theses enzymes are able to catalyze the transesterification reaction independently of the water content of the system, thus facilitating the subsequent separation of glycerol. Apart from that they can completely convert the contained FFAs to alkyl esters, but on the other hand the costs of an enzymatic catalyst are comparatively high ***(6,96)***.

## *3.2.4 SUPERCRITICAL TRANSESTERIFICATION*

Supercritical transesterification is a non-catalytic alcoholysis and is working at high temperatures and pressures instead, thus allowing the esterification of FFAs and the transesterification of triglycerides simultaneously. Apart from the high energy demands also an increased amount of alcohol is needed (molar ratios of up to 42 to 1) ***(5,6)***.

# 3.3 FEEDSTOCKS FOR BIODIESEL PRODUCTION

About 85% of the worldwide biodiesel production is based on rapeseed oil which is certainly due to its comparatively good combustion characteristics, its oxidative stability and its cold temperature behavior. Other common feedstocks are sunflower seed oil, soybean oil and palm oil ***(5)***.
As mentioned above lipid wastes constitute a promising alternative, not only because they do not come into conflict with the food chain, but also because of their low prices compared to food-grade vegetable oils. Moreover this concept helps solving the waste problems of some branches of industry (e.g. rendering industry, food processing facilities) ***(6)***.
The types of fatty acids which are present in the feedstock have a major impact on the physical and chemical properties of the produced biodiesel, depending on their chain length and their saturation level (Table 3-1). In general high numbers of carbon atoms and a low unsaturation level lead to an increase of viscosity, density, cetane number and heating value as well as to an insufficient cold temperature behavior ***(6)***.
Biodiesel based on low-quality feedstocks like yellow grease (consists of less than 15% FFAs), brown grease (consists of more than 15% FFAs), tallow or lard contains notably more saturated fatty acids than biodiesel based on vegetable oils ***(6)***.

***Table 3-1*** *Fatty acid distribution of different biodiesel feedstocks. Source:* (**6**).

| **feedstock** | **fatty acids [mass%] [number of carbon atoms : number of double bonds]** | | | | | | | **saturation level** |
|---|---|---|---|---|---|---|---|---|
| | **14:0** | **16:0** | **16:1** | **18:0** | **18:1** | **18:2** | **18:3** | **[%]** |
| sunflower | - | 6.08 | - | 3.26 | 16.93 | 73.73 | - | 9.34 |
| rapeseed | - | 3.49 | - | 0.85 | 64.4 | 22.3 | 8.23 | 4.34 |
| soybean | - | 10.58 | - | 4.76 | 22.52 | 52.34 | 8.19 | 15.34 |
| soybean soap stock | - | 17.2 | - | 4.4 | 15.7 | 55.6 | 7.1 | ~17 |
| used frying oil | - | 12 | - | - | 53 | 33 | 1 | ~12 |
| tallow | 3 to 6 | 24 to 32 | - | 20 to 25 | 37 to 43 | 2 to 3 | - | 47 to 63 |
| lard | 1 to 2 | 28 to 30 | - | 12 to 18 | 4 to 50 | 7 to 13 | - | 41 to 50 |
| yellow grease | 2.43 | 23.24 | 3.79 | 12.96 | 44.32 | 6.97 | 0.67 | 38.63 |
| brown grease | 1.66 | 22.83 | 3.13 | 12.54 | 42.36 | 12.09 | 0.82 | 37.03 |

# 3.4 FEEDSTOCK PRETREATMENT

In most cases, before the actual transesterification can take place, one or more pretreatment steps of the lipid feedstock are necessary in order to remove impurities like phosphatides, free fatty acids, water or waxes. Below several examples of such pretreatments are discussed.

- **Degumming**: Through the addition of water, soluble phosphatides are hydrated and separated from the lipid feedstock together with the aqueous phase *via* centrifugation. Water insoluble phosphatides are removed by decomposing them in acid solutions ***(5)***.
- **Deacidification**: Since a high amount of FFAs causes problems during alkaline transesterification, the acid number needs to be reduced to values below 1 mg KOH/g. Apart from the already discussed acid catalyzed pre-esterification of FFAs, this can also be achieved through neutralization with an alkali or through distillation ***(5)***.
- **Bleaching**: This step has the goal of removing colorants using bleaching earth, silica gel or activated carbon ***(5)***.
- **Dehydration**: Depending on the transesterification method the presence of water may have a negative impact on the production process, thus the removal of water from the raw material *via* distillation under reduced pressure may be required ***(5)***.

# 3.5 PROPERTIES AND SPECIFICATIONS OF BIODIESEL

Biodiesel based on vegetable oils has very similar properties as petrol-based diesel (Table 3-2). But because of the wide range of possible feedstocks for biodiesel production, a specification of several properties is necessary in order to ensure the needed quality.

- The **viscosity** is important for the injection and the combustion process of the fuel ***(6,96)***.
- The **flash point** is the temperature needed for ignition when the fuel is exposed to a flame or a spark and is of importance for storage and transportation ***(6,96)***.
- The **cold flow properties** are important for the cold weather performance and can be defined *via* cold filter plugging point (**CFPP**) which is the temperature beneath which the fuel starts to clog the fuel lines and filters or *via* **pour point**, which is the temperature which marks the limit of the fuel's ability to flow or to be pumped ***(6,96)***.
- The **density** is important for the fuel injection properties and also influences the exhaust emissions ***(6)***
- The **cetane number** is a measure of the delay between the start of the fuel injection and the first pressure increase during combustion and an indicator of the ignition properties and the combustion efficiency ***(6)***.
- The **acid number** is related to the presence of FFAs and the thermal degradation of the fuel. It is the amount of alkali necessary to neutralize the fuel and allows an assessment of the ageing properties ***(96)***.
- The **carbon residue** is correlated to the amount of the contained FFAs, glycerides, soaps, polymers, higher unsaturated fatty acids and inorganic impurities and describes the tendency of depositing carbons ***(96)***.
- The **content of alcohols** is connected to the deterioration of rubber seals and gaskets ***(96)***.
- The **content of mono-, di- and triglycerides** is related to fuel filter plugging ***(96)***.
- The **oxidative stability** is a measure of the tendency of the fuel to deteriorate over time because of oxidative reactions. It is connected to the degree of unsaturation and is important for long term storage ***(96,6)***.

***Table 3-2*** *Selected properties of diesel fuel and different biodiesel fuels. Source:* (**6**).

| fuel | kinematic viscosity (at 40°C) [mm²/s] | density (at 21°C) [g/cm³] | cetane number [-] | flash point [°C] | cloud point [°C] | pour point [°C] |
|---|---|---|---|---|---|---|
| diesel fuel | 2 to 4.5 | 0.82 to 0.86 | 51 | 55 | -18 | -25 |
| soybean methyl ester | 4.08 | 0.884 | 50.9 | 131 | -0.5 | -10.8 |
| rapeseed methyl ester | 4.83 | 0.882 | 52.9 | 155 | -4 | -10.8 |
| sunflower methyl ester | 4.6 | 0.88 | 49 | 183 | 1 | -7 |
| tallow methyl ester | 5 | 0.877 | 58.8 | 150 | 12 | 9 |
| yellow grease methyl ester | 5.16 | 0.873 | 62.6 | n.d. | 9 | 12 |
| soap stock methyl ester | 4.3 | 0.885 | 51.3 | 169 | 6 | n.d. |

The specifications for biodiesel quality in the USA and in Europe are listed in Table 3-3 and Table 3-4.

***Table 3-3*** *Specifications for biodiesel quality in the USA (ASTM D 6751). Source:* (**6**).

| property | unit | limits |
|---|---|---|
| kinematic viscosity (at 40°C) | [$mm^2/s$] | 1.9 to 6 |
| cetane number | [-] | ≥ 47 |
| flash point (closed cup) | [°C] | ≥ 130 |
| water and sediment | [vol%] | ≤ 0.05 |
| sulfated ash | [mass%] | ≤ 0.02 |
| sulfur | [mass%] | ≤ 0.05 |
| copper strip corrosion | [-] | ≤ no. 3 |
| carbon residue (100% sample) | [mass%] | ≤ 0.05 |
| acid number | [mg KOH/g] | ≤ 0.8 |
| free glycerol | [mass%] | ≤ 0.02 |
| total glycerol | [mass%] | ≤ 0.24 |
| phosphorus content | [mass%] | ≤ 0.001 |
| distillation temperature (90% recovered) | [°C] | ≤ 360 |

***Table 3-4*** *Specifications for biodiesel quality in Europe (EN 14214). Source:* (**6**).

| property | unit | limits |
|---|---|---|
| kinematic viscosity (at 40°C) | [$mm^2/s$] | 3.5 to 5 |
| density (at 15°C) | [$kg/m^3$] | 860 to 900 |
| cetane number | [-] | ≥ 51 |
| flash point | [°C] | ≥ 120 |
| water | [mg/kg] | ≤ 500 |
| sulfated ash | [mass%] | ≤ 0.02 |
| sulfur | [mg/kg] | ≤ 10 |
| copper strip corrosion (3h at 50°C) | [-] | no. 1 |
| carbon residue (10% sample) | [mass%] | ≤ 0.3 |
| acid number | [mg KOH/g] | ≤ 0.5 |
| free glycerol | [mass%] | ≤ 0.02 |
| total glycerol | [mass%] | ≤ 0.25 |
| phosphorus content | [mg/kg] | ≤ 10 |
| iodine number | [-] | ≤ 120 |
| oxidative stability (at 110°C) | [h] | ≥ 6 |
| monoglyceride content | [mass%] | ≤ 0.8 |
| diglyceride content | [mass%] | ≤ 0.2 |
| triglyceride content | [mass%] | ≤ 0.2 |

# 4 MATERIALS AND METHODS

## 4.1 CULTIVATION OF STRAINS

For all experiments the strain *Cupriavidus necator DSM 545*, obtained from "Deutsche Sammlung von Mikroorganismen und Zellkulturen", was used. The culture maintenance was done on agar plates of a Küng medium which contained 10 g/L glucose as carbon source (c.f. Table 4-1). Before the start of each experiment, a fresh agar plate was prepared, from which the precultures were inoculated. These consisted of two small shaking flasks containing 150 mL of the respective medium. Additionally one blank was prepared. After breeding of the precultures in the incubation room at a temperature of 37°C, they were checked for mono sepsis, motility and presence of cell debris or inclusions under an Olympus BH-2 microscope, using a magnification of 1:1000. Their optical density (OD) was measured with a Genesys 10S UV-VIS spectrophotometer by Thermo Scientific at a wavelength of 420 nm against deionized water as reference. Depending on the results one preculture was selected for the inoculation of the big shaking flasks.

***Table 4-1*** *Recipes for glucose plates after Küng and for trace element solution „SL6".*

| Küng glucose (plates) | | | |
|---|---|---|---|
| I) | $Na_2HPO_4$ | 3.589 | g/L |
| | $KH_2PO_4$ | 1.5 | g/L |
| | agar-agar | 15 | g/L |
| II) | $(NH_4)_2SO_4$ | 2 | g/L |
| | $MgSO_4.7H_2O$ | 0.2 | g/L |
| III) | $CaCl_2.2H_2O$ | 0.02 | g/L |
| | $NH_4Fe(III)Citrate$ | 0.05 | g/L |
| | SL6 | 1 | mL |
| IV) | glucose | 10 | g/L |

| SL6 | | |
|---|---|---|
| $ZnSO_4*7H_2O$ | 0.1 | g/L |
| $H_3BO_3$ | 0.3 | g/L |
| $CuSO_4$ | 0.006 | g/L |
| $Na_2MoO_4*2H_2O$ | 0.03 | g/L |
| $MnCl_2*2H_2O$ | 0.025 | g/L |

All liquid cultures were prepared based on Küng medium, but with different kinds of carbon sources (Table 4-2) and their pH value was always set to 7.0. The sterilization of the media and the necessary equipment was conducted *via* steam autoclaving. Their breeding was carried out on a shaker (Pilot Shake by Adolf Kühner AG, set to 110 orbits per minute) in a 37°C incubation room. The PHA accumulation phase was induced by nitrogen limitation for all experiments.

***Table 4-2*** *Used recipes for Küng media with different C-sources.*

| Küng glucose | | | |
|---|---|---|---|
| I) | $Na_2HPO_4$ | 3.589 | g/L |
| | $KH_2PO_4$ | 1.5 | g/L |
| II) | $(NH_4)_2SO_4$ | 2 | g/L |
| | $MgSO_4.7H_2O$ | 0.2 | g/L |
| III) | $CaCl_2.2H_2O$ | 0.02 | g/L |
| | $NH_4Fe(III)Citrate$ | 0.05 | g/L |
| | SL6 | 1 | mL |
| IV) | **glucose** | **10** | **g/L** |

| Küng glycerol | | | |
|---|---|---|---|
| I) | $Na_2HPO_4$ | 3.589 | g/L |
| | $KH_2PO_4$ | 1.5 | g/L |
| II) | $(NH_4)_2SO_4$ | 2 | g/L |
| | $MgSO_4.7H_2O$ | 0.2 | g/L |
| III) | $CaCl_2.2H_2O$ | 0.02 | g/L |
| | $NH_4Fe(III)Citrate$ | 0.05 | g/L |
| | SL6 | 1 | mL |
| IV) | **glycerol** | **10** | **g/L** |

| Küng biodiesel | | | |
|---|---|---|---|
| I) | $Na_2HPO_4$ | 4.8 | g/L |
| | $KH_2PO_4$ | 2 | g/L |
| II) | $(NH_4)_2SO_4$ | 3 | g/L |
| | $MgSO_4.7H_2O$ | 0.5 | g/L |
| III) | $CaCl_2.2H_2O$ | 0.02 | g/L |
| | $NH_4Fe(III)Citrate$ | 0.05 | g/L |
| | SL6 | 5 | mL |
| IV) | **biodiesel** | **5** | **g/L** |

# 4.2 ANALYSIS OF SAMPLES

## 4.2.1 MICROBIAL GROWTH

In order to monitor the microbial growth during the experiment the OD of each sample was measured the same way as for the precultures and also the condition of the bacteria was checked *via* microscope.
In addition the substrate was analyzed with the help of several fast tests, to get a fast overview of the actual state of the strain. Depending on the experiment, following colorimetric fast tests were used to determine the pH value, the $NH_4^+$ concentration and the glucose concentration of the medium: Universal indicator paper pH 1-14 by Macherey-Nagel, Merckoquant Ammonium Test 1.10024.0001 by Merck and Diabur-Test 5000 by ACCU-CHECK.

After the experiment the concentration of the cell dry mass (CDM) was determined.
As part of each sampling two weighted glass tubes were filled with 5 mL of medium respectively and centrifuged with a Heraeus SEPATECH Megafuge 1.0R at a speed of 4000 rpm for 10 minutes at 4°C. The supernatant was either discarded or kept for the later ammonia determination. After the closing of the experiment the glass tubes containing the biomass pellets were lyophilizated using a Christ Alpha 1-4 LOC 1-m freeze dryer and afterwards they were weighted again to allow the evaluation of the CDM.

If necessary the development of the ammonia content of the medium was determined using the supernatant of the sample tubes. Normally this was done with an ORION 95-12 ammonia electrode. Only if biodiesel was present in the medium this method was substituted by the photometric Spectroquant Ammonium Test 1.14752.0001 by Merck, because the biodiesel would have damaged the membrane of the electrode.

## 4.2.2 ANALYSIS OF PHA ACCUMULATION

To be able to determine the composition of the PHA content of each sample *via* gas chromatography (GC), it is necessary to convert the polymer into volatile derivatives of its building blocks. This was achieved *via* acidic methanolysis, as described by ***(39)***.
To this end the biomass pellets in the glass tubes were transesterified in presence of chloroform to extract the PHA from the cells and of sulfuric acid which acted as catalyst during methanolysis. In addition the transesterification mixture contained caproic acid as internal standard. After methanolysis sodium bicarbonate was added to the mixture in order to obtain a liquid phase which dissolves impurities and methanol. To achieve a sufficient phase separation the glass tubes were centrifuged at 4000 rpm for 10 minutes at 4°C, afterwards the lower organic phase was transfered into GC vials.
The samples were then analyzed with an Agilent Technologies 6850 series GC system equipped with an Agilent Technologies 6850 series auto sampler, an Agilent Technologies 19091J-433E, 30m x 0.25mm x 0.25 µm capillary column and a flame ionization detector. Helium (purity of 4.6) was used as carrier gas with a total flow rate of 17.1 mL/min, hydrogen (purity of 5.0) and synthetic air (purity: "free of hydrocarbons") were used as detector gases with 35 and 400 mL/min respectively and nitrogen (purity of 5.0) was used as makeup gas with a flow rate of 28 mL/min. The sample volume which was injected was 1 µL, the applied split ratio was 10:1. The initial oven temperature was 50°C which was first increased at a rate of 15°C/min to 60°C, then at a rate of 2°C/min to 80°C. Afterwards the post run was conducted for 5 minutes at a temperature of 300°C.
The evaluation of the GC measurement was carried out with the software GC Chem Station by Agilent Technologies. For the calculations the reported areas of the relevant peaks were taken. In Table 4-3 their retention times for the applied settings are specified.

***Table 4-3*** *GC retention times of different compounds (HX = caproic acid).*

| compound | 3HB | 4HB | HX | 3HV |
|---|---|---|---|---|
| retention time [min] | ~4.4 | ~5.3 | ~5.6 | ~6.5 |

The concentration of PHA was calculated starting from the mean area of caproic acid

$$\overline{HX} = \frac{\sum_1^n HX_i}{n}[pAs], \qquad \textit{Formula 1}$$

which was used to correct the area of the PHA peaks, e.g.

$$3HB_{corr,i} = 3HB_i \frac{\overline{HX}}{HX_i}[pAs], \qquad \textit{Formula 2}$$

thus allowing the calculation of the actual PHA concentration according to

$$3HB_i = 3HB_{corr,i}\, k \left[\frac{g}{L}\right], \qquad \textit{Formula 3}$$

with k being the slope of the standard line.

After determination of the PHA concentrations, the residual biomass ($X_r$) was calculated with

$$X_{r,i} = CDM_i - PHA_i \left[\frac{g}{L}\right]. \qquad \textit{Formula 4}$$

The production rates ($r_i$) and the specific production rates ($q_i$) for CDM, $X_r$, PHA, 3HB, 3HV and 4HB were calculated according to

$$r_{CDM,i} = \frac{CDM_i - CDM_{i-1}}{t_i - t_{i-1}}\left[\frac{g}{L\ h}\right] \qquad \textit{Formula 5}$$

and

$$q_{CDM,i} = \frac{r_{CDM,i}}{\overline{X_{r,i}}}\left[\frac{1}{h}\right]. \qquad \textit{Formula 6}$$

The volumetric production rate of PHA and the specific production rate of PHA were calculated both for the whole fermentation ($V_{prod}$, spec $V_{prod}$) and for accumulation phase ($V_{prod,acc}$, spec $V_{prod,acc}$) using the formulas

$$V_{prod} = \frac{PHA_{end} - PHA_0}{t_{end}}\left[\frac{g}{L\ h}\right] \qquad \textit{Formula 7}$$

and

$$spec\ V_{prod} = \frac{V_{prod}}{\overline{X_r}}\left[\frac{1}{h}\right]. \qquad \textit{Formula 8}$$

The maximum specific growth rate ($\mu_{max}$) was obtained starting from

$$\mu = \frac{1}{X}\frac{dX}{dt} \qquad \textit{Formula 9}$$

which provides the linear equation for exponential growth phase after integration

$$\ln X_r = \mu_{max}\, t + C. \qquad \textit{Formula 10}$$

The yield factors ($Y_{product/substrate}$) were calculated according to

$$Y_{product/substrate} = \frac{Product_{end} - Product_0}{Substrate_{\,added}}\left[\frac{g}{g}\right]. \qquad \textit{Formula 11}$$

# 4.3 BIOREACTOR EQUIPMENT

The bioreactor experiments were carried out in a Labfors 3 fermenter by Infors with a working volume of 5 L (Figure 4-1). It was equipped with an Oxyferm VP 225 $pO_2$ electrode by Hamilton and with an Easyferm plus K8 200 pH electrode by Hamilton.
Each experiment was carried out as fed-batch fermentation and every few hours 50 mL of sample were taken in order to determine the further feeding strategy.

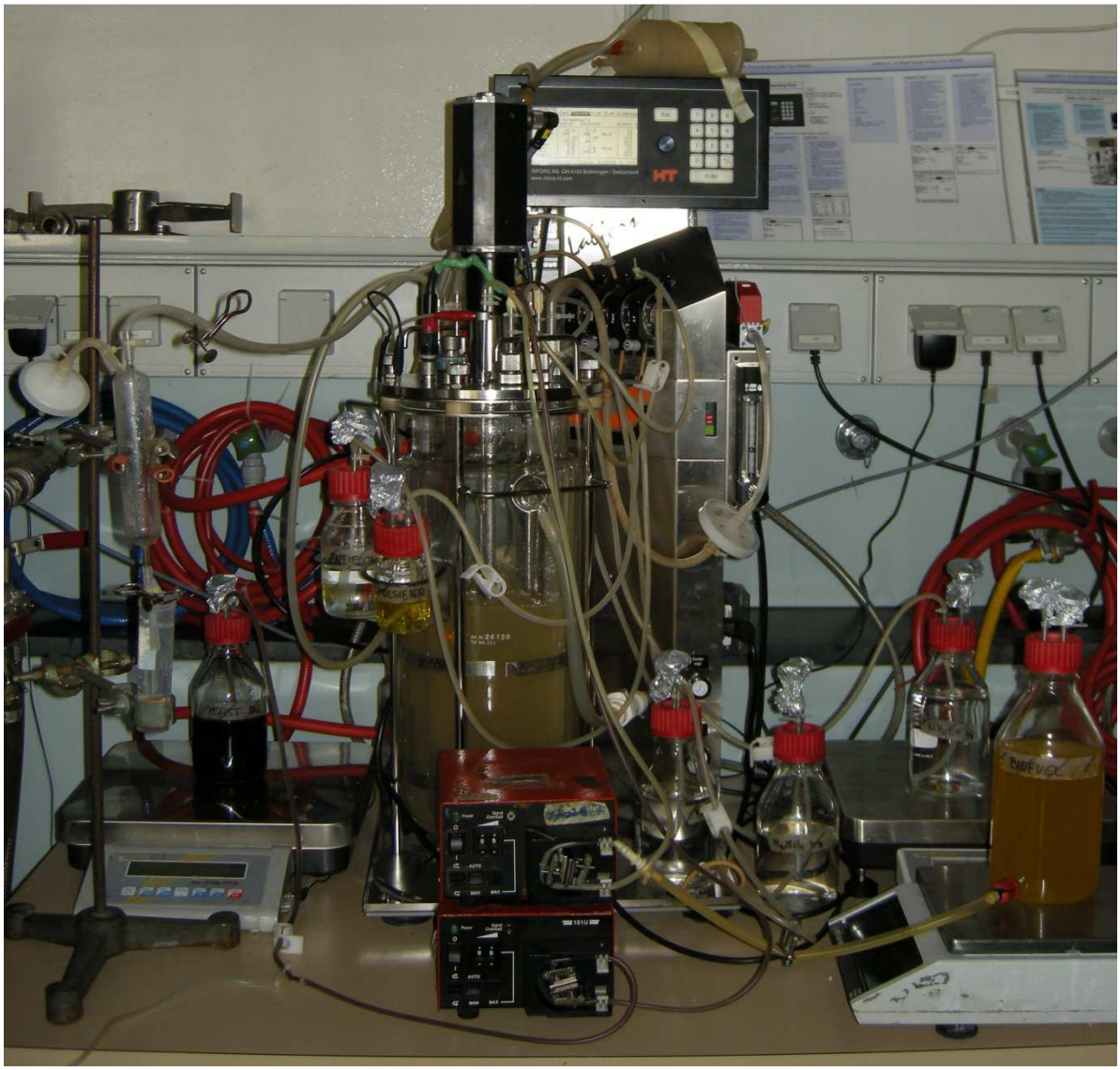

***Figure 4-1*** *Labfors 3 bioreactor during a fermentation.*

During the fermentation temperature, stirrer speed, air flow, $pO_2$ and pH values were constantly recorded *via* the software IRIS NT by Infors. Additionally this program was used in order to keep the pH value and the temperature constant. For adjusting the pH value a 20% $H_2SO_4$ solution, a 25% $NH_4OH$ solution and a 25% NaOH solution were connected to the respective automatic feed pumps of the fermenter. To induce the start of PHA accumulation the $NH_4OH$ solution, which had been used both as base and as nitrogen source during growth phase, was exchanged with the NaOH solution in order to achieve nitrogen limitation. The weights of the bottles containing the precursor, the carbon and the nitrogen sources were noted after each sampling and each manual refeeding.
From the 50 mL of sample two times 5 mL were taken and analyzed in terms of OD, $NH_4^+$, CDM, PHA content etc., as described above.
To get exact values of the concentration of carbon source and precursor soon after sampling, for some experiments the substrate was additionally analyzed with a high pressure liquid chromatograph (HPLC).

To this end a Shimadzu prominence system was used, consisting of a DGU-20$A_3$ degasser, an LC-20AD solvent delivery module, an SIL-20AC auto sampler, a CTO-20AC column oven, an RID-10A refractive index detector (RID) and an Aminex HPX-87C carbohydrate column with 300 mm x 7.8 mm. As eluent water was used with a flow rate of 0.6 mL per minute. The injection volume was 10 µL, the temperature of the column oven was set to 75°C and the temperature of the detector cell was 45°C. The evaluation of the HPLC mearurement was done with the Lab Solutions LCSolution Software by Shimadzu, the retention times of the relevant peaks are shown in Table 4-4.

***Table 4-4 HPLC retention times for different compounds.***

| compound | γ-butyrolactone | glycerol | glucose |
|---|---|---|---|
| retention time [min] | ~21 | ~15 | ~9 |

# 4.4 METHODS OF PHA ISOLATION

For analysis of the properties of the obtained polymer the biomass was centrifuged with a Du Pont Sorvall RC-5B centrifuge using a PTI FAs 10c rotor at a speed of 5000 rpm for 15 minutes at 4°C. After lyophilization the pellets were homogenized and the fivefold amount of ethanol was added to the obtained powder. This mixture was stirred for 24 hours to remove as much lipids from the biomass as possible. If needed this step was repeated a second time. Afterwards the degreased biomass was filtrated and air dried. In the next step the PHA was extracted from the cells both *via* batch extraction and *via* soxhlet extraction in parallel.

- **Batch extraction**
  First enough chloroform to dissolve the polymer was added to the dried biomass (approximately the same amount as that of ethanol used for degreasing). This mixture was protected from light by covering the flask with aluminum foil and it was put on a magnetic stirrer over night.
- **Soxhlet extraction**
  Again chloroform was added to the biomass to dissolve the polymer, but instead of a flask and a magnetic stirrer a soxhlet apparatus was used over night, thus allowing several extraction cycles, instead of only one.

The next day the obtained solution was filtrated to remove the residual biomass. In a parallel setup this was also achieved, then twice as much cooled ethanol as chloroform had been used was added to the filtrate to precipitate the polymer. This mixture was stirred again over night, but this time at a temperature of 4°C, then it was filtrated.
The obtained pure polymer was dried and sent to the Centre of Polymer and Carbon Materials in Zabrze where it was analyzed *via* gel permeation chromatography (GPC), nuclear magnetic resonance ($^{1}$H and $^{13}$C) (NMR), elemental analysis (EA), thermogravimetric analysis (TGA) and multistage mass spectrometry (ESI-MS).

# 5 SHAKING FLASK EXPERIMENTS

## 5.1 SCREENING OF EMULSIFIERS

### 5.1.1 AIM OF THE EXPERIMENT

Since biodiesel is water insoluble, the application of an emulsifier is essential in order to achieve a satisfying distribution of the carbon source within the medium which is a prerequisite for optimal microbial growth by avoiding carbon limitation. Thus in a first step of developing a fermentation strategy which aims at using biodiesel as sole carbon source, eleven different emulsifiers were screened for their growth promoting effects in a shaking flask experiment.
In addition two different biodiesel types were tested for their suitability as carbon source. One was the saturated FAME fraction directly obtained from project partner Argent Energy which already had been distilled once. The other one was basically the same, but had been distilled for a second time by project partner University of Graz (KFU), thus removing impurities which might inhibit microbial growth (Figure 5-1).

***Figure 5-1*** *Biodiesel from Argent (on the left) and biodiesel from KFU (on the right).*

### 5.1.2 EXPERIMENTAL SETUP

The experiment was conducted in big shaking flasks containing 200 mL of Küng medium with 3 g/L of biodiesel as sole carbon source and one of eleven different emulsifiers from several manufacturers. In addition two flasks without emulsifier were prepared as a reference for both examined biodiesel types. The preculture was grown in a Küng glucose medium. After 21 hours the experiment was ended.
The samples that were taken were only used for determination of the actual OD in order to monitor the growth. No other analyses were conducted.

### 5.1.3 RESULTS AND DISCUSSION

The obtained OD values are shown in Figure 5-2 and Figure 5-3. It is important to mention that the presence of biodiesel in the medium definitely had an effect on the OD because of its different light scattering properties. Thus the obtained values are not completely accurate, but still reliable enough to show a trend of the occurring microbial growth which allows the evaluation of the different biodiesel and emulsifiers.

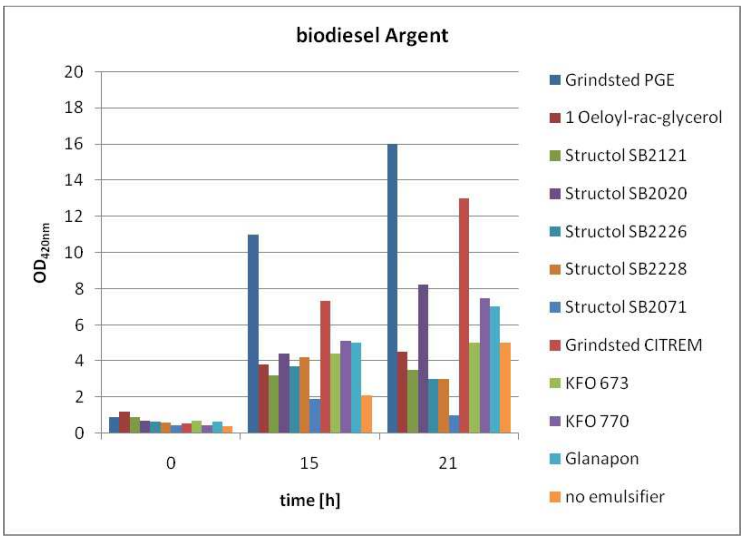


***Figure 5-2*** *Effect of different emulsifiers on the growth of DSM 545, using single distilled biodiesel from Argent Energy as carbon source.*

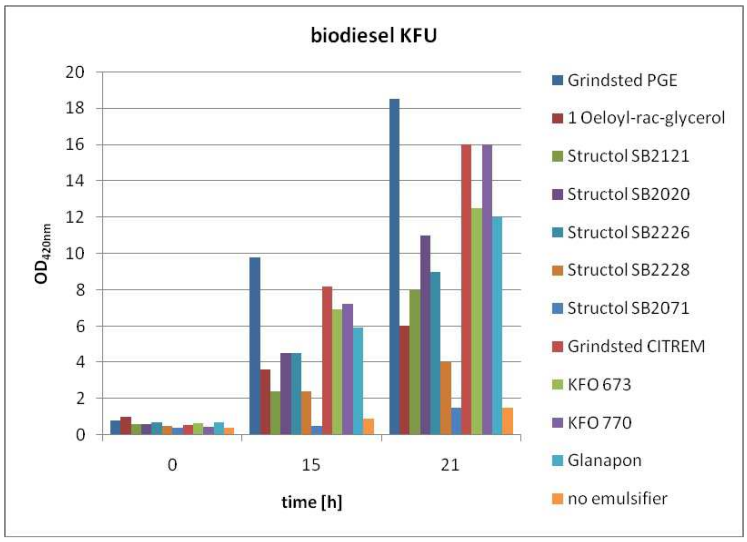


***Figure 5-3*** *Effect of different emulsifiers on the growth of DSM545, using double distilled biodiesel from University of Graz as carbon source.*

The cultures grown on twice distilled biodiesel achieved higher optical densities after 21 hours (average OD of 9.7) than those grown on one time distilled biodiesel (average OD of 6.4). In addition the emulsifier Grindsted PGE was shown to be the most effective for both substrates.

The reference flasks, which were prepared without any emulsifier, only showed reduced microbial growth, especially those containing double distilled biodiesel. This may be due to the fact that some of the impurities which were removed during the second distillation step had emulsifying properties. But still the flask containing biodiesel from Argent Energy without additional emulsifier did not even reach the average OD at the end of the experiment.

Based on these findings Grindsted PGE O 18 was used as emulsifier and double distilled biodiesel from University of Graz as carbon source for all following biodiesel experiments.

# 5.2 EFFECTS OF MARGARIC ACID ON MICROBIAL GROWTH

## 5.2.1 AIM OF THE EXPERIMENT

This experiment was conducted in order to determine the toxic influence of the novel 3HV precursor margaric acid on microbial growth on the one hand and its effect on PHA accumulation during growth phase on the other hand. In addition it was tried to gain a first insight concerning the applicability of CGP and the saturated FAME fraction from biodiesel production as carbon sources compared to the frequently used carbon source glucose.

## 5.2.2 EXPERIMENTAL SETUP

Three different Küng media, one containing 10 g/L glucose, the other 10 g/L CGP and the third 2g/L biodiesel as carbon sources, were prepared. 24 big shaking flasks were filled with 250 mL of one of the media and a different amount of margaric acid (0.2 g/L, 0.8 g/L, 1.4 g/L and 2.0 g/L) was added to each of the flasks. After 36 hours the experiment was terminated.
The growth of the strains was controlled after each sampling *via* OD measurement. After the end of the experiment the CDM of the samples was evaluated and they were transesterified for GC analysis in order to determine their 3HB and 3HV contents.

## 5.2.3 RESULTS AND DISCUSSION

All shaking flasks containing CGP as carbon source showed a decrease in biomass concentration in the course of the experiment, while accumulating insignificant amounts of PHB without any 3HV units being detected (data not shown; obtained maximum values and yields are listed in Table 5-1). Based on these results it can be concluded that the unrefined CGP contains toxic compounds which inhibit microbial growth. In order to allow its use as carbon source for fermentation, some kind of purification would be needed.
The fermentation patterns of the different glucose flasks show a very similar course, independent from the added amount of margaric acid. The same is true for the flasks which contained biodiesel as carbon source (Figure 5-4). Thus no toxic influence on microbial growth of margaric acid in a concentration ranging from 0.2 to 2.0 g/L was observed.

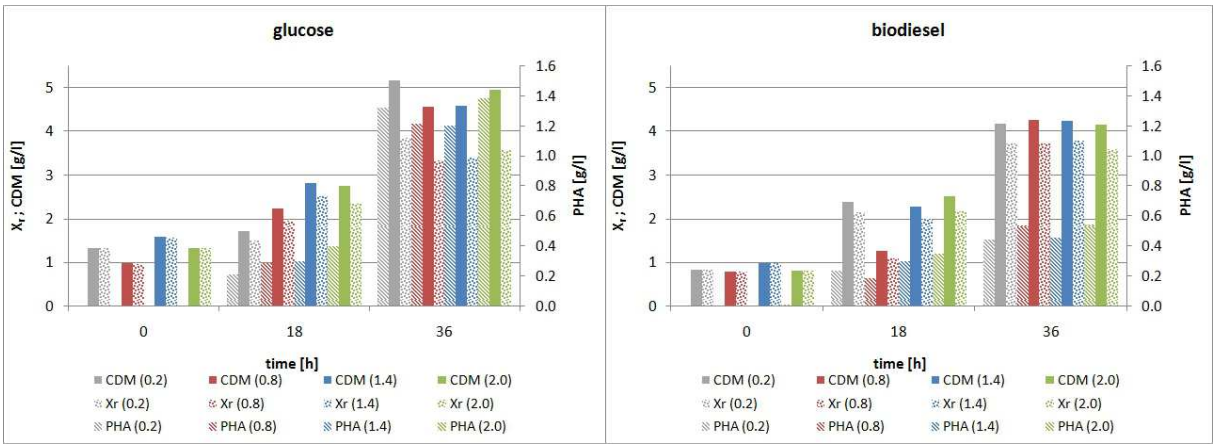


***Figure 5-4*** *Effects of margaric acid on microbial growth. Comparison of fermentation patterns for 10 g/L glucose and 2 g/L biodiesel.The values in brackets indicate the respective concentration of margaric acid.*

The direct comparison of the biodiesel and the glucose flasks in terms of achieved concentrations of cell dry mass, residual biomass and PHA show comparable results. Only the PHA concentration after 36 hours shows a significant difference. By then the biodiesel cultures solely had accumulated about half the amount of polymer compared to the glucose cultures (Figure 5-4). But a closer look at the composition of the produced PHA reveals the fact that the cultures grown on biodiesel produced definitely more 3HV building blocks than those grown on glucose, even if the outliers at a concentration of 0.8 g/L are excluded (Figure 5-5). The yield of 3HV per margaric acid is 3.5 times higher for biodiesel than for glucose. Additionally the achieved maximum yield of PHA per biodiesel even exceeds that of PHA per glucose by a factor 2 which is also due to the fact that the initial concentration of glucose was five times higher than that of biodiesel (Table 5-1).

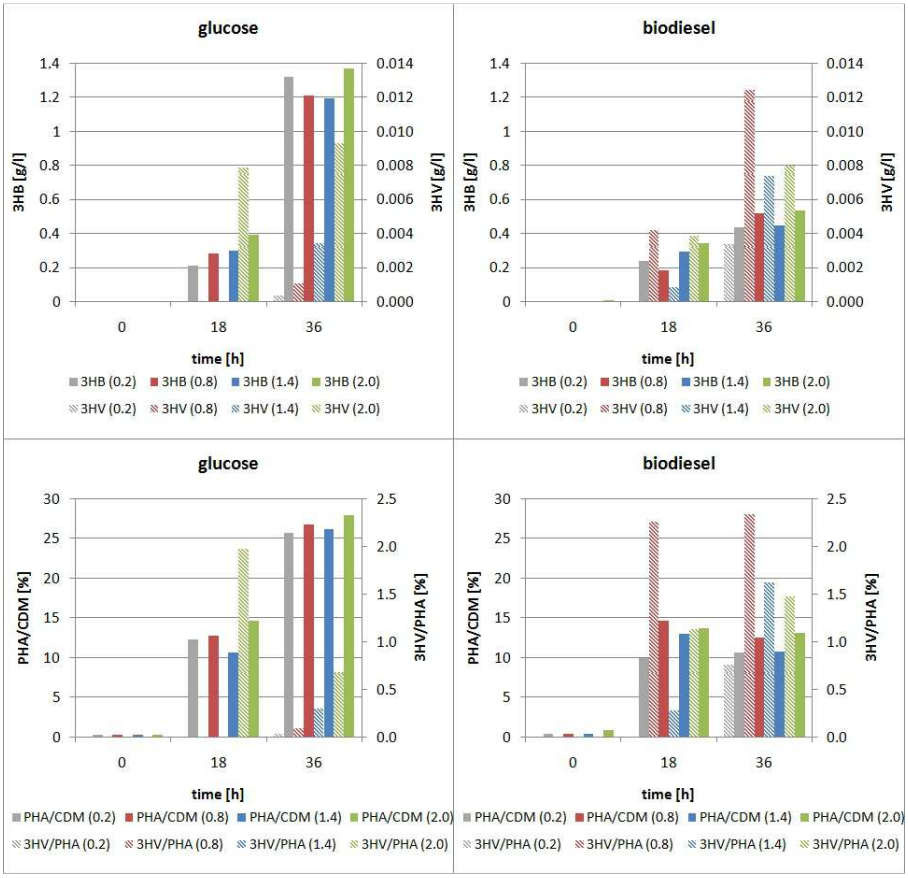


***Figure 5-5*** *Effects of margaric acid on microbial growth. Comparison of PHA production for 10 g/L glucose and 2 g/L biodiesel.The values in brackets indicate the respective concentration of margaric acid.*

***Table 5-1*** *Effects of margaric acid on microbial growth. Comparison of maximum values and yields for 10 g/L glucose, 2 g/L biodiesel and 10 g/L CGP (MA = margaric acid).*

| | | glucose | | | biodiesel | | | CGP | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | max. value | $c_{MA}$ [g/L] | time [h] | max. value | $c_{MA}$ [g/L] | time [h] | max. value | $c_{MA}$ [g/L] | time [h] |
| **CDM** | **[g/L]** | 5.2 | 0.2 | 36 | 4.2 | 0.8 | 36 | 1.4 | 2.0 | 18.0 |
| **$X_r$** | **[g/L]** | 3.8 | 0.2 | 36 | 3.8 | 1.4 | 36 | 1.4 | 2.0 | 18.0 |
| **PHA** | **[g/L]** | 1.4 | 2.0 | 36 | 0.5 | 2.0 | 36 | 0.0 | 0.2 | 36.0 |
| **PHA/CDM** | **[%]** | 27.9 | 2.0 | 36 | 14.6 | 0.8 | 18 | 1.8 | 0.8 | 36.0 |
| **3HV/PHA** | **[%]** | 2.0 | 2.0 | 18 | 2.3 | 0.8 | 36 | 0 | - | - |
| **$Y_{CDM/C\text{-}source}$** | **[g/g]** | 0.4 | 0.2 | - | 1.7 | 0.8 | - | -0.1 | 0.2 | - |
| **$Y_{PHA/C\text{-}source}$** | **[g/g]** | 0.14 | 2.0 | - | 0.27 | 2.0 | - | 0.001 | 0.8 | - |
| **$Y_{3HV/MA}$** | **[g/g]** | 0.005 | 2.0 | - | 0.017 | 0.2 | - | 0 | - | - |
| **$V_{prod}$** | **[g/Lh]** | 0.038 | 2.0 | - | 0.015 | 2.0 | - | 0.0002 | 0.8 | - |

# 5.3 EFFECTS OF MARGARIC ACID DURING ACCUMULATION PHASE

## 5.3.1 AIM OF THE EXPERIMENT

Since during growth phase no toxic influence of margaric acid had been found, its positive impact on the building of 3HV units during accumulation phase was investigated in a next step. To obtain as significant results as possible for the intended adaption to reactor scale, different carbon sources were compared. Thus the saturated FAME fraction as well as glycerol and glucose were used as substrates. Because of the not very promising results of the first margaric acid shaking flask experiment regarding the applicability of CGP as carbon source, pure p.a. glycerol was used instead, to investigate the general impact of glycerol on PHA accumulation in comparison to glucose and biodiesel (Figure 5-6).

***Figure 5-6*** *CGP from Argent (on the left) and p.a. glycerol (on the right).*

## 5.3.2 EXPERIMENTAL SETUP

In order to let the accumulation phase start at the same time for all shaking flasks, two different media were prepared. One was used for growth phase and contained 10 g/L glucose as carbon source. The other one was prepared like a normal Küng medium with 10 g/L glucose, 10 g/L glycerol or 3 g/L biodiesel as carbon sources, but without adding the nitrogen source ammonium sulfate. Instead to half of the flasks containing accumulation medium, 2 g/L of margaric acid were added, the others were used as references.
24 hours after the inoculation of 12 big shaking flasks containing 250 mL of growth medium, the cultures were already well grown and the contents of each flask were filled in a centrifuge beaker under sterile conditions. To balance their weights a 0.9% solution of sodium chloride was used, then they were centrifuged for 10 minutes at a speed of 5000 rpm at 4°C. The supernatant was discarded and 100 mL of the sodium chloride solution were added to the pellet. This mixture was centrifuged again for 10 minutes at 5000 rpm and 4°C to remove residues of the growth medium from the pellet. After discarding the supernatant each type of prepared accumulation medium was filled into one centrifugation beaker which was shaken until the pellet was dissolved. Then the first sample was taken. After 16.5 hours the cultures were refed with 4 g/L glucose, 4 g/L CGP or 1.2 g/L biodiesel, after 40 hours the experiment was ended.

## 5.3.3 RESULTS AND DISCUSSION

On the whole the concentration of the residual biomass of each flask was constant during the experiment which was to be expected since the absence of nitrogen inhibited the production of biomass. Only for glucose a decrease of $X_r$ could be observed after 22 hours. This was also associated with a decline of the PHA concentration and marks the time when glucose was depleted and the culture started to degrade the accumulated polymer. Nevertheless these cultures had produced the highest amount of PHA at the end of the experiment (Figure 5-7).
Generally the flasks containing pure glycerol showed promising results. Only in terms of 3HV accumulation they could not compete with the others, although their PHA composition was similar to that of the glucose flasks (Figure 5-7).

The biodiesel cultures utilized the carbon source only hesitantly and did not accumulate as much polymer as those grown on glucose or glycerol. Thus the producing strain needed to be adapted much better to biodiesel. Still they showed the best results by far in terms of generating 3HV building blocks, although after 22 hours a drop in the 3HV concentration was observed. Apart from that the cultures grown on biodiesel were the only ones which accumulated 3HV without the presence of margaric acid. This is due to the fact that this carbon source contains several odd numbered FAMEs which act as precursor (Figure 5-7).

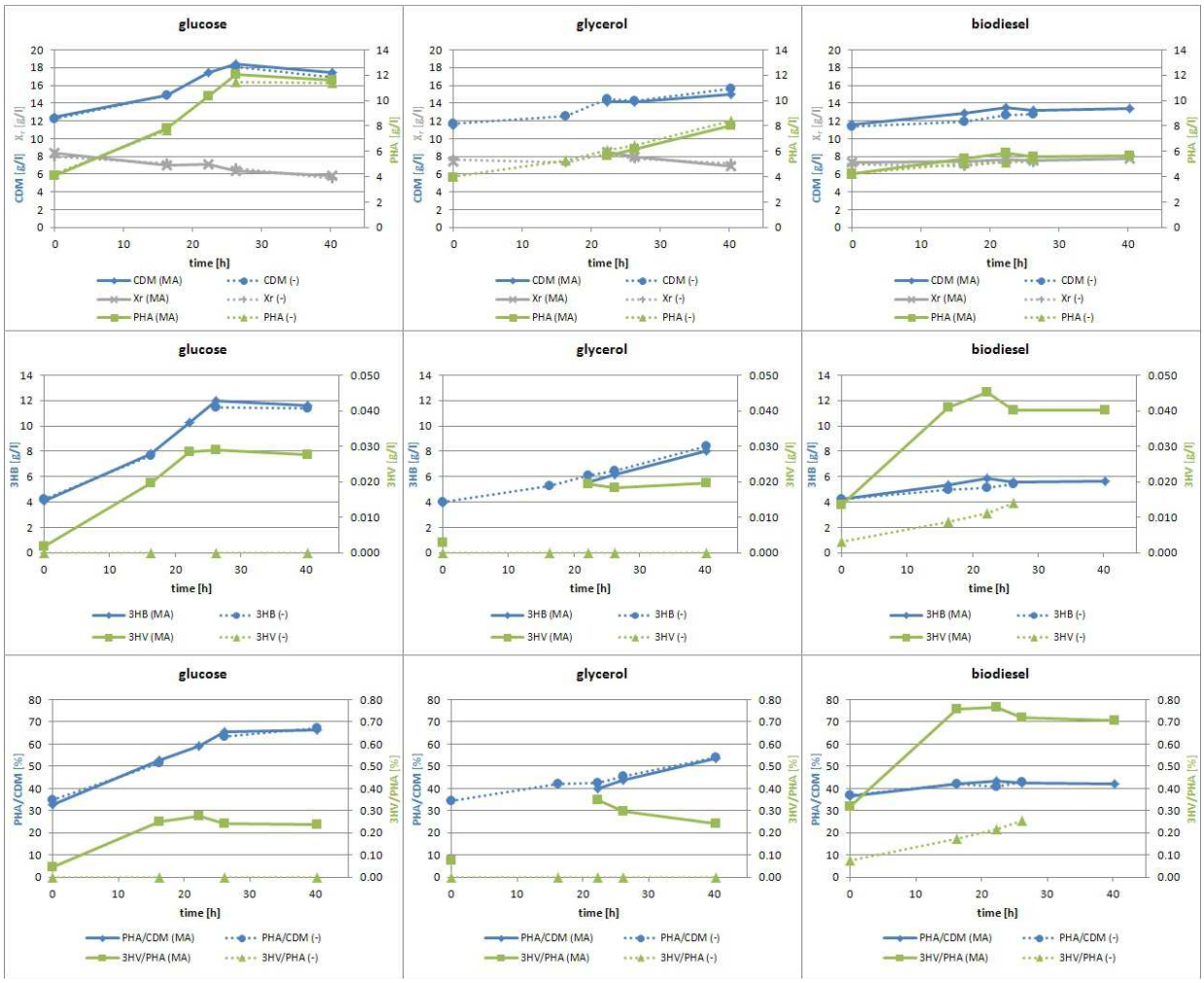


***Figure 5-7*** *Effects of margaric acid on growth phase. Comparison of fermentation patterns and PHA accumulation for 10 g/L glucose, 10 g/L CGP and 3 g/L biodiesel: (MA): flasks containing 2 g/L margaric acid; (-): flasks containing no margaric acid.*

The achieved yields of 3HV per margaric acid concentration are within the same range for all three substrates, including biodiesel. But if the outlier of the 3HV concentration of the biodiesel cultures at the beginning of the experiment is corrected the resulting $Y_{3HV/MA}$ doubles (Table 5-**2**).
The PHA per carbon source yields of biodiesel are in between those of glycerol and glucose. It is important to state that the glucose cultures, in contrast to the other two carbon sources, had already reached carbon limitation by the end of the experiment and that only biodiesel was used in a concentration of 3 g/L. Thus the yields of glucose in reality were slightly higher than shown in Table 5-**2** and the yields of glycerol compared to those of biodiesel supposedly would have achieved better results if both would have reached carbon limitation.

***Table 5-2** Effects of margaric acid during growth phase. Comparison of maximum values and yields for10 g/L glucose, 10g/L CGP and 3 g/L biodiesel (MA = margaric acid).*

| | | | glucose | | biodiesel | | glycerol | |
|---|---|---|---|---|---|---|---|---|
| | | | maximum value | time [h] | maximum value | time [h] | maximum value | time [h] |
| with margaric acid | CDM | [g/L] | 18.38 | 26 | 13.54 | 22 | 14.97 | 40 |
| | $X_r$ | [g/L] | 8.39 | 0 | 7.77 | 40 | 8.49 | 22 |
| | PHA | [g/L] | 12.04 | 26 | 5.89 | 22 | 8.05 | 40 |
| | PHA/CDM | [%] | 66.40 | 40 | 43.48 | 22 | 53.78 | 40 |
| | 3HV/PHA | [%] | 0.27 | 22 | 0.77 | 22 | 0.35 | 22 |
| | $Y_{CDM/C\text{-}source}$ | [g/g] | 0.36 | - | 0.60 | - | 0.25 | - |
| | $Y_{PHA/C\text{-}source}$ | [g/g] | 0.54 | - | 0.47 | - | 0.29 | - |
| | $Y_{3HV/MA}$ | [g/g] | 0.01 | - | 0.01 | - | 0.01 | - |
| | $V_{prod}$ | [g/Lh] | 0.19 | - | 0.03 | - | 0.10 | - |
| without margaric acid | CDM | [g/L] | 18.10 | 26 | 12.72 | 26 | 15.59 | 40 |
| | $X_r$ | [g/L] | 7.98 | 0 | 7.46 | 22 | 8.29 | 22 |
| | PHA | [g/L] | 11.45 | 26 | 5.43 | 26 | 8.42 | 40 |
| | PHA/CDM | [%] | 67.26 | 40 | 42.71 | 26 | 54.03 | 40 |
| | 3HV/PHA | [%] | 0.00 | - | 0.26 | - | 0.00 | - |
| | $Y_{CDM/C\text{-}source}$ | [g/g] | 0.34 | - | 0.45 | - | 0.28 | - |
| | $Y_{PHA/C\text{-}source}$ | [g/g] | 0.51 | - | 0.41 | - | 0.31 | - |
| | $Y_{3HV/MA}$ | [g/g] | - | - | - | - | - | - |
| | $V_{prod}$ | [g/Lh] | 0.18 | - | 0.05 | - | 0.11 | - |

# 6 BIOREACTOR EXPERIMENTS

## 6.1 GLUCOSE FERMENTATION

### *6.1.1 AIM OF THE EXPERIMENT*

This experiment was conducted in order to determine the optimal adjustments of fermentation parameters like temperature, pH value and speed of stirrer when working with *Cupriavidus necator DSM 545* in a Labfors 3 fermenter. For this reason glucose was chosen as carbon source since the working group already had much experiences in using it as substrate for this strain. Additionally the project partners in Pisa, Zabrze and Ljubljana needed samples of the copolymer P(3HB-co-4HB) to analyze them for their properties. Thus the 4HB precursor γ-butyrolactone was added during accumulation phase.

### *6.1.2 EXPERIMENTAL SETUP*

After autoclaving the bioreactor was filled with 4 L of Küng glucose medium, but its components had been calculated for 5 L. This was the total content after adding the inoculum which was started in six big shaking flasks containing 250 mL of Küng glucose medium each. The glucose concentration of all prepared media was 20 g/L. After 24 hours the growth of the inoculum flasks was checked *via* spectrophotometer and microscope. Based on these results four of them were chosen to inoculate the bioreactor and directly afterwards the first sample was taken.
The temperature was set to 37°C, the pH value to 7.0 and the initial stirrer speed was 200 rpm. The latter was increased during the fermentation depending on the $pO_2$ which was tried to be kept below a value of 50%.
After 14 hours, when an OD of 100 had been reached, the refeeding of the nitrogen source was stopped to induce accumulation phase, which was reached 2.5 hours later. At the same time the manual feeding of the 4HB precursor γ-butyrolactone was started.
After each sampling the actual glucose and γ-butyrolactone concentrations were determined *via* HPLC to allow an adequate addition of these substrates. As glucose refeed a 50% solution was used, γ-butyrolactone was added in its pure form to maintain a precursor concentration of 2 to 3 g/L, the actually added amounts are shown in Table 6-1.
After 24 hours an OD of 300 had been reached with a final stirrer speed of 850 rpm and the fermentation was ended.

***Table 6-1*** *Additions of glucose and γ-butyrolactone during the fermentation.*

| time [h] | glucose [g/L] | γ-butyrolactone [g/L] |
|---|---|---|
| 8.2 | 14.1 | 0.0 |
| 14.0 | 10.1 | 0.0 |
| 14.7 | 10.0 | 0.0 |
| 16.3 | 0.0 | 3.2 |
| 16.7 | 10.0 | 0.0 |
| 18.2 | 9.9 | 0.0 |
| 20.1 | 9.1 | 1.1 |
| 22.3 | 10.8 | 1.3 |
| 22.9 | 11.2 | 0.0 |

## 6.1.3 RESULTS AND DISCUSSION

In Table **6-2** the main results of the fermentation are summarized.

***Table** 6-2 Maximum values, volumetric productivities and yield factors of the glucose fermentation.*

| | value | time |
|---|---|---|
| $CDM_{max}$ [g/L] | 35.69 | 24 h |
| $X_{r,max}$ [g/L] | 12.35 | 17 h |
| $PHA_{max}$ [g/L] | 25.84 | 24 h |
| $3HB_{max}$ [g/L] | 25.83 | 24 h |
| $4HB_{max}$ [g/L] | 0.006 | 24 h |
| $PHA/CDM_{max}$ [%] | 72.39 | 24 h |
| $4HB/PHA_{max}$ [%] | 0.023 | 24 h |
| $\mu_{max}$ [1/h] | 0.16 | - |

| | value |
|---|---|
| $V_{prod}$ [g/Lh] | 1.07 |
| $V_{prod,acc}$ [g/Lh] | 2.44 |
| spec $V_{prod}$ [1/h] | 0.13 |
| spec $V_{prod,acc}$ [1/h] | 0.15 |
| $Y_{CDM/GLY}$ [g/g] | 0.31 |
| $Y_{PHA/GLY}$ [g/g] | 0.24 |
| $Y_{4HB/y\text{-butyrolactone}}$ [g/g] | 0.001 |
| $Y_{Xr/ammonia\ sulfate}$ [g/g] | 0.56 |

After the induction of accumulation phase the concentration of ammonium sulfate decreased rapidly and at the same time the graph of residual biomass concentration became constant with a value of 11.6 g/L. This was to be expected since once a culture is limited in nitrogen, the carbon flux is redirected towards PHA accumulation. Thus also the graph of PHA concentration was affected, starting to increase linearly with a volumetric productivity of 2.44 g/Lh until the end of the fermentation. This shows that the strain had still been actively accumulating PHA and it would have been possible to achieve higher maximum values and yields if the experiment would have been carried on (Figure 6-1).

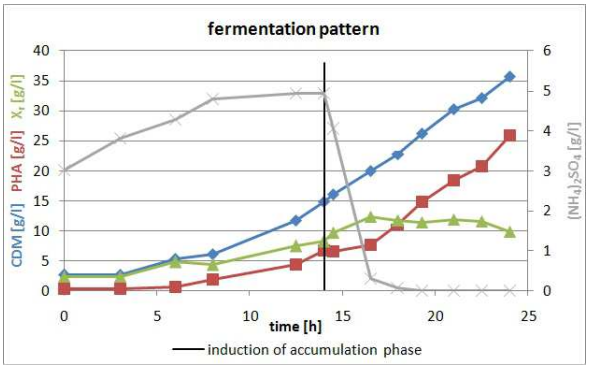


***Figure 6-1** Fermentation pattern of the glucose fermentation.*

The formation rates of residual biomass indicate that, after the biomass generation had already stopped entirely after 17 hours, the strain became soon limitated in carbon. Thus a drop of 3HB formation rates together with an increase of biomass production can be observed for this time (Figure 6-2).

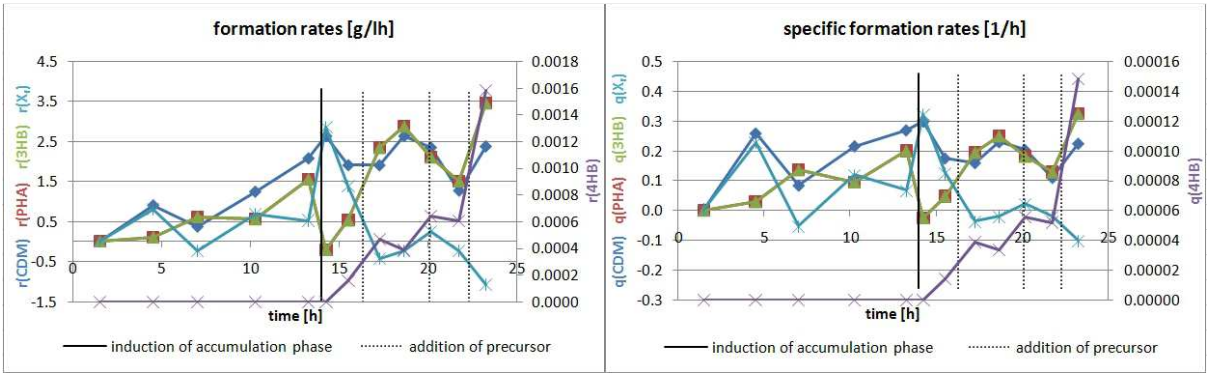


***Figure 6-2** Formation rates and specific formation rates of the glucose fermentation.*

This is also confirmed by the diagram showing the utilization of substrates (Figure 6-4) which was calculated from the data obtained by HPLC measurement. It also demonstrates that after 20 hours the carbon limitation was resolved which also matches the course of the formation rates of PHA and $X_r$. Additionally Figure 6-2 shows that after each precursor addition the 4HB formation rate significantly increased.
Figure 6-3 indicates that already after 6 hours the PHA synthesis started to rise.

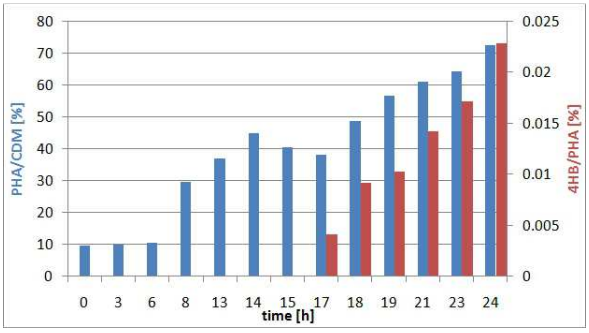


***Figure 6-3*** *PHA/CDM and 4HB/PHA shares of the glucose fermentation.*

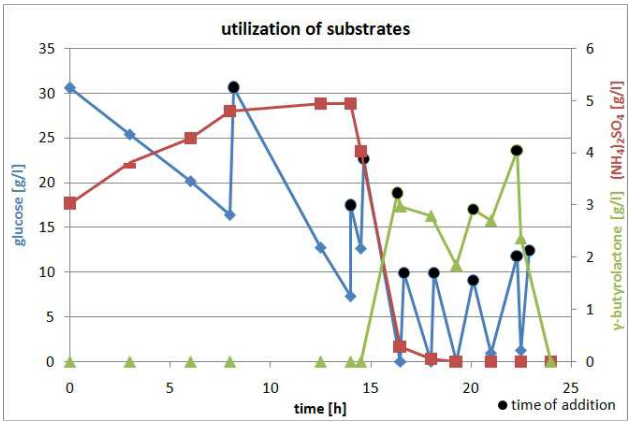


***Figure 6-4*** *Addition and utilization of substrates during glucose fermentation.*

# 6.2 GLYCEROL FERMENTATION

## *6.2.1 AIM OF THE EXPERIMENT*

After the results of the shaking flask experiments utilizing pure glycerol as carbon source, this fermentation was planned in order to see if the strain *C. necator DSM 545* would also use this substrate on reactor scale. Additionally the project partners in Croatia needed kinetic data for mathematical modeling, thus only p.a. glycerol could be utilized to produce pure PHB. This homopolymer was expected to show comparatively low molecular masses, since glycerol is known to cause the termination of the chain propagation reaction ***(****101****)***.

## *6.2.2 EXPERIMENTAL SETUP*

The fermenter was filled with 4 L of Küng medium containing 10 g/L glycerol as sole carbon source and calculated for a total volume of 5 L. The inoculum consisted of four out of six prepared shaking flasks, containing 250 mL of Küng glycerol medium each. They had been inoculated from a glucose preculture two days before.

After 19 hours an OD of 135 had been accomplished and nitrogen limitation was induced by exchanging the base ammonium sulfate with sodium hydrogen. Four hours later the start of accumulation phase had been reached.
Since the pH value of the culture did not shift to an acidic milieu, as observed in the glucose fermentation, the nitrogen source ammonium sulfate had to be added manually twice during growth phase. Apart from that only glycerol was refed after determining the actual concentration in the bioreactor *via* HPLC. The added amounts are shown in Table 6-3.
30 hours after the start of the fermentation an OD of 330 had been reached and the experiment was ended.

***Table 6-3*** *Additions of ammonium sulfate and glycerol during the fermentation.*

| time | $(NH_4)_2SO_4$ | glycerol |
|---|---|---|
| [h] | [g/L] | [g/L] |
| 8.8 | 0.0 | 5.1 |
| 13.0 | 0.8 | 0.0 |
| 13.3 | 0.0 | 4.2 |
| 17.2 | 0.0 | 3.2 |
| 19.0 | 1.9 | 0.0 |
| 19.1 | 0.0 | 9.6 |
| 20.5 | 0.0 | 6.0 |
| 21.8 | 0.0 | 12.9 |
| 23.6 | 0.0 | 10.9 |
| 25.2 | 0.0 | 11.1 |

## 6.2.3 RESULTS AND DISCUSSION

The volumetric productivities of PHA, the yield factors and the maximum values which had been achieved are listed in Table 6-4.

***Table 6-4*** *Maximum values, volumetric productivities and yield factors of the glycerol fermentation.*

| | value | time |
|---|---|---|
| $CDM_{max}$ [g/L] | 45.28 | 30 h |
| $X_{r,max}$ [g/L] | 17.68 | 24 h |
| $PHA_{max}$ [g/L] | 29.45 | 30 h |
| $PHA/CDM_{max}$ [%] | 65.04 | 24 h |
| $\mu_{max}$ [1/h] | 0.11 | |

| | |
|---|---|
| $V_{prod}$ [g/Lh] | 0.98 |
| $V_{prod,acc}$ [g/Lh] | 2.80 |
| spec $V_{prod}$ [1/h] | 0.09 |
| spec $V_{prod,acc}$ [1/h] | 0.17 |
| $Y_{CDM/GLY}$ [g/g] | 0.60 |
| $Y_{PHA/GLY}$ [g/g] | 0.40 |
| $Y_{Xr/ammonia\ sulfate}$ [g/g] | 2.90 |

Figure 6-5 shows fluctuations of the ammonium sulfate graph during growth phase which is due to the manual addition. After induction of accumulation phase it decreases as usual. This is connected to the residual biomass concentration reaching the constant value of approximately 16.5 g/L and the linear increase of PHA showing a volumetric productivity of 2.8 g/Lh. Again the fermentation was stopped before stationary phase had been reached (Figure 6-5).

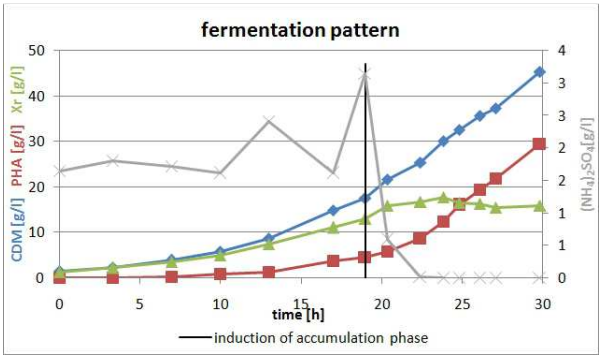


***Figure 6-5*** *Fermentation pattern of the glycerol fermentation.*

The formation rates of PHA increased steadily after accumulation phase had been induced, while those of $X_r$ were declining until after 24 hours no biomass production was detected. Less than one hour later the carbon source was almost limited (Figure 6-7) and the PHA formation rates started to decrease for some time (Figure 6-6).

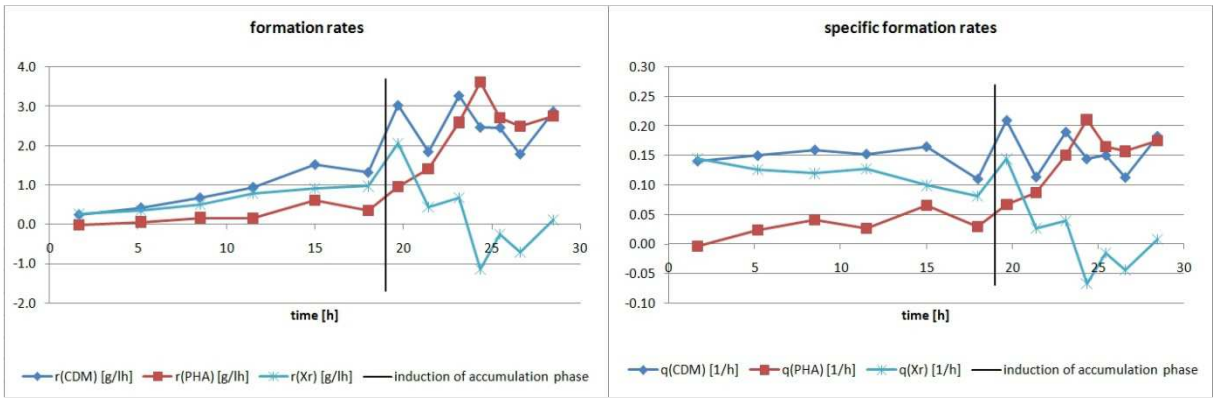


***Figure 6-6*** *Formation rates and specific formation rates of the glycerol fermentation.*

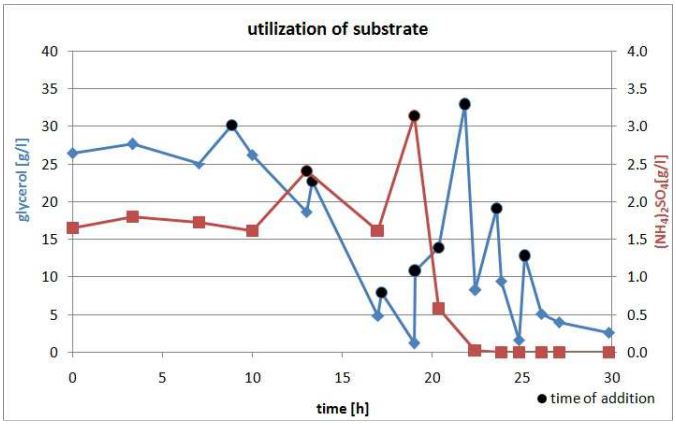


***Figure 6-7*** *Addition and utilization of substrate during glycerol fermentation.*

Until nitrogen limitation was reached the amount of PHA per CDM remained constant in general, although after 10 and after 17 hours jumps were detected. Afterwards the ratio started to increase because of the predominant PHA accumulation (Figure 6-8).

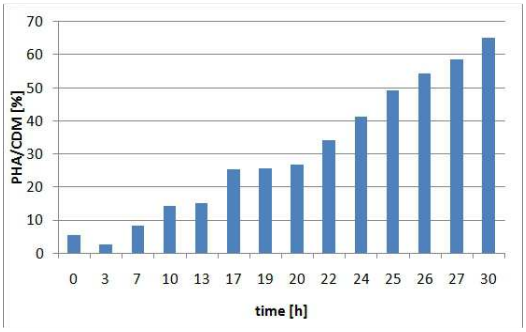


***Figure 6-8*** *PHA/CDM shares of the glycerol fermentation.*

# 6.3 BIODIESEL FERMENTATION

## 6.3.1 AIM OF THE EXPERIMENT

The aim of this experiment was to establish a fermentation which utilizes solely the saturated FAME fraction as carbon source and margaric acid, which is contained in animal lipids, as 3HV precursor. The achievement of this objective constitutes an important step for the project ANIMPOL.

## 6.3.2 EXPERIMENTAL SETUP

Before the experiment could be conducted, first the strain *C. necator DSM 545* had to be adapted to biodiesel as sole carbon source. This was achieved by growing two cultures in parallel in small shaking flasks containing 150 mL of Küng medium with 3 g/L biodiesel. Once they were well grown the more active looking culture was transferred to two new shaking flasks. This procedure was repeated several times while gradually increasing the biodiesel concentration until even 5 g/L were well tolerated by the strain.
For the fermentation the bioreactor was filled with 2.5 L of Küng medium lacking any carbon source and calculated for a volume of 5 L. As inoculum 14 big shaking flasks containing 250 mL of Küng biodiesel medium were prepared. After 72 hours they were well grown and ten of them were chosen to inoculate the fermenter. Previously 25 g biodiesel had been added to the fermentation medium from the refeed bottle, thus a starting concentration of 5 g/L was achieved.
In order to ensure an optimal $pO_2$ value of 50% maximum, the stirrer speed was set to be automatically regulated according to the actual $pO_2$. In contrast to the other two fermentations a lot of foam was produced during this experiment, thus the automatic addition of antifoam (Glanapon 2000 Konz.) was required. Because HPLC analysis is not possible when working with biodiesel, the decisions regarding refeeding could only be made based on the visual impression of the sample tubes after they had been centrifuged (Figure 6-9; the added amounts are shown in Table 6-5).

***Figure 6-9 Sample tubes after centrifugation. Left: Biodiesel layer visible on top. Right: No Biodiesel layer visible.***

29 hours after the start of the fermentation the OD had reached a value of 110. At this time accumulation phase was induced and ten hours later nitrogen limitation was achieved.
Already after 31 hours margaric acid was added in a concentration of 2 g/L, this was repeated after 10 hours, this time with a concentration of 4 g/L, then the tube which connected the fermenter to the margaric acid bottle broke. This was due to the fact that the 20% margaric acid solution, which was used, solidified at room temperature, which was encountered by using a magnetic stirrer to heat the solution, but the remains inside the tube were clogging. Thus no additional margaric acid could be fed anymore. To prevent the achieved 3HV/PHA concentration from decreasing again also the feeding of biodiesel had to be stopped and after 49 hours, when an OD of 230 had been reached, the experiment was ended.

***Table 6-5*** *Additions of biodiesel and margaric acid during the fermentation.*

| time [h] | biodiesel [g/L] | margaric acid [g/L] |
|---|---|---|
| 0.0 | 5.0 | 0.0 |
| 15.8 | 4.2 | 0.0 |
| 19.3 | 2.2 | 0.0 |
| 21.3 | 4.2 | 0.0 |
| 24.3 | 4.3 | 0.0 |
| 26.3 | 0.6 | 0.0 |
| 29.3 | 1.9 | 0.0 |
| 31.0 | 10.5 | 2.1 |
| 38.6 | 4.2 | 0.0 |
| 41.3 | 4.2 | 4.0 |

## 6.3.3 RESULTS AND DISCUSSION

The main results of this fermentation are shown in Table 6-6.

***Table 6-6*** *Maximum values, volumetric productivities and yield factors of the biodiesel fermentation.*

| | value | time |
|---|---|---|
| $CDM_{max}$ [g/L] | 43.58 | 49 h |
| $X_{r,max}$ [g/L] | 33.16 | 45 h |
| $PHA_{max}$ [g/L] | 12.74 | 49 h |
| $3HB_{max}$ [g/L] | 12.20 | 49 h |
| $3HV_{max}$ [g/L] | 0.54 | 49 h |
| $PHA/CDM_{max}$ [%] | 29.30 | 48 h |
| $3HV/PHA_{max}$ [%] | 4.32 | 48 h |
| $\mu_{max}$ [1/h] | 0.05 | - |

| | value |
|---|---|
| $V_{prod}$ [g/Lh] | 0.25 |
| $V_{prod,acc}$ [g/Lh] | 0.26 |
| spec $V_{prod}$ [1/h] | 0.01 |
| spec $V_{prod,acc}$ [1/h] | 0.01 |
| $Y_{CDM/BD}$ [g/g] | 0.99 |
| $Y_{PHA/BD}$ [g/g] | 0.30 |
| $Y_{3HV/margaric\ acid}$ [g/g] | 0.09 |
| $Y_{Xr/ammonia\ sulfate}$ [g/g] | 1.73 |

Unlike the other two fermentations there was practically no PHA formed before induction of accumulation phase after 29 hours. Then the ammonium sulfate concentration started to decrease comparatively slowly, while PHA generation started to increase, but not as fast as had been observed for the other two substrates. Thus in this process PHA production occurs in a not growth associated way. The volumetric PHA productivity of the whole process with a value of 0.25 g/Lh is approximately only a fourth of the productivities achieved with glucose or glycerol, the volumetric productivity of the accumulation phase is only a tenth. This time the experiment was ended when the PHA graph already had started to flatten out due to the stop of refeeding of the carbon source (Figure 6-10).

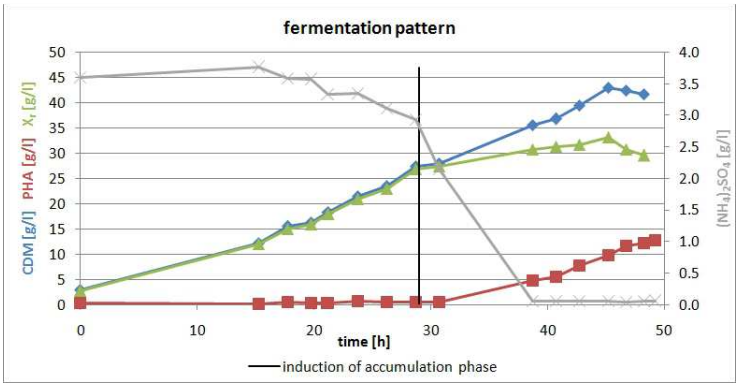


***Figure 6-10*** *Ferementation pattern of the biodiesel fermentation.*

The decreases of residual biomass formation rates during growth phase indicate a substrate limitation, which also explains why almost no PHA had been accumulated during this time.
At the end of the experiment there was definitely a carbon restriction since 3HB and 3HV formation rates decreased, while those of CDM and $X_r$ increased (Figure 6-11).

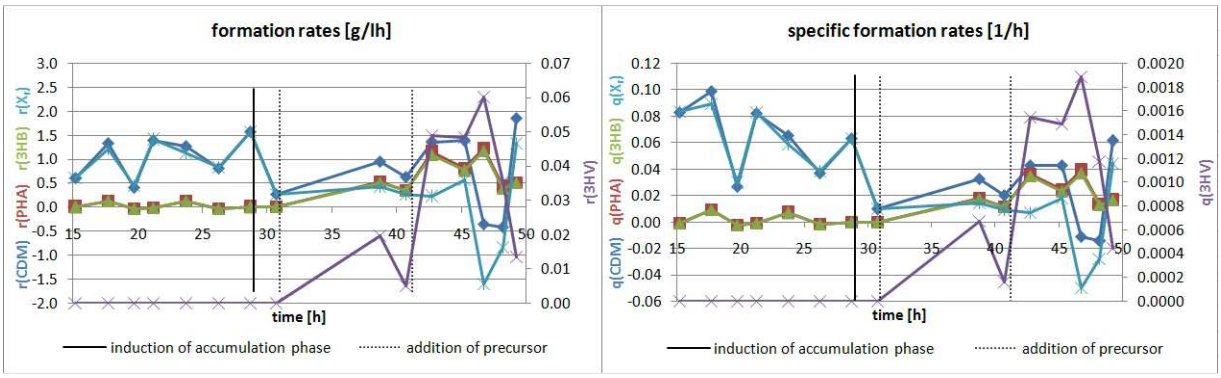


***Figure 6-11*** *Formation rates and specific formation rates of the biodiesel fermentation.*

Figure 6-12 shows that during growth phase the PHA/CDM ratio was constant, because the carbon was mainly utilized for microbial growth. A very interesting fact is that the 3HV content per PHA jumps from 0 to 3.3% only after the second addition of margaric acid. Before that no 3HV was detected.

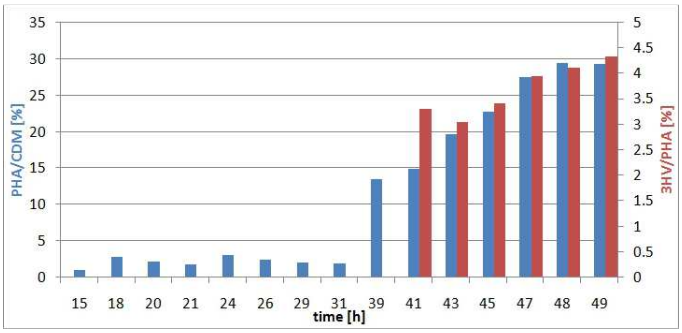


***Figure 6-12*** *PHA/CDM and 3HV/PHA shares of the biodiesel fermentation.*

# 7 CONCLUSION

The results of the glucose fermentation using γ-butyrolactone as 4HB precursor are not very satisfying regarding the formation of special building blocks with only 0.02% of the accumulated PHA being 4HB and a yield of 4HB per γ-butyrolactone of 0.001. But this fermentation yielded a rather high PHA per CDM content of 72% (Table 7-1).

Although the glycerol fermentation showed a lower PHA/CDM share than glucose with a value of 65%, its achieved PHA concentration of 29% is higher than that of glucose. Its production rate of PHA and its maximum specific growth rate, with values of 1 g/Lh and 0.11 1/h respectively, are very similar to those of the glucose fermentation. The yield of 0.6 of CDM per carbon source is even twice that of glucose (Table 7-1).
These numbers underline that pure glycerol is gernerally a rather promising substrate when working with *C. necator*. Nevertheless its endcapping effect should not be overlooked.
The results of the shaking flask experiments indicate that the direct utilization of CGP from biodiesel industry is not possible, because the contained impurities have a toxic influence on the producing strain. Thus a preliminary purification of the CGP waste stream would be needed before it could be utilized as carbon source.

The biodiesel fermentation achieved by far the lowest values for maximum specific growth rate and production rate of PHA with 0.05 1/h and 0.25 g/Lh respectively. Still, due to the adaption of the strain to this new substrate, a distinct improvement concerning the utilization of biodiesel could be observed. The yield of PHA per carbon source with a value of 0.3 is even higher thant that of the glucose fermentation. Additionally ther performance of the novel 3HV precursor margaric acid is rather promising with a content of 4.3% 3HV per PHA (Table 7-1).
Based on these results a next objective would be to allow the utilization of biodiesel in the same concentrations as glucose together with an increase of the 3HV content in PHA *via* optimizing the feeding strategy.

***Table 7-1*** *Comparison of the results of the different fermentations (glucose: 10g/L, glycerol: 10g/L, biodiesel: 5g/L).*

| | | glucose | glycerol | biodiesel |
|---|---|---|---|---|
| $CDM_{max}$ | [g/L] | 35.69 | 45.28 | 43.58 |
| $X_{r,max}$ | [g/L] | 12.35 | 17.68 | 33.16 |
| $PHA_{max}$ | [g/L] | 25.84 | 29.45 | 12.74 |
| $3HB_{max}$ | [g/L] | 25.83 | 29.45 | 12.20 |
| $4HB_{max}$ | [g/L] | 0.01 | - | - |
| $3HV_{max}$ | [g/L] | - | - | 0.54 |
| $PHA/CDM_{max}$ | [%] | 72.39 | 65.04 | 29.30 |
| $4HB/PHA_{max}$ | [%] | 0.02 | - | - |
| $3HV/PHA_{max}$ | [%] | - | - | 4.32 |
| $\mu_{max}$ | [1/h] | 0.16 | 0.11 | 0.05 |
| $V_{prod}$ | [g/Lh] | 1.07 | 0.98 | 0.25 |
| $V_{prod,acc}$ | [g/Lh] | 2.44 | 2.80 | 0.26 |
| spec $V_{prod}$ | [1/h] | 0.13 | 0.09 | 0.01 |
| spec $V_{prod,acc}$ | [1/h] | 0.15 | 0.17 | 0.01 |
| $Y_{CDM/C\text{-}source}$ | [g/g] | 0.31 | 0.60 | 0.99 |
| $Y_{PHA/C\text{-}source}$ | [g/g] | 0.24 | 0.40 | 0.30 |
| $Y_{4HB/\gamma\text{-}butyrolactone}$ | [g/g] | 0.001 | - | - |
| $Y_{3HV/margaric\ acid}$ | [g/g] | - | - | 0.089 |
| $Y_{Xr/ammonia\ sulfate}$ | [g/g] | 0.56 | 2.90 | 1.73 |

# 8 BIBLIOGRAPHY


1. Niederl, A., Narodoslawsky, M. (2006) Life cycle assessment of the production of biodiesel from tallow and used vegetable oil. *ACS Symposium Series, 921 (Feedstocks for the Future)*, 239-252.
2. Kettl, K., Titz, M., Koller, M., Shahzad, K., Schnitzer, H., Narodoslawsky, M. (2011) Process Design and Evaluation of Biobased Polyhydroxyalkanoates (PHA) Production. *Chemical Engineering Transactions* **25**, 983-987.
3. Harding, K. G., Dennis, J. S., von Blottnitz, H., Harrison, S. TL. (2007) Environmental analysis of plastic production processes: comparing petroleum-based polypropylene and polyethylene with biologically-based poly-beta-hydroxybutyric acid using life cycle analysis. *Journal of biotechnology* **130(1)**, 57-66.
4. Koller, M., Atlic, A., Dias, M., Reiterer, A., Braunegg, G. (2010) Microbial PHA production from waste raw materials. In Chen, G. Q., ed. : *Plastics from Bacteria - Natural functions and applications*. 85-119.
5. Mittelbach, M., Remschmidt, C., eds. (2006) *Biodiesel: The Comprehensive Handbook*. Mittelbach, M, Graz (Austria).
6. Canakci, M., Sanli, H. (2008) Biodiesel production from various feedstocks and their effects on the fuel properties. *Journal of Industrial Microbiology & Biotechnology* **35(5)**, 431-441.
7. Luzier, W. D. (1992) Materials derived from biomass/biodegradable materials. *Proceedings of the National Academy of Sciences of the United States of America* **89(3)**, 839-842.
8. Holmes, P. A. (1985) Applications of PHB - a microbially produced biodegradable thermoplastic. *Physics in Technology* **16(1)**, 32-36.
9. Koller, M., Salerno, A., Dias, M., Reiterer, A., Ingolic, E., Braunegg, G. (2009) Raw materials as a crucial factor for the economical feasible production of polyhydroxyalkanoate biopolyesters. *Biologi Italiani* **7**, 18-22.
10. Braunegg, G., Lefebvre, G., Genser, K. F. (1998) Polyhydroxyalkanoates, biopolyesters from renewable resources: Physiological and engineering aspects. *Journal of Biotechnology* **65(2-3)**, 127-161.
11. Sudesh, K., Abe, H., Doi, Y. (2000) Synthesis, structure and properties of polyhydroxyalkanoates: Biological polyesters. *Progress in Polymer Science* **25(10)**, 1503-1555.
12. Lee, S. Y. (1996) Bacterial polyhydroxyalkanoates. *Biotechnology and Bioengineering* **49(1)**, 1-14.
13. Chen, G. Q. (2010) Introduction of bacterial plastics PHA, PLA, PBS, PE, PTT and PPP. In Chen, G. Q., ed. : *Plastics from Bacteria - Natural functions and applications*. 1-16.
14. Nonato, R. V., Mantelatto, P. E., Rossell, C. E. (2001) Integrated production of biodegradable plastic, sugar and ethanol. *Applied Microbiology and Biotechnology* **57(1-2)**, 1-5.
15. Koller, M., Salerno, A., Miranda de Sousa Dias, M., Reiterer, A., Braunegg, G. (2010) Modern Biotechnological Polymer Synthesis: A Review. *Food Technology and Biotechnology* **48**, 255-269.
16. Chen, G. Q. (2009) A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry. *Chemical Society Reviews* **38(8)**, 243-2446.
17. Williamson, D. H., Wilkinson, J. F. (1958) The isolation and estimation of the poly-β-hydroxybutyrate inclusions of Bacillus species. *Journal of General Microbiology* **19(1)**, 198-209.
18. Lemoigne, M. (1923) Production d'acide β-oxybutyrique par certaines bactéries du groupe du B. subtilis. *Comptes rendus hebdomadaires des séances de l'Académie des sciences* **176**, 1761-1763.
19. Lemoigne, M. (1924) Sur le mécanisme de la production de l'acide β-oxybutyrique par voie biochimique. *Comptes rendus hebdomadaires des séances de l'Académie des sciences* **178**, 1093-1095.
20. Lemoigne, M. (1925) Sur l'origine de l'acide β-oxybutyrique obtenu par processus microbien. *Comptes rendus hebdomadaires des séances de l'Académie des sciences* **180**, 1539-1541.
21. Lemoigne, M. (1927) Études sur l'autolyse microbienne: Origine de l'acide β-oxybutyrique formé par

autolyse. *Annales de l'Institut Pasteur* **41**, 148-165.

22. Lenz, R. W., Marchessault, R. H. (2005) Bacterial Polyesters: Biosynthesis, Biodegradable Plastics and Biotechnology. *Biomacromolecules* **6(1)**, 1-8.
23. Lemoigne, M., Delaporte, B., Croson, M. (1944) Contribution à l'étude botanique et biochimique des bactéries du genre Bacillus. 2. Valeur du test des lipides β-hydroxybutyriques pour la charactérisation des espèces. *Annales de l'Institut Pasteur* **70**, 224.
24. Weibull, C. (1953) Characterization of the protoplasmic constituents of Bacillus megaterium. *Journal of Bacteriology* **66**, 696-702.
25. Macrae, R. M., Wilkinson, J. F. (1958) Poly-β-hydroxybutyrate metabolism in washed suspensions of Bacillus cereus and Bacillus megaterium. *Journal of General Microbiology* **19(1)**, 210-222.
26. Macrae, R. M., Wilkinson, J. F. (1958) The influence of cultural conditions on poly-β-hydroxybutyrate synthesis in Bacillus megaterium. *Proceedings of the Royal Society of Edinburgh* **27**, 73-78.
27. Forsyth, W. GC., Hayward, A. C., Roberts, J. B. (1958) Occurrence of poly-β-hydroxybutyric acid in aerobic gram-negative bacteria. *Nature* **182**, 800-801.
28. Doudoroff, M., Stainer, R. Y. (1959) Role of poly-β-hydroxybutyric acid in the assimilation of organic carbon by bacteria. *Nature* **183**, 1440-1442.
29. Alper, R., Lundgren, D. G., Marchessault, R. H., Cote, W. A. (1963) Properties of poly-β-hydroxybutyrate. I. General considerations concerning the naturally occurring polymer. *Biopolymers* **1(6)**, 545-556.
30. Lundgren, D. G., Pfister, R. M., Merrick, J. M. (1964) Structure of poly(β-hydroxybutyric acid) granules. *Journal of General Microbiology* **34**, 441-446.
31. Lundgren, D. G., Alper, R., Schnaitman, C. (1965) Characterization of poly-β-hydroxybutyrate extracted from different bacteria. *Journal of Bacteriology* **89(1)**, 245-251.
32. Oeding, V., Schlegel, H. G. (1973) β-Ketothiolase from Hydrogenomonas eutropha H16 and its significance in the regulation of poly-(β-hydroxybutyrate) metabolism. *Biochemical Journal* **134(1)**, 239-248.
33. Senior, P. J., Dawes, E. A. (1973) Regulation of poly-(β-hydroxybutyrate) metabolism in Azotobacter beijerinckii. *Biochemical Journal* **134(1)**, 225-238.
34. Merrick, J. M., Doudoroff, M. (1964) Depolymerization of poly(β-hydroxybutyrate) by an intracellular enzyme system. *Journal of Bacteriology* **88(1)**, 60-71.
35. Merrick, J. M., Delafield, F. P., Doudoroff, M. (1962) Hydrolysis of poly-beta-hydroxybutyrate by intracellular and extracellular enzymes. *Federation Proceedings* **21**, 228.
36. Bergmeyer, H. U., Gawehn, K., Klotzsch, H., Krebs, H. A., Williamson, D. H. (1967) Purification and properties of crystalline 3-hydroxybutyrate dehydrogenase from Rhodopseudomonas spheroides. *Biochemical Journal* **102(2)**, 423-431.
37. Wallen, L. L., Davis, E. N. (1972) Biopolymers of activated sludge. *Environmental Science and Technology* **6(2)**, 161-164.
38. Wallen, L. L., Rohwedder, W. K. (1974) Poly-β-hydroxyalkanoate from activated sludge. *Environmental Science and Technology* **8(6)**, 576-579.
39. Braunegg, G., Sonnleitner, B., Lafferty, R. M. (1978) A rapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid in microbial biomass. *European Journal of Applied Microbiology and Biotechnology* **6(1)**, 29-37.
40. Findlay, R. H., White, D. C. (1983) Polymeric beta-hydroxyalkanoates from environmental samples and Bacillus megaterium. *Applied and Environmental Microbiology* **45(1)**, 71-78.
41. De Smet, M. J., Eggink, G., Witholt, B., Kingma, J., Wynberg, H. (1983) Characterization of intracellular inclusions formed by Pseudomonas oleovorans during growth on octane. *Journal of Bacteriology* **154(2)**, 870-878.
42. Doi, Y., Kunioka, M., Nakamura, Y., Soga, K. (1988) Nuclear magnetic resonance studies on unusual bacterial copolyesters of 3-hydroxybutyrate and 4-hydroxybutyrate. *Macromolecules* **21(9)**, 2722-2727.
43. Baptist, J. (1962) Process for preparing poly-β-hydroxybutyric acid. *US Patent No. 3,036,959*.
44. Baptist, J. (1962) Process for preparing poly-β-hydroxybutyric acid. *US Patent No. 3,044,942*.

45. Orts, W. J., Nobes, G. AR., Kawada, J., Nguyen, S., Yu, G., Ravenelle, F. (2008) Poly(hydroxyalkanoates): biorefinery polymers with a whole range of applications. The work of Robert H. Marchessault (1). *Canadian Journal of Chemistry* **86(6)**, 628-640.
46. Steinbuechel, A. (2006) Polymeric and low molecular weight hydrophobic chemicals produced by microorganisms from renewables. *Renewable Resources & Biorefineries Conference*, 1-34.
47. Byrom, D. (1987) Polymer synthesis by microorganisms: technology and economics. *Trends in Biotechnology* **5(9)**, 246-250.
48. Marchessault, R. H. (2009) Polyhydroxyalkanoate (PHA) History at Syracuse University and Beyond. *Cellulose* **16**, 357-359.
49. Chen, G. Q. (2010) Indutstrial Production of PHA. In Chen, G. Q., ed. : *Plastics from Bacteria - Natural functions and applications*..
50. Metabolix. Metabolix Purchases Biopol Assets From Monsanto. Retrieved: October, 2011. Available at: http://ir.metabolix.com/releasedetail.cfm?ReleaseID=211102
51. Metabolix. Metabolix and ADM Enter Strategic Alliance to Commercialize PHA Natural Polymers. Retrieved: October, 2011. Available at: http://ir.metabolix.com/releasedetail.cfm?ReleaseID=211091
52. Borealis group. Standortgeschichte. Von den Stickstoffwerken zu Borealis. Retrieved: October, 2011. Available at: http://www.borealisgroup.com/linz/ueber-borealis/stickstoffwerken-zur-borealis/
53. Biomer. Hintergründe. Retrieved: October, 2011. Available at: http://biomer.de/IndexD.html
54. Koller, M., Hesse, P., Kutschera, C., Bona, R., Nascimento, J., Ortega, S., Agnelli, J. A., Braunegg, G. (2010) Sustainable embedding of the bioplastic poly-(3-hydroxybutyrate) into the sugarcane industry: Principles of a future-oriented technology in Brazil. In Eyerer, P., Weller, M., Hübner, C., eds. : *Polymers - Opportunities and Risks II* **12**. 81-96.
55. Biocycle. Company History. Retrieved: October, 2011. Available at: http://www.biocycle.com.br/site.htm
56. DSM. DSM China, News. DSM invests in development of bio-based materials in China. Retrieved: October, 2011. Available at: http://www.dsm.com/nl_NL/html/dcn/DSM_invests_in_development_of_bio-based_materials_in_China.htm
57. Choi, J., Lee, S. Y. (1997) Process analysis and economic evaluation for poly (3-hydroxybutyrate) production by fermentation. *Bioprocess and Biosystems Engineering* **17(6)**, 335-342.
58. Makkar, N. S., Casida, L. E. (1987) Cupriavidus necator gen. nov., sp. nov.: a Nonobligate Bacterial Predator of Bacteria in Soil. *International Journal of Systematic Bacteriology* **37(4)**, 323-326.
59. Yabuuchi, E., Kosako, Y., Yano, I., Hotta, H., Nishiuchi, Y. (1995) Transfer of two Burkholderia and an Alcaligenes species to Ralstonia gen. nov: proposal of Ralstonia pickettii (Ralston, Palleroni and Doudoroff 1973) comb. nov., Ralstonia solanacearum (Smith 1896) Comb. Nov. and Ralstonia eutropha (Davis 1969) comb. nov. *Microbiology and Immunology* **39(11)**, 897-904.
60. Vaneechoutte, M., Kampfer, P., De Baere, T., Falsen, E., Verschraegen, G. (2004) Wautersia gen. nov., a novel genus accommodating the phylogenetic lineage including Ralstonia eutropha and related species, and proposal of Ralstonia [Pseudomonas] syzygii (Roberts et al. 1990) comb. nov. *International Journal of Systematic and Evolutionary Microbiology* **54(2)**, 317-327.
61. Vandamme, P., Coenye, T. (2004) Taxonomy of the genus Cupriavidus: a tale of lost and found. *International Journal of Systematic and Evolutionary Microbiology* **54(6)**, 2285-2289.
62. Fritsche, W. (1990) *Mikrobiologie*. Gustav Fischer Verlag Jena.
63. Schlegel, H. G. (1992) *Allgemeine Mikrobiologie*. Georg Thieme Verlag Stuttgart New York.
64. Dawes, E. A., Senior, P. J. (1973) The role and regulation of energy reserve polymers in micro-organisms. *Advances in microbial physiology* **10**, 135.
65. Koller, M., Bona, R., Chiellini, E., Fernandes, E. G., Horvat, P., Kutschera, C., Hesse, P., Braunegg, G. (2008) Polyhydroxyalkanoate production from whey by Pseudomonas hydrogenovora. *Bioresource Technology* **99(11)**, 4854-4863.
66. Chen, G. Q., ed. (2010) Natural Functions of Bacterial Polyhydroxyalkanoates. In : *Plastics from Bacteria - Natural functions and applications*. 39-61.
67. Anderson, A. J., Dawes, E. A. (1990) Occurrence, metabolism, metabolic role, and industrial uses of

bacterial polyhydroxyalkanoates. *Microbiological Reviews* **54(4)**, 450-472.

68. Zhao, Y. H., Li, H. M., Qin, L. F., Wang, H. H., Chen, G. Q. (2007) Disruption of the polyhydroxyalkanoate synthase gene in Aeromonas hydrophila reduces its survival ability under stress conditions. *FEMS Microbiology Letters* **276(1)**, 34-41.
69. Kadouri, D., Jurkevitch, E., Okon, Y., Castro-Sowinski, S. (2005) Ecological and agricultural significance of bacterial polyhydroxyalkanoates. *Critical Reviews in Microbiology* **31(2)**, 55-67.
70. Kawaguchi, Y., Doi, Y. (1992) Kinetics and mechanism of synthesis and degradation of poly(3-hydroxybutyrate) in Alcaligenes eutrophus. *Macromolecules* **25(9)**, 2324-2329.
71. Lefebvre, G., Rocher, M., Braunegg, G. (1997) Effects of low dissolved-oxygen concentrations on poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) production by Alcaligenes eutrophus. *Applied and Environmental Microbiology* **63(3)**, 827-833.
72. Doi, Y., Kunioka, M., Nakamura, Y., Soga, K. (1987) Biosynthesis of copolyesters in Alcaligenes eutrophus H16 from carbon-13 labeled acetate and propionate. *Macromolecules* **20(12)**, 2988-2991.
73. E, V. H., G, Z., A, S., A, S. (1995) Metabolic pathway for biosynthesis of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) from 4-hydroxybutyrate by Alcaligenes eutrophus. *European Journal of Biochemistry* **227(1-2)**, 43-60.
74. Lee, Y. H., Kang, M. S., Jung, Y. M. (2000) Regulating the molar fraction of 4-hydroxybutyrate in poly (3-hydroxybutyrate-4-hydroxybutyrate) biosynthesis by Ralstonia eutropha using propionate as a stimulator. *Journal of Bioscience and Bioengineering* **89(4)**, 380-383.
75. Doi, Y., Segawa, A., Kunioka, M. (1990) Biosynthesis and characterization of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) in Alcaligenes eutrophus. *International Journal of Biological Macromolecules* **12(2)**, 106-111.
76. Chen, G. Q., Wu, Q. (2005) Microbial production and applications of chiral hydroxyalkanoates. *Applied Microbiology and Biotechnology* **67(5)**, 593-299.
77. Chen, G. Q. (2010) Plastics completely synthesized by bacteria: Polyhydroxyalkanoates. In Chen, GQ., ed. : *Plastics from Bacteria - Natural functions and applications*. 17-37.
78. Barnard, G. N., Sanders, J. KM. (1989) The poly-β-hydroxybutyrate granule in vivo. A new insight based on NMR spectroscopy of whole cells. *Journal of Biological Chemistry* **264(6)**, 3286-3291.
79. Kunasundari, B., Sudesh, K. (2011) Isolation and recovery of microbial polyhydroxyalkanoates. *eXPRESS Polymer Letters* **5(7)**, 620-634.
80. King, P. P. (1982) Biotechnology. An industrial view. *Journal of Chemical Technology and Biotechnology* **32(1)**, 2-8.
81. Cornibert, J., Marchessault, R. H. (1975) Conformational isomorphism. General 21 helical conformation for poly(β-alkanoates). *Macromolecules* **8(3)**, 296-305.
82. Chen, G. Q., Wu, Q. (2005) The application of polyhydroxyalkanoates as tissue engineering materials. *Biomaterials* **26(33)**, 6565-6578.
83. Bluhm, T. L., Hamer, G. K., Marchessault, R. H., Fyfe, C. A., Veregin, R. P. (1986) Isodimorphism in bacterial poly(β-hydroxybutyrate-co-β-hydroxyvalerate). *Macromolecules* **19(11)**, 2871-2876.
84. Kunioka, M., Tamaki, A., Doi, Y. (1989) Crystalline and thermal properties of bacterial copolyesters: poly(3-hydroxybutyrate-co-3-hydroxyvalerate) and poly(3-hydroxybutyrate-co-4-hydroxybutyrate). *Macromolecules* **22(2)**, 694-697.
85. Nakamura, S., Doi, Y., Scandola, M. (1992) Microbial synthesis and characterization of poly(3-hydroxybutyrate-co-4-hydroxybutyrate). *Molecular Microbiology* **25(17)**, 4237-4241.
86. Braunegg, G., Bona, R., Koller, M. (2004) Sustainable Polymer Production. *Polymer-Plastics Technology and Engineering* **43(6)**, 1779-1793.
87. Madison, L. L., Huisman, G. W. (1999) Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic. *Microbiology and Molecular Biology Reviews* **63(1)**, 21-53.
88. Agus, J., Kahar, P., Abe, H., Doi, Y., Tsuge, T. (2006) Molecular weight characterization of poly[(R)-3-hydroxybutyrate] synthesized by genetically engineered strains of Escherichia coli. *Polymer Degradation and Stability* **91(5)**, 1138-1146.
89. Bureau of Labor Statistics. CPI Inflation Calculator. Retrieved: October, 2011. Available at: http://bls.gov/data/inflation_calculator.htm

90. U.S. Energy Information Administration. Cushing, OK Crude Oil Future Contract1 (Dollars per Barrel). Retrieved: October, 2011. Available at: http://www.eia.gov/dnav/pet/hist/LeafHandler.ashx?n=pet&s=rclc1&f=d
91. Choi, J., Lee, S. Y. (1999) Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation. *Applied Microbiology and Biotechnology* **51(1)**, 13-21.
92. Zhang, X., Luo, R., Wang, Z., Deng, Y., Chen, G. Q. (2009) Application of (R)-3-Hydroxyalkanoate Methyl Esters Derived from Microbial Polyhydroxyalkanoates as Novel Biofuels. *Biomacromolecules* **10(4)**, 707-711.
93. Liu, Q., Ouyang, S., Kim, J., Chen, G. Q. (2007) The impact of PHB accumulation on L-glutamate production by recombinant Corynebacterium glutamicum. *Journal of Biotechnology* **132(3)**, 273-279.
94. Mirel: Media & Events, Hi-res images for press usage. Retrieved: October, 2011. Available at: http://www.mirelplastics.com/news/default.aspx?ID=1895
95. Zinn, M., Witholt, B., Egli, T. (2001) Occurrence, synthesis and medical application of bacterial polyhydroxyalkanoate. *Advanced Drug Delivery Reviews* **53(1)**, 5-21.
96. Meher, L. C., Vidya Sagar, D., Naik, S. N. (2006) Technical aspects of biodiesel production by transesterification - a review. *Renewable and Sustainable Energy Reviews* **10**, 248-268.
97. Eisentraut, A. Sustainable production of second-generation biofuels - Potential and perspectives in major economies and developing countries. Retrieved: October, 2011. Available at: http://www.iea.org/papers/2010/second_generation_biofuels.pdf
98. Demirbas, M. F. (2011) Biofuels from algae for sustainable development. *Applied Energy* **88(10)**, 3473-3480.
99. Energy: Biofuels and other renewable energy in the transport sector - European Commission. Retrieved: October, 2011. Available at: http://ec.europa.eu/energy/renewables/biofuels/biofuels_en.htm
100. Plass, L., Reimelt, S. (2007) Status und Zukunft der Biotreibstoffe. *Chemie Ingenieur Technik* **79(5)**, 561-568.
101. Madden, L. A., Anderson, A. J., Shah, D. T., Asrar, J. (1999) Chain termination in polyhydroxyalkanoate synthesis: Involvement of exogenous hydroxy-compounds as chain transfer agents. *International Journal of Biological Macromolecules* **25(1-3)**, 43-53.