Integrated Master in Chemical Engineering

PHA production by a mixed culture under dissolved oxygen limitation

Master Thesis

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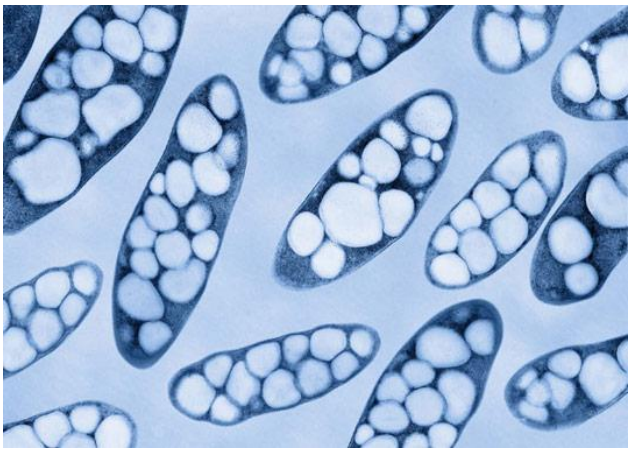

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Abstract

Polymer storing bacteria have been studied by their ability to store plastic-like polymers. Polyhydroxyalkanoates (PHA) have been the focus of several studies over the last 50 years due to their plastic-like properties and by the fact that they are degradable. Commercial production of PHAs is currently based on pure culture processes using either PHA producing bacteria or genetically modified bacteria. Pure substrates and sterile equipment are used in the production of the PHAs which leads to high energy costs and as a consequence expensive products.

Mixed culture technology using non sterile substrate and equipments offers a good advantage on the PHA production. Although the costs are significantly lower, the production process requires optimization for higher cellular PHA contents to be able to compete with pure culture processes.

To optimize the polymer storage a two step process: a culture enrichment and growth step and a PHA production step were used. The enrichment culture strategy consisted in alternating a feast phase (presence of carbon source) and a famine phase (lack of carbon source). PHA storing bacteria have high substrate uptake rate and have a balanced growth way throughout the feast and famine regime so those bacteria would become dominant and survive while the other starved and died. In the PHA production step, excess of carbon source and ammonium free medium to prevent growth were used to reach a maximum value of polymers.

A sequencing batch reactor (SBR) was the reactor used because it is the type of reactor that is ideal to achieve the dynamic conditions of a feast-famine regime in a continuous way. The carbon source used in this thesis was glycerol and the stored polymers were polyglucose and polyhydroxybutyrate (PHB).

Dissolved oxygen limitation is going to be the aim of this study. Using lower stirring speeds and changing how the air flow is introduced in the reactor will lower the mass transfer coefficient and result in a system with lower oxygen supply. Oxygen is a key component in the bacteria storage system so the lack of it will bring new results regarding the polyglucose/PHB ratio.

Key words: PHAs, polyglucose, mass transfer coefficient, dissolved oxygen limitation

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Nomenclature

AcCoA	Acetyl coenzime A
ATP	Adenosine triphosphate
C	Concentration of oxygen in the liquid phase (mg O_2 L^{-1})
C^*	Saturating concentration of oxygen in the gas phase (mg O_2 L^{-1})
C/N	Carbon/Nitrogen
CO_2	Carbon dioxide
DO	Dissolved oxygen (%) or (mg O_2/L)
DW	Dry weight
FISH	Fluorescence in situ hybridization
$f_{L,i}$	Diluting factor for each time point
$f_{L,i-1}$	Diluting factor for the previous time point
$f_{L,t(i)}$	New dilution factor for each time point
HCl	Hydrochloric acid
i	Indicator of number or component
Kla	Mass transfer coefficient (h^{-1})
NAD^+	Nicotinamide adenine dinucleotide, oxidizing agent
$NADH_2$	Nicotinamide adenine dinucleotide, reduction agent
NaOH	Sodium Hydroxide
NH_3	Ammonia
O_2	Oxygen
OTR	Oxygen Transfer Rate (mg O_2 L^{-1} h^{-1})
OUR	Oxygen Uptake Rate (mg O_2 L^{-1} h^{-1})
PHA	Polyhydroxyalkanoates
PHB	Polyhydroxybutyrate
q_{CO2}	Biomass specific CO_2 production rate (Cmmol/Cmmol/h)
$q_{glycerol}$	Biomass specific glycerol consumption rate (Cmmol/Cmmol/h)
q_{NH4+}	Biomass specific NH_4^+ consumption rate (Cmmol/Cmmol/h)
q_{O2}	Biomass specific O_2 consumption rate (Cmmol/Cmmol/h)
q_{PHB}	Biomass specific PHB production rate (Cmmol/Cmmol/h)
$q_{Polyglucose}$	Biomass specific polyglucose production rate (Cmmol/Cmmol/h)

SBR	Sequencing Batch Reactor
SRT	Solid Retention Time (days)
t	Time
T_{cycle}	Length of cycle
TSS	Total Suspended Solids (g/L)
TSS^{Effl}	Total suspended solids in the effluent (g/L)
$TSS^{R}(t_{end})$	Total suspended solids in the reactor in the end of the cycle (g/L)
TSS^{Sludge}	Total suspended solids in the sludge (g/L)
$V_{acid,t(i)}$	Volume of added acid into the reactor in each time point (L)
$V_{acid,t(i-1)}$	Volume of added acid into the reactor in the previous time point (L)
$V_{base,t(i)}$	Volume of added base into the reactor in each time point (L)
$V_{base,t(i-1)}$	Volume of added base into the reactor in the previous time point (L)
$V_{cum\ dil,t(i)}$	Volume of cumulative dilution value in each time point (L)
$V_{cum\ dil,t(i-1)}$	Volume of cumulative dilution value in the previous time point (L)
V^{Effl}	Volume of Effluent withdrawn form the reactor (L)
V_L^R	Volume of liquid inside the reactor (L)
$V^R_{L\ true,t(i)}$	True volume of liquid inside the reactor in each time point (L)
V^{Sludge}	Volume of Sludge withdrawn from the reactor (L)
X	Biomass
X_{active}	Active biomass
$Y_{CO2/Glycerol}$	Observed CO_2 yield relative to glycerol (Cmmol/Cmmol)
$Y_{CO2/O2}$	Observed CO_2 yield relative to O_2 (Cmmol/Cmmol)
$Y_{CO2/PHB}$	Observed CO_2 yield relative to PHB (Cmmol/Cmmol)
$Y_{CO2/Polyglucose}$	Observed CO_2 yield relative to polyglucose (Cmmol/Cmmol)
$Y_{PHB/Glycerol}$	Observed PHB yield relative to glycerol (Cmmol/Cmmol)
$Y_{Polyglucose/Glycerol}$	Observed polyglucose yield relative to glycerol (Cmmol/Cmmol)
$Y_{X/Glycerol}$	Observed X yield relative to glycerol (Cmmol/Cmmol)
$Y_{X/PHB}$	Observed X yield relative to PHB (Cmmol/Cmmol)
$Y_{X/Polyglucose}$	Observed X yield relative to polyglucose (Cmmol/Cmmol)
δ	Delta

1 Introduction

1.1 Theory

1.1.1 Plastics and bioplastics

Over the last century, the world's population has been more aware of the need of the production and the use of renewable energies. The excess talk and studies about green house gases, global warming, CO_2 excess and more, created a demand for new, green and safer energy production. Not only energy has been looked at, but also products that come from oil treatment.

The petroleum-based plastics are amongst the most used materials in the world. Plastics have a range of unique properties: they can be used at a very wide range of temperatures, are chemical- and light- resistant and they are very strong and tough, but can be easily worked as a hot melt. It is this range of properties together with their low cost that has driven the annual worldwide demand for plastics to reach 245 million tonnes in 2009 (www.plasticseurope.org, accessed on March 2010). The concern about the climate change and also the need to reduce the oil consumption has led to several studies and the production of a new type of plastics: the bioplastics. Bio-based plastics may offer important contributions by reducing the dependence on fossil fuels and the related environmental impacts.

Bioplastics are industrially produced, renewable, biodegradable and eco-friendly plastics, formed by biomass. Most of the bioplastics are used for packaging applications, including soluble films for industrial packaging, films for bags and sacks, and loose fills. The emerging applications are components for automobiles (e.g., tyre fillers, panels), electronic devices, household appliances and other durable applications. Also, bioplastics are used for shopping bags, strings, straws, tableware, tapes, technical films, trays and wrap film (www.biotec.de).

Using different feedstock like starch, cellulose, sugars, acetate, glycerol and others, it was possible to study the different plastic-like polymers stored by the microorganisms. Bioplastics production employs pure culture of microorganisms and therefore requires well-defined feedstocks and sterile process conditions. Moreover, these processes often have to be

performed as batch processes. These conditions result in expensive equipment and high energy demand, making industrial biotechnology based on pure cultures unfavourable for the large scale production of relatively cheap bulk biochemicals and biomaterials.

Figure 1 - Example of the use of bioplastics

1.1.2 Mixed Cultures

The use of pure cultures has proven to be too expensive to perform in an industrial scale due to its extreme need for sterile material. An alternative method is the use of a mixed culture biotechnology. A mixed culture is defined by a group of different bacteria species, living in the same conditions. Mixed culture biotechnology aims at using undefined bacteria species to produce products using a mixture that combines environmental biotechnology and industrial biotechnology in order to achieve its goals. These mixed cultures have been mainly applied in waste treatment (biological wastewater treatment plants, anaerobic digesters and others).

The principle of mixed culture biotechnology is based on natural selection and competition rather than on genetic or metabolic engineering (Kleerebezem and van Loosdrecht 2007). By choosing the substrate and medium composition, the ecosystem is controlled rather than the organisms. The bacteria are obliged to adapt to the feeding conditions and as a consequence, a natural selection occurs in every cycle as the bacteria with higher substrate uptake rate are the ones that survive while the others starve. Some of the reasons why the mixed culture biotechnology is so attractive are the fact that reactors are able to work on non-sterile conditions, which leads to less energy and equipment costs. Also the fact that the risk of

contaminations being almost non-existent, the technology not being protected by patents and the waste streams being able to use non pure substrates make mixed culture biotechnology much more economically attractive than pure culture biotechnology. During this thesis, the use of fluorescence in situ hybridization (FISH), made it possible to observe the different species present inside the reactor at different time points and allowed to see the evolution of the dominant specie.

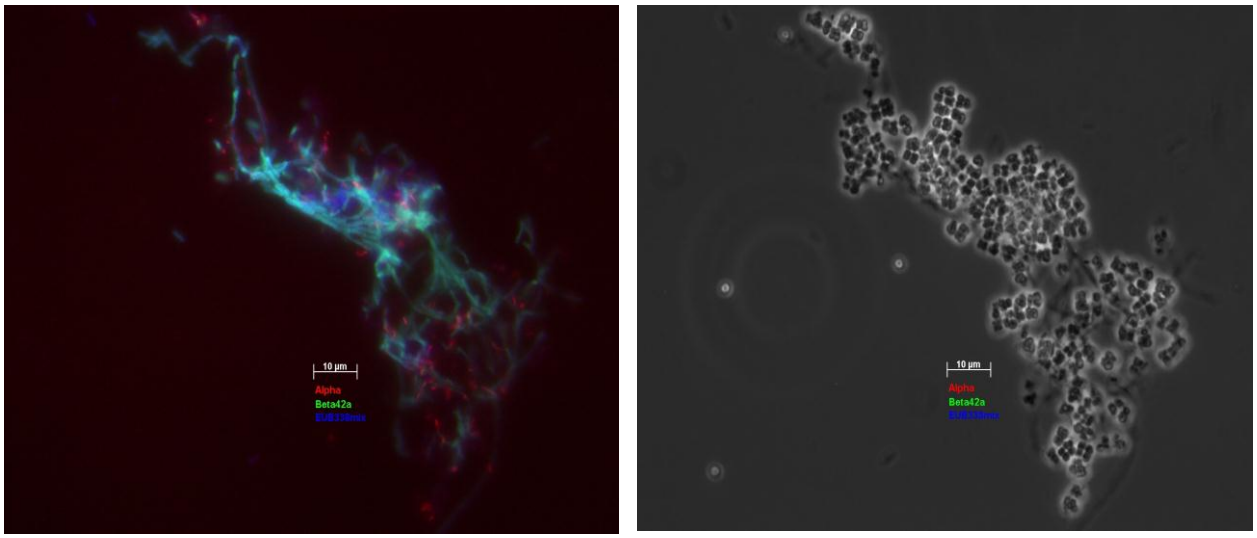

Figure 2 – FISH images performed by Yang Jiang in April 2010. The left picture shows a mixed culture with 3 different species (3 different colours). By changing the contrast, it is possible to see on the picture on the right that there is dominant specie, although there is no probe yet that can identify that specie.

1.1.3 PHA and polyglucose

The carbon source used in this thesis is glycerol and the polymers that results from the glycerol uptake PHAs and polyglucose.

Polyhydroxyalkanoates (PHAs) are biopolymers of hydroxyl fatty acids which are naturally produced by many different bacteria as an intracellular carbon and energy reserve material (Wältermann and Steinbüchel 2005). The occurrence of PHAs in bacteria has been known since 1920s, when Lemoigne reported the formation of polyhydroxybutyrate (PHB) inside bacteria (Lemoigne 1926). However, the high cost of producing these bioplastics and the availability of low-cost petrochemical-derived plastics led to bioplastics being ignored for a long time.

The most produced bioplastic is PHB. The monomer composition of the polymer, the chains length of the polymer and the length of the side chains influence the properties of the polymer.

PHAs are interesting as bioplastics as they can exhibit thermoplastic and/or elastomeric properties, are non-toxic, biocompatible, and made from renewable sources and biodegradable (Steinbüchel 2001). With the conditions used in this thesis, the PHA stored is PHB.

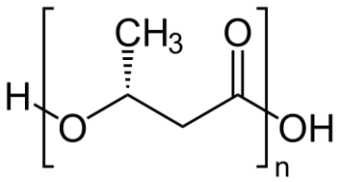

Figure 3 - PHB chemical structure

Glycogen or polyglucose is a polysaccharide that contains only glucose with a 1,4 linkages and is branched via a 1 ,6 linkages that has been found in many bacteria as well as in eukaryotic organisms. It is generally considered to be a storage compound providing both carbon and energy for the microbes that accumulate them (Kumar, Ghosh et al. 1989).

In certain conditions, bacteria accumulate relatively large amounts of polyglucose compounds with properties similar to those of animal glycogen. An interpretation of bacterial "glycogen" production is that it provides a food and/or energy reserve for the organisms in unfavourable environments, in other words, in starvation periods, bacteria rich in glycogen should survive longer than bacteria without such reserves (Strange 1968).

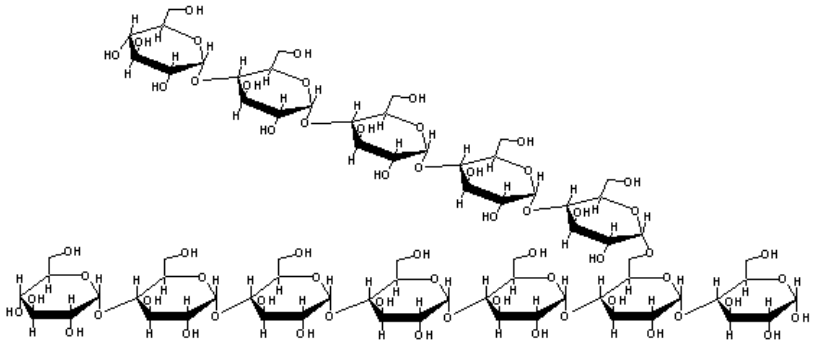

Figure 4 – Polyglucose chemical structure

To better understand the chemical reactions occurring inside the bacteria, the next figure shows the metabolic pathway from glycerol to the production of PHB, glycogen, ATP and growth:

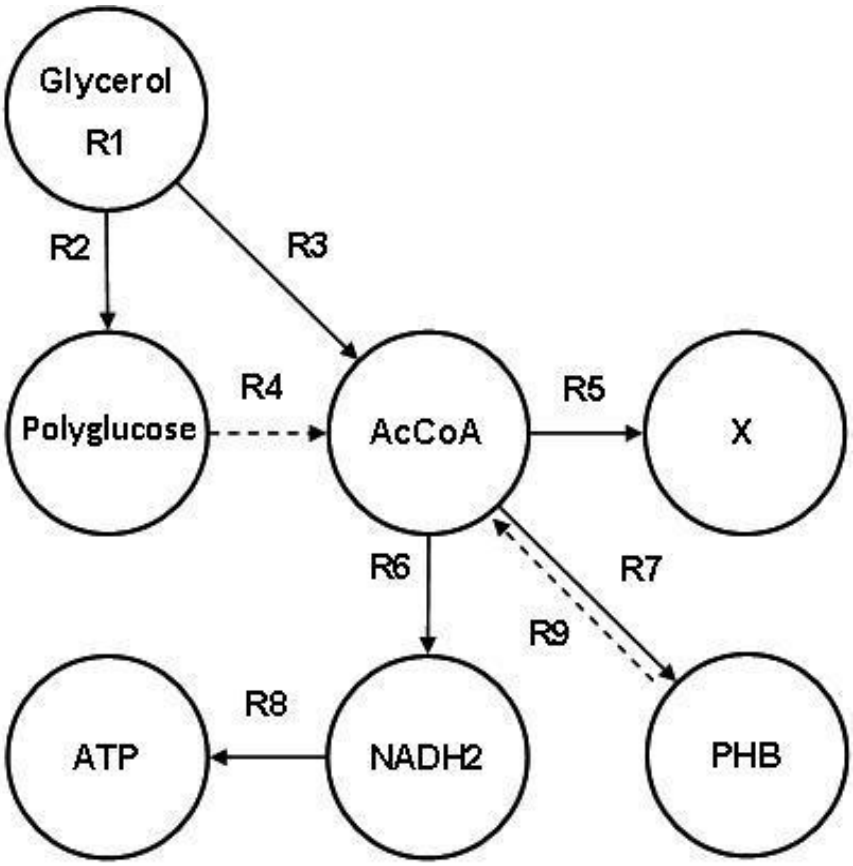

Figure 5 – Scheme of the metabolic pathway from glycerol to the different components of the bacteria

Table 1 – Reaction and stoichiometry of the metabolic pathway from glycerol to the different components of the bacteria

	Reaction	Stoichiometry
1	Glycerol uptake	1 Glycerol → 1 **Glycerol**
2	Polyglucose production	1 **Glycerol** + 0.5 **ATP** → 1 Polyglucose + 1/3 **$NADH_2$**
3	AcCoA production	1 **Glycerol** → 2/3 **AcCoA** + 1/3 **CO_2** + **$NADH_2$**+ 1/3 **ATP**
4	Conversion from Polyglucose to AcCoA	1 Polyglucose → 2/3 **AcCoA** + 1/3 **CO_2** + 2/3 **ATP** + 2/3 **$NADH_2$**
5	X production	1.267 **AcCoA** + 0.2 NH3 + 2.16 **ATP** → 1 X + 0.267 **CO_2**+ 0.434 **$NADH_2$**
6	TCA cycle	1 **AcCoA** → 1 **CO_2**+2 **$NADH_2$**
7	PHB production	1 **AcCoA** + 0.25 **$NADH_2$** → 1 PHB
8	Oxidative phosphorylation	1 **$NADH_2$** + 0.5 **O_2** → δ **ATP**
9	PHB consumption	1 PHB + 0.25 **ATP** → 1 **AcCoA** + 0.25 **$NADH_2$**

The previous figure and table show the metabolic pathway from glycerol to the different components of the bacteria. Taking a look at the polymers storage, one can observe that in the polyglucose production, not only ATP is used but there is also the production of 1/3 moles of $NADH_2$. In both pathways to PHB production and biomass growth (X production), there is higher use of ATP and production of $NADH_2$ compared to the polyglucose pathway. Oxygen is needed not only on ATP formation but also as an electron acceptor on the oxidization of $NADH_2$ to NAD^+. It is this oxidizing reaction and the ATP formation why oxygen is so important in PHAs production.

1.1.4 PHA producing mixed culture enrichment strategy

The most important step for a successful mixed culture PHA production process is the enrichment of a mixed culture with microorganism that have a superior ability for faster substrate uptake and to have high storage capacity for PHA accumulation. One of the ways to optimize this storage is the enrichment of the mixed culture by alternating a feast phase (presence of carbon source) and a famine phase (lack of carbon source) during each cycle.

This enrichment strategy can use several substrates like acetate, glycerol, butyrate and others as carbon source. Once the carbon source is supplied to the reactor, a feast phase will occur, giving the chance to the bacteria to either grow and/or store PHA or polyglucose. After the feast phase ends, i.e., there is a total consumption of the carbon source, there will be a phase of carbon starvation (famine), where the bacteria that previously stored polymers, will continue to grow in this phase while the bacteria that didn't store polymers will starve and die. By removing an excess of biomass at the end of each feast-famine cycle, we are assuring that PHA storing bacteria that have faster substrate uptake rate, become dominant in the mixed culture and assure higher amounts of substrate so they can produce bigger amounts of biomass. According to Johnson et al. (2008), PHA storing bacteria can take up the carbon source very fast and they can still continue to grow in the long famine phase, so, the bacteria survival in the feast-famine system is generally based on substrate uptake rate instead of growth rate.

Once the PHA storing bacteria have been enriched, the feast-famine system would continue, now to have a biomass production step that favours PHA storing bacteria. When this system is stable, one other step would be done with the objective of reaching a maximum value of PHA. In this accumulation step, biomass collected at the end of the cycle would be supplied with an excess of carbon source and with medium with nitrogen limitation, to make sure that biomass growth is prevented.

The reactor type used for the biomass enrichment and production step was a sequencing batch reactor (SBR), as this type of reactor is ideal to achieve the dynamic conditions of a feast-famine regime in a continuous way.

Figure 6 shows a picture of the SBR set-up. The big bottles in the foreground contained the concentrated carbon source (glycerol) and the medium content. Water, carbon source and nutrient source were mixed and automatically pumped together into the reactor by the two upper pumps on the left side. The third pump was used for effluent/biomass withdrawal. The smaller glass bottles under the bench contained acid and base for pH control, both pumped via the biocontroller standing under the reactor on the right side.

Figure 6 – Reactor set-up

2 Scope and outline of this thesis

Oxygen has been found to be closely related with ATP production. Under dissolved oxygen limitation conditions, the biomass has a limited amount of ATP for all metabolisms. As aforementioned, PHA synthesis and polyglucose synthesis pathway require different amount of ATP. The PHA/polyglucose ratio is therefore expected to be affected by the dissolved oxygen level. In this study, the influence of oxygen on the biomass storage mechanism will be studied. Oxygen is also used as an oxidizing agent, so the influence of the lack of this molecule will be studied in order to understand the storing mechanisms.

The mass transfer coefficient was studied by using different stirring speeds and different air flow values. This way, it was possible to select the conditions necessary to have the oxygen limitation conditions inside the reactor.

2.1 Chapter review

In chapter 3 the background is explained. Some of the prior studies and experiments are presented and some of the ideas on how to perform the work on this thesis are introduced.

In chapter 4 the materials and methods used are described. For all the experiments done, the sample and instruments used are presented and also explained on how to use them. The protocol for all different samples is explained and all the procedures and methods are also explained.

Chapter 5 has all the results from all the performed experiments are shown. The samples evolution and results are explained and compared. A general discussion compares both types of experiments, their main results and the results discussion.

In chapter 6 the conclusions of the work performed and the results obtained are presented

In chapter 7 some of the work for the future is introduced as well as some of the work's bottlenecks and some of the ideas on were this work could be implemented on.

Chapter 8 has the references of all the literature that was used in this thesis.

3 Background

As the main objective of the present project is to study the influence of dissolved oxygen limitation conditions in PHA production, it is interesting to talk about the studies that have been made on PHA producing bacteria.

Several parameters have been studied to achieve the highest percentage of stored PHAs by bacteria. These parameters are, for example, temperature, pH, substrates, medium, solid retention times (SRT), C/N limitations and several more. So far, the genetically altered bacteria, like recombinant *E. coli*, have shown to have the highest PHB accumulation percentage. It has been reported to reach up to 80–90 wt% (Slater, Voige et al. 1988). The new focus of studies is using mixed cultures of bacteria. These cultures have been reported to result in up to 65 wt% cellular PHB content (Dias, Lemos et al. 2006). In a more recent research, a maximum of 89% of PHB content was achieved. By changing the temperature, pH, SRTs and C/N limitations, the results prove that mixed cultures can have a very high PHB storage and can compete with pure cultures and genetically modified bacteria. These results are present in the paper of Katja Johnson (2010).

Nowadays, several reactors have been used to study different parameters in the PHA production by bacteria. The research was performed in Kluyverlaboratorium, Delft.

A mixed microbial culture has been enriched with glycerol as carbon source without dissolved oxygen limitation. The results obtained in this reactor will be used as reference to compare with the results obtained in this thesis. This reactor (due to its normal day-to-day work and disposition around the laboratory, will be called fermentor 5) has been operated since November 2008 and its original inoculum was from the Dokhaven wastewater treatment plant in Rotterdam, the Netherlands. The medium content is the same as the medium used in this thesis (Appendix A). The SRT is of 2 days with 24 hour cycle (see Appendix B), the same used in fermentor 7 (reactor used for the enrichment of the bacteria culture used in this thesis). The operating conditions were of 30° Celsius, 400-450 rpm, pH 7 and the air flow is sparged into the reactor at 1 l/min. Several enrichment and production experiments have been conducted in the past years, to evaluate different working conditions.

The objective of this work is to study the influence of dissolved oxygen limitation in the PHA production by the bacteria. To assure this oxygen limitation, special attention should be given to the mass transfer.

The oxygen transfer rate (OTR) expresses the concentration of oxygen transferred from the air into the liquid per amount of time. Its formula is:

$$\mathrm{OTR} = \mathrm{Kla}(\mathrm{C}^{*} - \mathrm{C}) \tag{3.1}$$

where the *OTR* is the oxygen transfer rate (mg O_2 L^{-1} h^{-1}), which is equal to the *OUR* (Oxygen Uptake Rate) at steady state, *Kla* being the mass transfer coefficient, C^* the saturating concentration of oxygen in the gas phase (mg O_2 L^{-1}), and *C* the concentration of oxygen in the liquid phase (mg O_2 L^{-1}).

Oxygen is a key product in the bioplastics production since it is used by the microorganisms to form ATPs that will be directed to several tasks, being some of them the storage of polymers and the microorganism growth.

The mass transfer coefficient (*Kla*) is very important to the oxygen transfer system as it varies according to different condition. The velocity of the stirrer and the air flow that is added to the reactor influences proportionally the value of the *Kla*. The higher the *Kla*, the quicker can an oxygen free reactor reach its oxygen saturation level.

In a non-limited reactor like fermentor 5, with a stirring speed of 400 rpm and an air flow of 1 l/min, the *Kla* value is 41,7 h^{-1}. In order to have less oxygen being transferred to fermentor 7, lower stirring speed, lower air flow and the air flow being added to the reactor's headspace were thought to have positive influence on reaching its goal. By sparging air, a big interfacial area is achieved with the air bubbles and by adding air into the reactor's headspace, the mass transfer area is limited to the liquid's surface area.

When performing a cycle characterization, the dissolved oxygen (DO) curve would be the same as a normal enrichment cycle. For the accumulation experiment, the bacteria would uptake the carbon source throughout the whole experiment, so the DO curve is expected to be a "never ending" feeding phase. The DO curve would decrease to value 0 when the experiment started and would never increase again until the end of the experiment. In the following figures, which were all performed by Helena Moralejo Garate in 2010, it is possible to see a cycle and accumulation experiment and its values, performed with the biomass of fermentor 5.

The cycle experiment was working with 30° Celsius, pH 7, 450 rpm and total air flow 1,132 L/min with 0,920 L/min of recycled air. The accumulation experiment was working with 30° Celsius, pH 7, 300 rpm and total air flow 1,487 L/min with 1,288 L/min of recycled air.

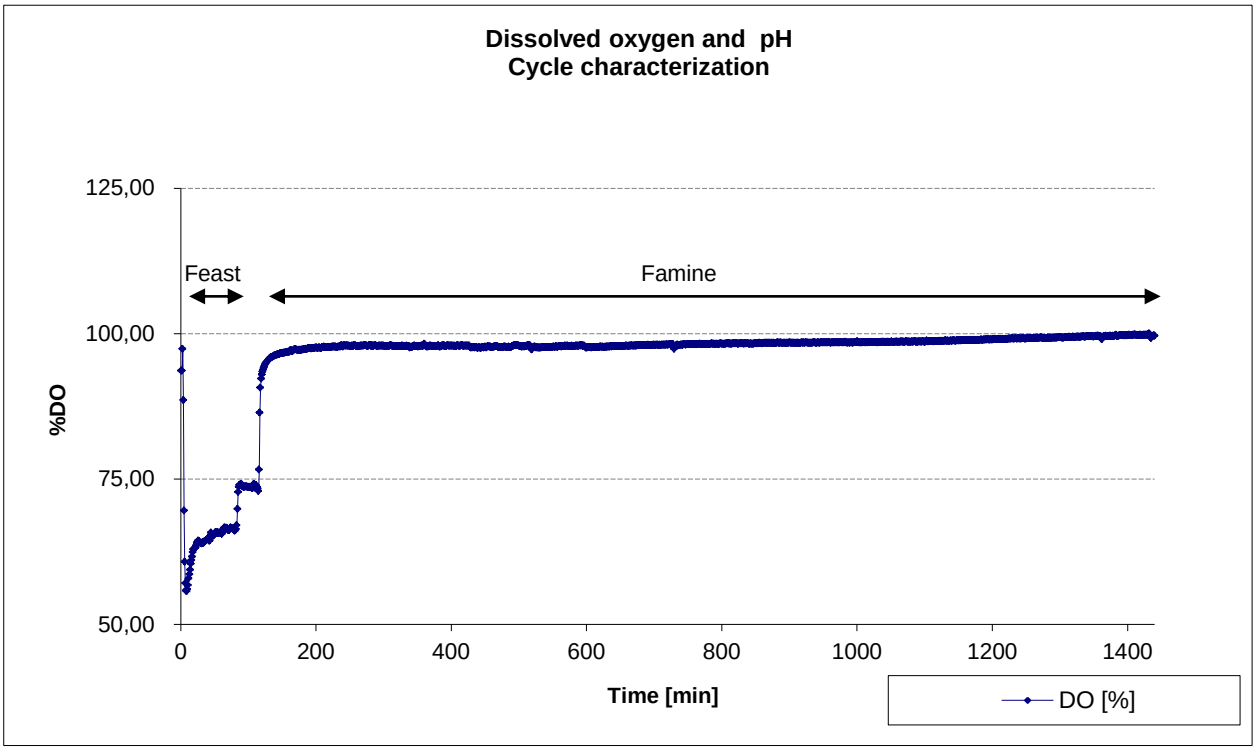

Figure 7 – DO curve from cycle experiment with fermentor 5's biomass

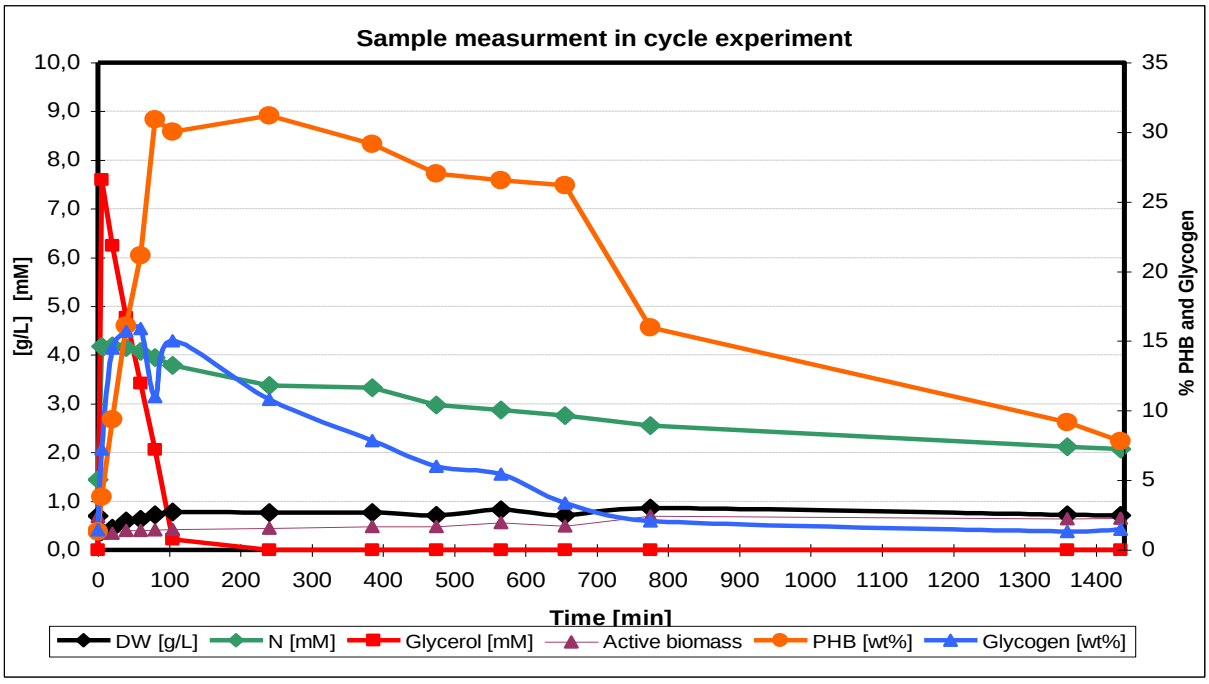

Figure 8 – Sample values evolution from cycle experiment with fermentor 5's biomass

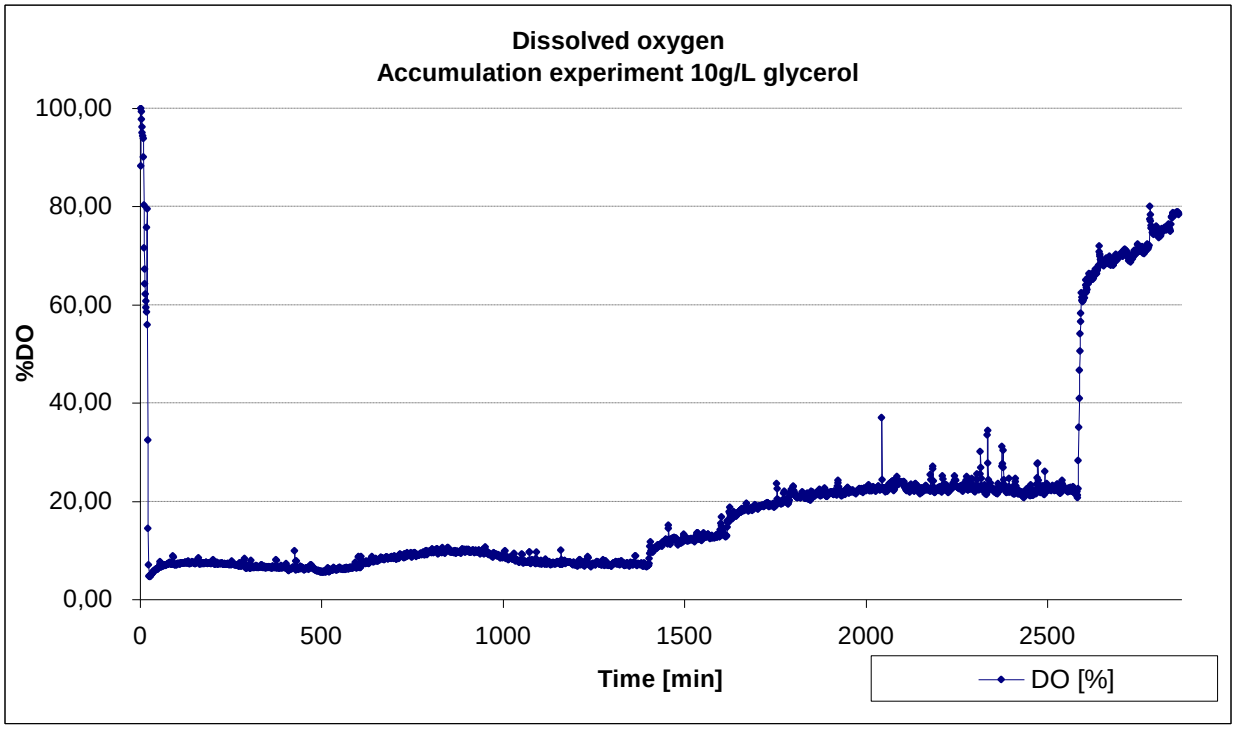

Figure 9 – DO curve from accumulation experiment with fermentor 5's biomass

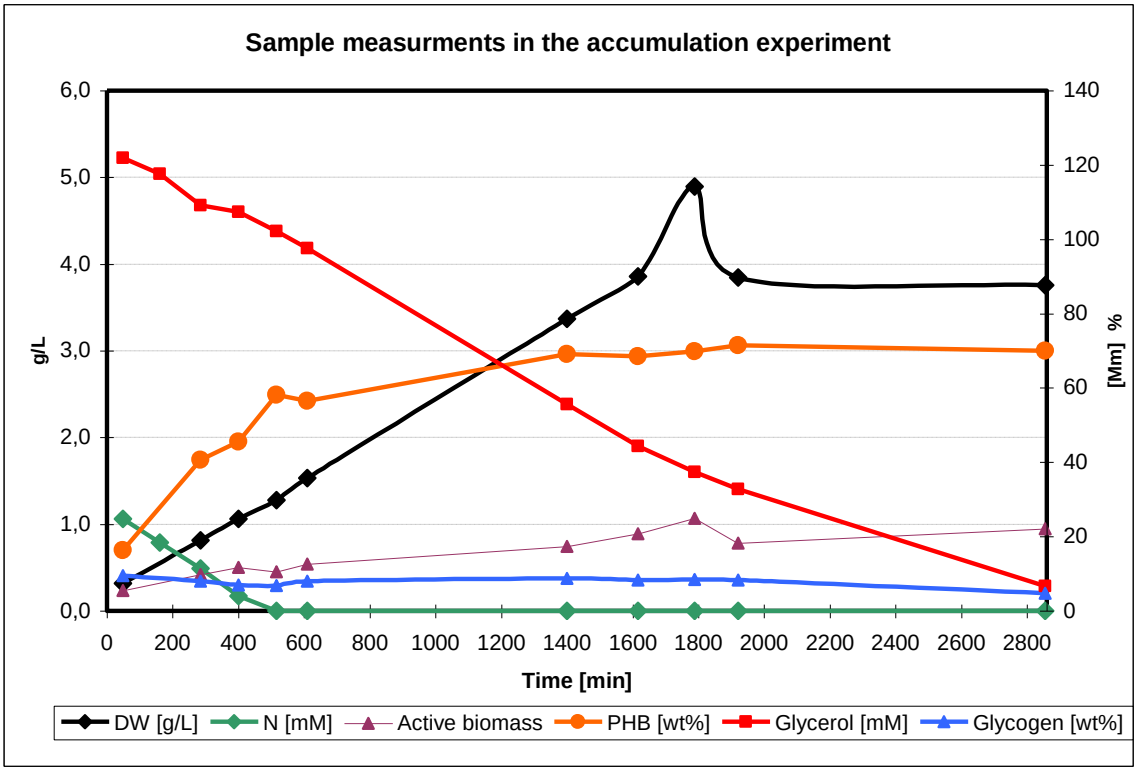

Figure 10 – Sample values evolution from accumulation experiment with fermentor 5's biomass

By observing the previous DO figures, it is possible to see that both are operated under no oxygen restriction, since both minimum values are higher than 0 %. Since there is no oxygen restriction, the polyglucose pathway is not the preferred pathway for storage, so the PHB values should are higher. As it is possible to confirm in the previous figures, in both cycle and accumulation experiments, the PHB value stored is higher than polyglucose. In the cycle experiment, the maximum PHB value stored was around 32 % of the biomass dry weight and the maximum polyglucose stored was around 16 %. In the accumulation experiment the maximum PHB stored was around 72 % and the maximum polyglucose stored was around 10 %.

4 Methods and materials

Adapted from Johnson, K. 2010

4.1 Sequencing batch reactor (SBR) for culture enrichment

A double jacket glass bioreactor with a working volume of 2 litres (Applikon, The Netherlands) was used for the cultivation of PHB producing bacterial cultures. Each SBR cycle consisted of several phases split up over 24 hours: (i) a short start phase in which the 2 litre reactor was half full, (ii) a very short filling phase in which the reactor was filled up with fresh carbon source and medium; the beginning of the filling phase was also the beginning of the feast phase, (iii) a long reaction phase consisting of the remaining feast phase (until carbon source depletion) and the famine phase, (iv) an effluent withdrawal phase in which the broth was withdrawn until the reactor was only half full and (v) a short idle phase . The last phase was immediately followed by the first phase of the next cycle resulting in a continuous sequence of batches with the biomass from the previous cycle being the inoculum for the next cycle.

The reactor was equipped with a stirrer with two standard geometry six blade turbines. The flow of air to the reactor was controlled with a mass flow controller (Brooks Instrument, USA). The experiments had off gas recycling, since the quality of the oxygen off gas measurements was improved significantly with this recycling. The depletion of oxygen in the off gas increases by a factor of 4-7 depending of the extent of the off gas recycling (Johnson 2010). The flow of air was kept between 1-1,5 lN/min with off gas recycling of 0.8-1.2 lN/min. All gas flows were checked with a gas flow analyzer before conducting experiments. Tubing between the reactor and the analyzer was kept as short and thin as possible in order to avoid delays in the measurement. The reactor was temperature controlled at 30°C by means of a water jacket and a thermostat bath (Lauda, Germany). The pH of the reactor liquid was maintained at 7.0 ± 0.1 using 0.5 M HCl and 0.5 M NaOH. The pumps and the pH were controlled by the MFCS/win software. On line measurements (dissolved oxygen, pH, temperature, acid dosage and base dosage) were acquired with the same software.

The amount of biomass removed from the reactor during the effluent withdrawal phase determined the SRT. The reactor was considered to be in a steady operation when, for at least

five days (i) the concentration of total suspended solids (TSS) at the end of the cycle, (ii) the measured SRT and (iii) the length of the feast phase as indicated by the DO changes were constant.

The initial inoculum of the SBR was 90% of aerobic activated sludge from the second aerobic stage of the Dokhaven wastewater treatment plant in Rotterdam, the Netherlands (February 2010) and 10% of biomass from fermentor 5. For each new experiment the biomass from the enrichment reactor was used as inoculum. Biomass was harvested from the reactor in order to study the PHA production capacity of the biomass in a production step.

4.2 SBR for PHA production

The biomass harvested in the enrichment reactor, once it was under stable operation, was used for PHB production. For this purpose the same set-up as for the culture selection was used. 1 L of culture from the end of a SBR cycle was mixed with 1 L of glycerol (in excess, 10 g/L) and ammonium-free medium (same composition as for the SBR, but with no NH_4Cl). Growth was limited in these experiments as no nitrogen source was supplied and only a small amount (if any) remaining nitrogen source from the previous SBR cycle was available. The progress of the experiments was monitored via online (DO, pH, acid and base dosage, off gas CO_2 and O_2) and offline (glycerol, PHA, ammonium, polyglucose) measurements.

4.3 Analytical methods

The concentration of dissolved oxygen (DO) in the reactor was measured with a DO electrode (Mettler Toledo, USA) as percentage of air saturation and the pH was monitored with a pH electrode (Mettler Toledo, USA). The temperature of the reactor broth was measured with a thermo element. The amount of acid or base dosed for pH control was measured online. Carbon dioxide and oxygen partial pressures in the gas entering and leaving the reactor were analyzed in dried gas with a gas analyzer (NGA 2000, Rosemount, USA). Gas measurements and calibrations were corrected with the actual atmospheric air pressure for standard conditions. Samples taken from the reactor for analysis of glycerol and ammonium were immediately filtered with a 0.45 µm pore size filter (PVDF membrane, Millipore, Ireland). The glycerol concentration in the supernatant was measured with a Chrompack CP 9001 gas chromatograph (Chrompack, The Netherlands) equipped with a FID, on a HP Innowax column. The ammonium concentration was either determined by spectrophotometry as ammonium-nitrogen with a cuvette test (Lange, Germany) or with a Chemiquik 2000 FIA machine. The biomass concentration was measured as total suspended solids (TSS) by filtration according to standard methods (Taras, Greenberg et al. 1971). The amount of PHB was subtracted from the TSS to calculate the concentration of active biomass. The active biomass concentration was converted from g/L into carbon moles per litre (Cmol/L) assuming a composition of $CH_{1.8}O_{0.5}N_{0.2}$ with a molecular weight including ash of 25.1 g/Cmol (Beun, Dircks et al. 2002).

Samples taken for PHA and polyglucose analysis were added to 50 ml tubes containing 5 drops of formaldehyde in order to stop all biological activity. Samples were subsequently centrifuged and freeze-dried. For PHA analysis, pure PHB (Sigma) was used as a standard in the analysis and treated alongside. Freeze-dried biomass samples and 3 standards were weighed with an analytical balance and transferred into tubes with screw caps. 1 mg of benzoic acid in 1-propanol was added as the internal standard. 1.5 mL of a mixture of concentrated HCl and 1-propanol (1:4) and 1.5 mL of dichloroethane was added. The closed tubes were heated for 2 h at 100°C. After cooling, free acids were extracted from the organic phase with 3 ml water. 1 mL of the organic phase was filtered over water-free sodium sulphate into GC vials. The PHAs in the organic phase were analyzed by gas chromatography (model 6890N, Agilent, USA) equipped with a FID, on a HP Innowax column. Results were expressed as weight percentage of PHB of the total solids.

For polyglucose samples, freeze-dried samples were weighted with the same analytical balance and transferred into tubes with screw caps. In order to break the polyglucose into its glucose monomers, an acidolysis step is needed. HCl 6 M is diluted to 0,6 M with a final volume of 4 or 5 mL. For this purpose, the dilution machine is used. Tubes containing the biomass and the known volume of 0,6 M HCl are heated during 3 h at 100°C using a block heater. After the tubes are cooled to room temperature, around 2,5 mL of each tube are added to Eppendorf tubes with the help of syringes with filters, to make sure that only the liquid phase is present. The liquid phase is analyzed with the HPLC. The concentration of glucose is measured. This glucose concentration indicates de amount of glucose that is present in the known 0,6 M acid volume. In this volume, the amount of biomass is known, so the amount of glucose per unit of biomass can be calculated. For the calculation of the polyglucose concentration, a molecular mass of 162 g/mol polyglucose is used (assuming that during the polymerization, each mol of glucose looses 1 mol of water).

4.4 Mass transfer coefficient

The oxygen limitation is achieved by low stirring and low air supply. In other similar systems, the air is usually sparged through a tube with small holes, so a big interfacial area is achieved with the air bubbles. In this research, the air was continuously added into the reactor's headspace. By doing so, the mass transfer area is limited to the liquid's surface area.

To calculate the *Kla*, 0,1 mL of medium and 1,9 mL of water were added into the reactor to make the *Kla* measurement more correct, as the normal daily cycle also uses practically the same amount of water and medium and also due to the absorbing properties of the medium salts. In an oxygen saturated liquid, nitrogen gas is added into the reactor's headspace while the stirring pump is working, resulting in a decrease of the DO. The different air flows are controlled by an air flow controller and the different stirring speeds are controlled by the stirring speed controller. Once the reactor is oxygen-free, nitrogen is replaced by air. By adding air, the DO starts to rise until it reaches the saturation level. The DO control was done as described before. Using the formula of the OTR it is possible to calculate the *Kla*:

$$\mathrm{OTR} = \mathrm{Kla}(\mathrm{C}^{*} - \mathrm{C}) \tag{4.1}$$

Using the dynamic method described by Garcia Ochoa (2009), the OTR is equal to the following formulas, for both absorption and desorption:

$$\begin{aligned} &\frac{dC}{dt} = Kla(C^* - C) \\ &\Leftrightarrow \int \frac{dC}{(C^* - C)} = Kla \int dt \\ &\Leftrightarrow \ln \frac{(C^* - C_2)}{(C^* - C_1)} = -Kla(t_2 - t_1) \quad \rightarrow \quad C_1 = C_0 \vee t_1 = 0 \\ &\Leftrightarrow C_2 = C^* \times \exp(-Kla.t) \end{aligned} \tag{4.2}$$

$$\begin{aligned} &\frac{dC}{dt} = Kla(C^* - C) \\ &\Leftrightarrow \int \frac{dC}{(C^* - C)} = Kla \int dt \\ &\Leftrightarrow \ln \frac{(C^* - C_2)}{(C^* - C_1)} = -Kla(t_2 - t_1) \quad \rightarrow \quad C_1 = 0 \vee t_1 = 0 \\ &\Leftrightarrow \ln(1 - \frac{C_2}{C^*}) = Kla.t \end{aligned} \tag{4.3}$$

The percentage of air saturation had to be converted into oxygen concentration. At a temperature of 30° Celsius and a pressure of 760 mmHg, the maximum value of oxygen solubility is 7,5 mg O_2 L^{-1} (Radtke, White et al. 1998).

5 Results and discussion

5.1 Mass transfer coefficient

In order to have full control over the reactor's behaviour, *Kla* measurements were performed in fermentor 7 before starting the enrichment of the bacteria culture. Different stirring speeds and different air flows were tested.

Using the method described in the previous chapter, the *Kla* values were determined for both adsorbing and desorbing curves. The absorbing and desorbing curves and their *Kla* values were the following:

Table 2- Kla values for 1 L/min air flow

Rpm	*Kla* (h^{-1})
300	1,708
350	5,751
400	6,198
450	12,008

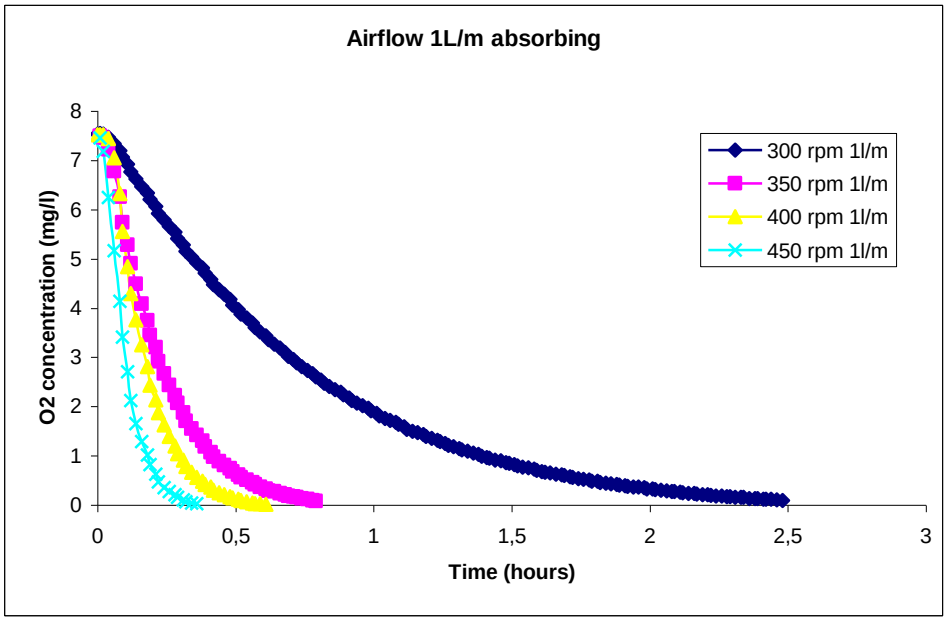

Figure 11 – DO curve for Kla measurement with 1 L/min air flow

Table 3 - Kla values for 2 L/min air flow

Rpm	*Kla* (h^{-1})
300	1,875
350	5,189
400	7,295
450	9,403

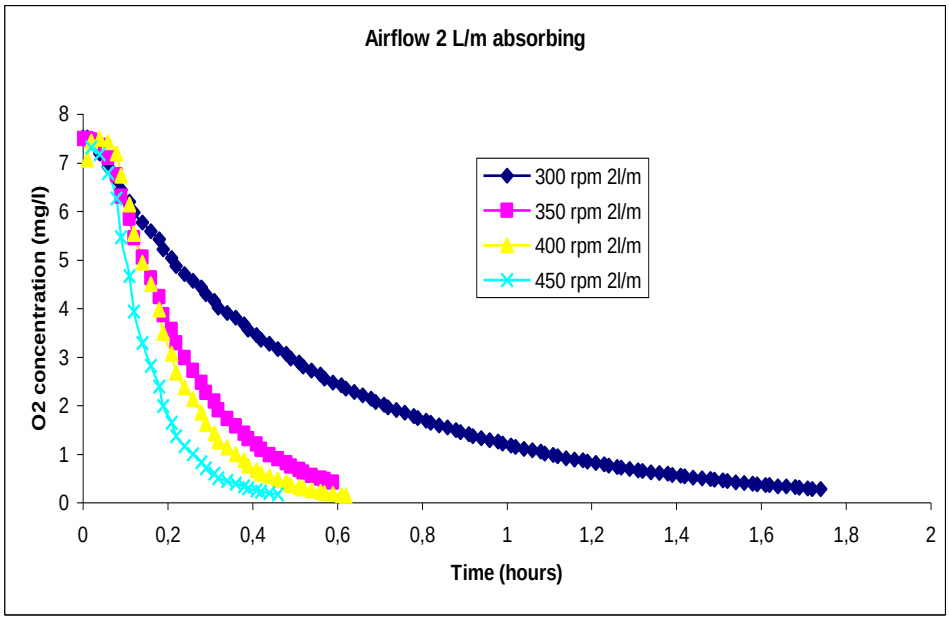

Figure 12 - DO curve for Kla measurement with 2 L/min air flow

Table 4 - Kla values for 1 L/min air flow

Rpm	*Kla* (h^{-1})
300	1,493
350	4,633
400	5,323
450	7,402

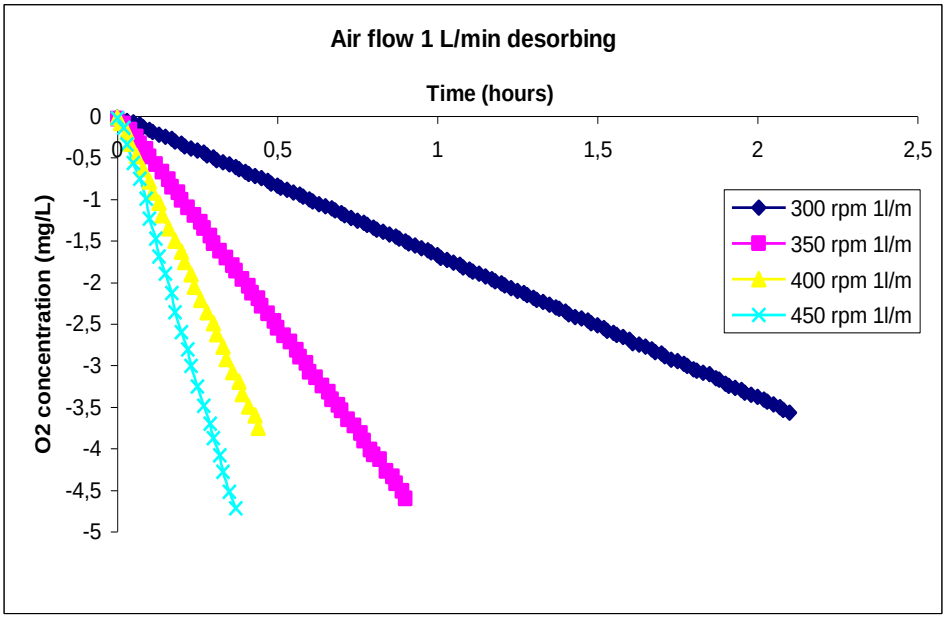

Figure 13 - DO curve for Kla measurement with 1 L/min air flow

Rpm	*Kla* (h^{-1})
300	1,692
350	5,106
400	6,766
450	9,183

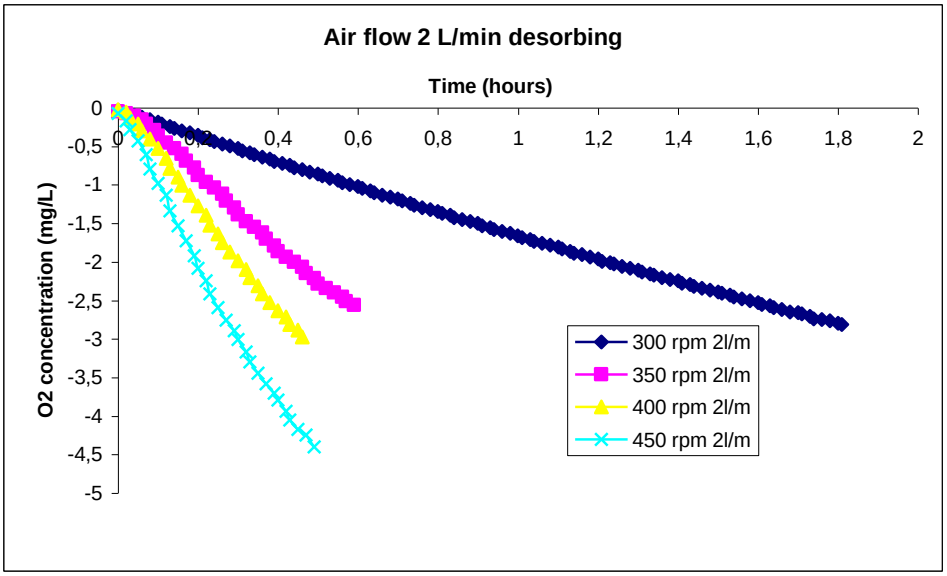

Table 5 - Kla values for 2 L/min air flow

Figure 14 - DO curve for Kla measurement with 2 L/min air flow

By comparing the values on both absorbing and desorbing tables, one can see that both absorbing and desorbing *Kla* values are very similar, as it was expected. Because the air flow was better controlled and had a better constant flow in the desorbing curves, these were the results taken into account. The increase of the stirring speed enhances the mass transfer, so the *Kla* values gets higher. The air flow doesn't have a big impact as it only affects the time of the headspace filling, i.e., when changing from nitrogen to air, the 1 L headspace will take 1 minute with 1 L/min air flow and 30 seconds with 2 L/min to get full of the added gas. It is possible to say that for every 50 rpm added, the *Kla* increases 1,5 fold. To make sure that there is a low oxygen supply to the reactor, the stirring speed was set at 400 rpm and 1 L/min of air flow.

5.2 Culture enrichment

Using all the conditions referred in the previous chapters, the enrichment of the culture started. In the following figure it is possible to see that during the initial cycles, the feast phase is longer because the microorganisms that are able of storing compounds are not dominant. The microorganisms that are present in the reactor are using the substrate directly for growth. The shortening of the feast phase is due to the higher capacity of substrate uptake rate by bacteria. Also, when the feast phase becomes stable, it symbolizes that the mixed culture has at least one dominant bacteria, capable of faster substrate uptake.

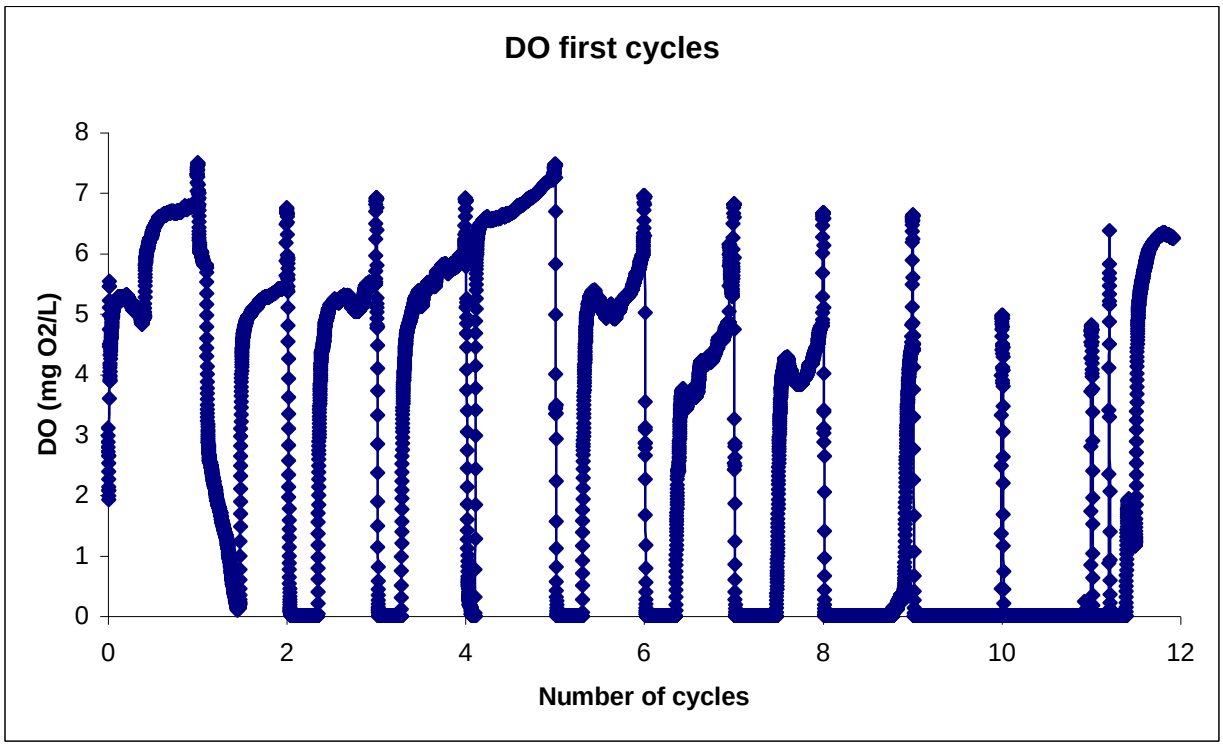

Figure 15 - DO curve for first cycles of culture enrichment

In the previous figure, one can observe that the reactor took some cycles to start stabilizing. The steady operation was reached a few cycles after the end of the ones show on the figure. As mentioned before, the DO curve is not the only key component of the stability of the reactor's operation. TSS and ammonium are also key components to check the stability of the reactor's operation, as well as the length of the feast phase. Before every experiment was performed, these stability conditions had to be checked. The tables in appendix A show the evolution of

some of these variables. Note that the cycle number symbolizes the number of cycles that the reactor had done since it started its culture enrichment.

Table 6 – Length of the feast phase evolution

Date	Cycles	Feast phase length (hours)
12-3-2010	21	4,5
13-3-2010	22	4,2
14-3-2010	23	4,4
15-3-2010	24	4,5
16-3-2010	25	4,1
17-3-2010	26	4,3
18-3-2010	27	4,4
19-3-2010	28	4,6
20-3-2010	29	4,5
21-3-2010	30	4,5
22-3-2010	31	4,7
23-3-2010	32	5,1

Date	Cycles	TSS (g/L)
8-3-2010	18	0,549
9-3-2010	19	0,641
10-3-2010	20	0,554
15-3-2010	25	0,608
16-3-2010	26	0,721
18-3-2010	28	0,675
19-3-2010	29	0,630
24-3-2010	34	0,822

Table 7 – TSS evolution in the early cycles

Table 8 – Ammonium evolution in the end of the feast phase and end of cycle

Taking a look at the previous tables, one can see that the length of the feast phase tends to stabilize after some days after the start of the culture enrichment. The average length of the feast phase is 4,48 ± 0,24. It is also possible to see that the TSS values tend to be stable around 0,65 ± 0,08 g/L. The ammonium values are more important because they indicate if the reactor is stable and they have information about the growth of bacteria according to the amount of ammonium consumed during each cycle. Since one of the objective of this thesis is that there is practically no growth during the feast phase, the ammonium value related to the end of the feast phase should be equal to the value of the end of the previous cycle plus the amount of ammonium added in the medium. With those results it is possible to analyze if the bacteria are focused on storing polymers during the feast phase and growth during the famine phase. The amount of ammonium present in the medium is around 20 mg N/L. With this value, it is possible to calculate the amount of ammonium uptake by the bacteria during the feast phase. In the first cycle of the table, the bacteria uptake around 23 mg N/L and later started to decrease the ammonium uptake, being 0,50 mg N/L in the last cycle of the table. Once all the variables kept almost constant values, it was possible to perform cycle characterization experiments and PHA accumulation experiments.

Date	2-3-2010	3-3-2010	4-3-2010	5-3-2010	8-3-2010	9-3-2010	10-3-2010	19-3-2010
Cycle	12	13	14	15	16	17	18	23
End of the cycle (mg N/L)	37,5	47,3	40,7	41,1	22,9	22,5	22,2	18,7
End of the feast phase (mg N/L)	34,6	50,7	4,3	47,2	36,2	36,7	41,7	30,2

In the next figure, one can observe two stable culture enrichment cycles where the length of the feast phase and the pH had almost the same values:

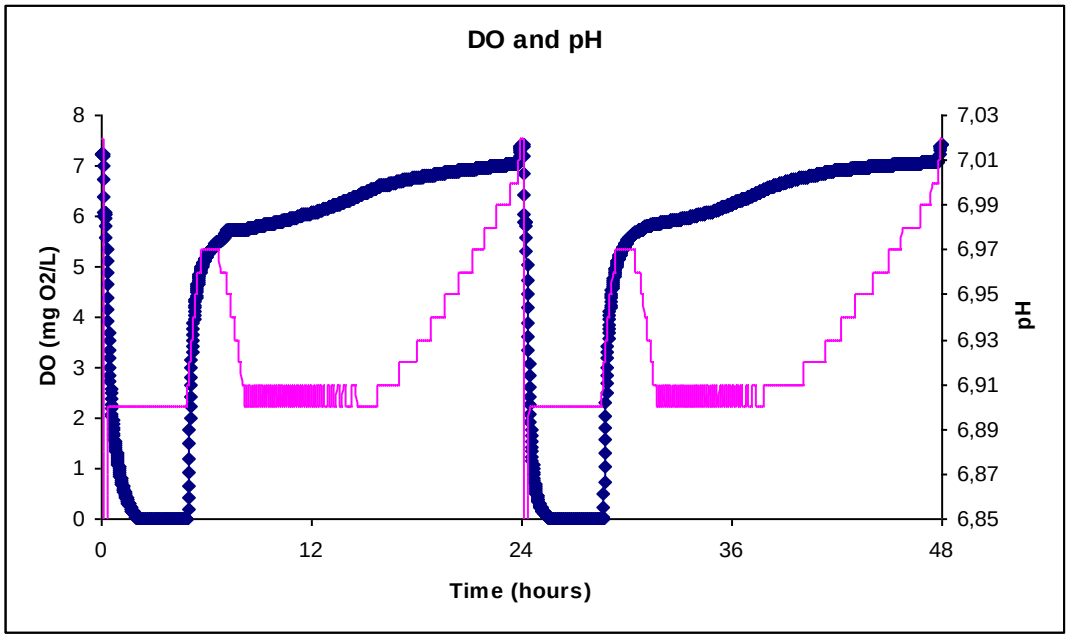

Figure 16 – Example of two cycles working on a steady operation

5.3 Cycle characterization and PHA accumulation

During this thesis, and because this is a relatively new process, some discoveries were found when some experiments failed reaching the oxygen limitation. Those breakthroughs will be referred in this chapter. Note that time is the bottleneck of this process, since small mistakes lead to an, at least, 24 hour delay and plus, the sample measurements requires time and patience.

From the beginning of this thesis in February, the first step was to study the *Kla*. Once the *Kla* values were determined, the culture enrichment started. Once the stability operation mode was reached, some cycle measurements were performed that ended falling or being cancelled. The reasons were some small and unforeseen errors occurring in the reactor, for example the temperature tube being under the reactor, leading to an excessive temperature rise, damaging the bacteria or a disconnected tube in the air flow controller or an error in one of the main solvents for PHA or the instability of the bacteria, leading to a bigger length of the feast phase, or a non ending feast phase. Having made these mistakes, it was possible to correct them in future experiments.

Two of these failed experiments that influenced the future ones will be shortly introduced here. The first experiment was an accumulation experiment performed on the 20th of April. The

conditions were 30° Celsius, 450 rpm, 1 L/min air flow and pH 7. The DO curve is present in the next figure:

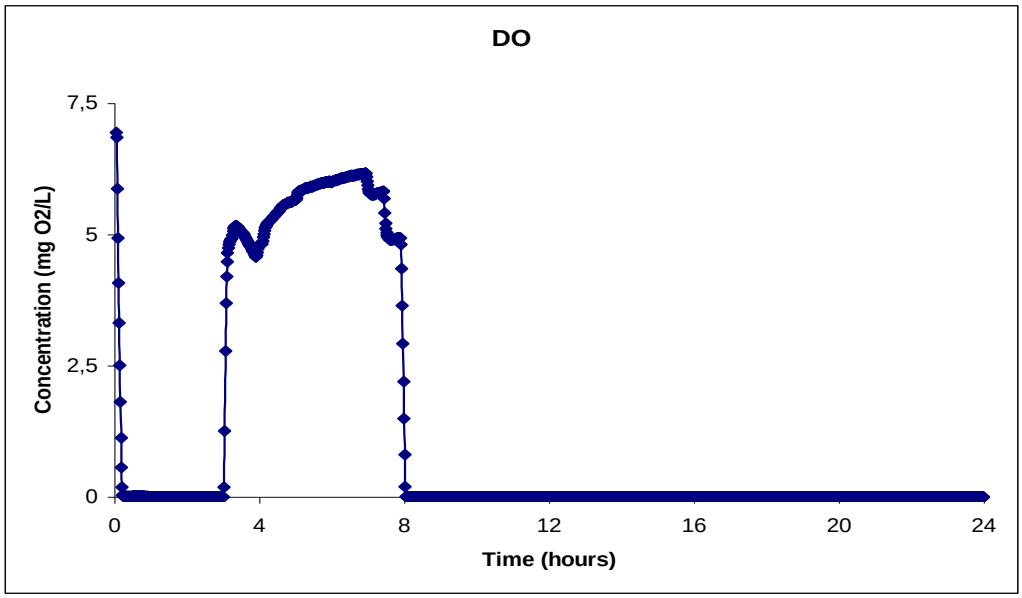

Figure 17 – DO curve for a cycle measurement with an oxygen spike

As mentioned in a previous chapter, the DO curves in accumulation experiments are suppose to have the value 0 throughout the whole experiment. As one can visualize in the previous figure, that doesn't happen in this experiment. When every sample was taken, the liquid level dropped a few centimetres. When a specific number of samples were taken, the liquid level dropped so much that it reached the blades height. When this happened, the mass transfer increased so much that an excess of oxygen was given to the system. As such, the bacteria had now an excess of oxygen to use, leading to an increase of the dissolved oxygen. To make sure that this was the reason why the DO curve had those results, *Kla* tests were performed using both 1 and 2 L headspace and see the influence of 1 and 2 blades. The stirring speed was kept at 450 rpm. The results are presented in the following table:

Table 9 – Kla measurements while changing the number of blades and headspace capacity

Headspace (L)	Number of blades	*Kla* (h^{-1})
1	1	5,394
2	1	1,185
1	2	8,177
2	2	1,25

With two blades and 1 L headspace, due to the proximity of the liquid level to the blades, turbulence was created leading to air bubbles formation and therefore, a big increase in the Kla. According to the literature, in order to have different mixing comparing to the use of 1 blade, it is necessary to assure that the space between the blades is bigger than the blade's diameter, otherwise they will work as only one blade (Puthli, Rathod et al. 2005). Comparing the blade results, it is possible to conclude that for 2 L headspace, the proximity of the liquid level to the blades is independent of the number of blades, so the *Kla* value is almost the same but for 1 L headspace, the distance of the blades make them act like described before, so there are distinct *Kla* values.

With this new information, a strategy was thought that could maintain the liquid level stable and the *Kla* constant. That strategy was a dilution method. When every biomass sample was withdrawn, the same amount of medium was added to the reactor. The addition of medium into the reactor wouldn't have any influence on the bacteria's activity. Only the pH changed since the amount of acid and base dosed to the reactor was higher due to the acidity of the medium. When the samples were analyzed, a correction was used on all the measured concentrations in the liquid phase to have the real values since the values in each sample were diluted (see Appendix C). The number of blades was kept the same, 2.

Now using the diluting method in all the next experiments, a cycle experiment was done on the 28th of May. The conditions were kept exactly the same as in fermentor 7: pH 7, 30° Celsius and 1 L/min air flow. The stirring speed was 450 rpm. The DO curve is presented in the following figure:

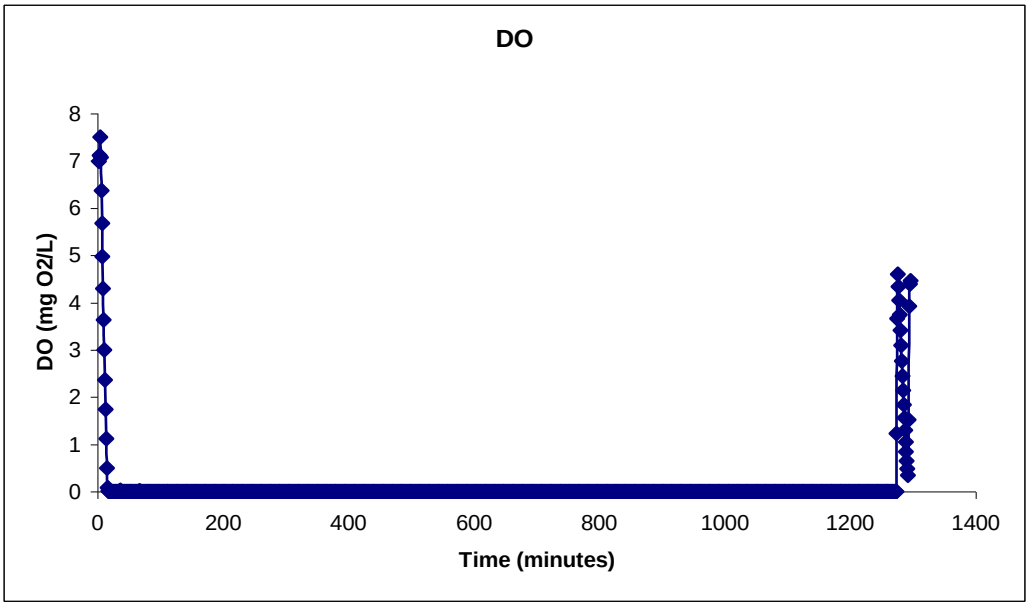

Figure 18 – DO curve of cycle experiment that failed

The expected time of the feast phase was of 4 hours but as it is seen the figure, the feast phase lasted almost the entire cycle. After analyzing the design of fermentor 2's lid, it was clear that the blades were clearly smaller than the ones used in fermentor 7, therefore, for the same conditions, the *Kla* was smaller in fermentor 2. To make sure that the same conditions and results were obtained in fermentor 2, fermentor 7's head was used in the following experiments in fermentor 2.

All the following experiments used these two changes. The first samples of all the following experiments were taken from fermentor 7 to minimise the dilution effect.

5.3.1 Cycle Characterization

The first cycle experiment using the previous changes was performed on the 3rd of May. The operating conditions were of 30° Celsius, pH 7, 1,455 L/min total air flow with 1,267 L/min recycled air, 950 g of biomass used and stirring speed of 450 rpm. The DO curve is presented in the following figure:

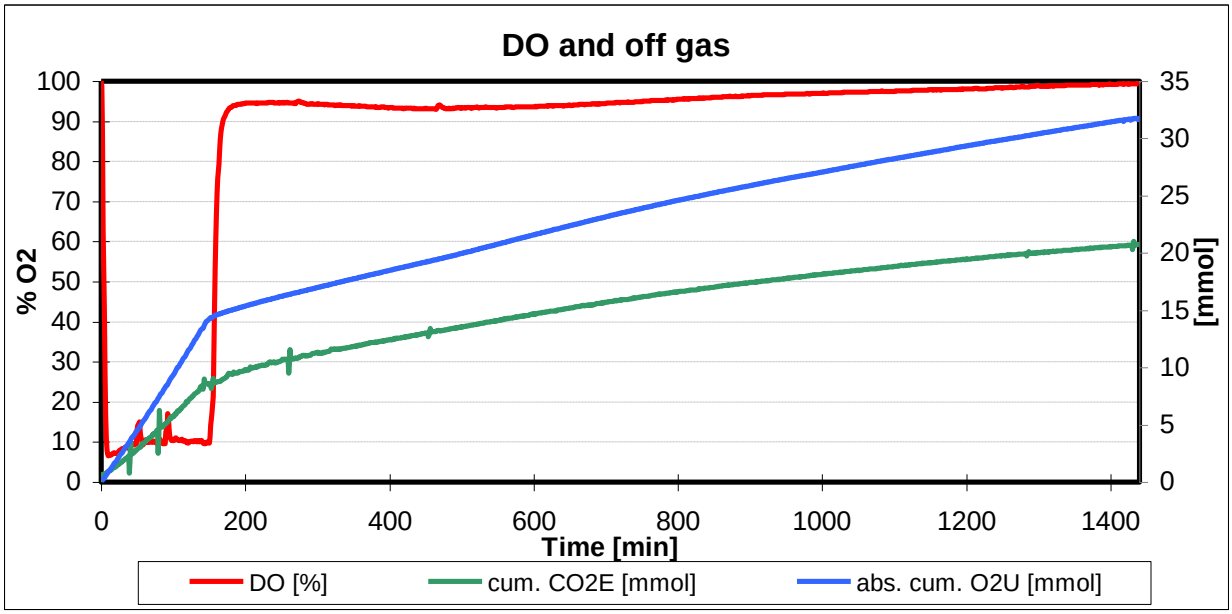

Figure 19 – DO curve for cycle experiment done on the 3rd of May

Analyzing figure 19, one can see that the DO concentration value never reached 0 of dissolved oxygen and every time a sample was taken, the DO rose, showing that the reactor wasn't operating on a dissolved oxygen limitation system. This might have happen due to a higher rotation speed than usual. Without oxygen limitation, this experiment acted like a normal experiment, without dissolved oxygen limitation. Compared to a normal cycle from fermentor 5, this reactor had an inferior *Kla*. These results were analyzed so it would be possible to compare future dissolved oxygen limitation results with these ones.

As described in a previous chapter, the samples were analyzed. In the following figure, it is possible to observe the evolution of the different components of the system:

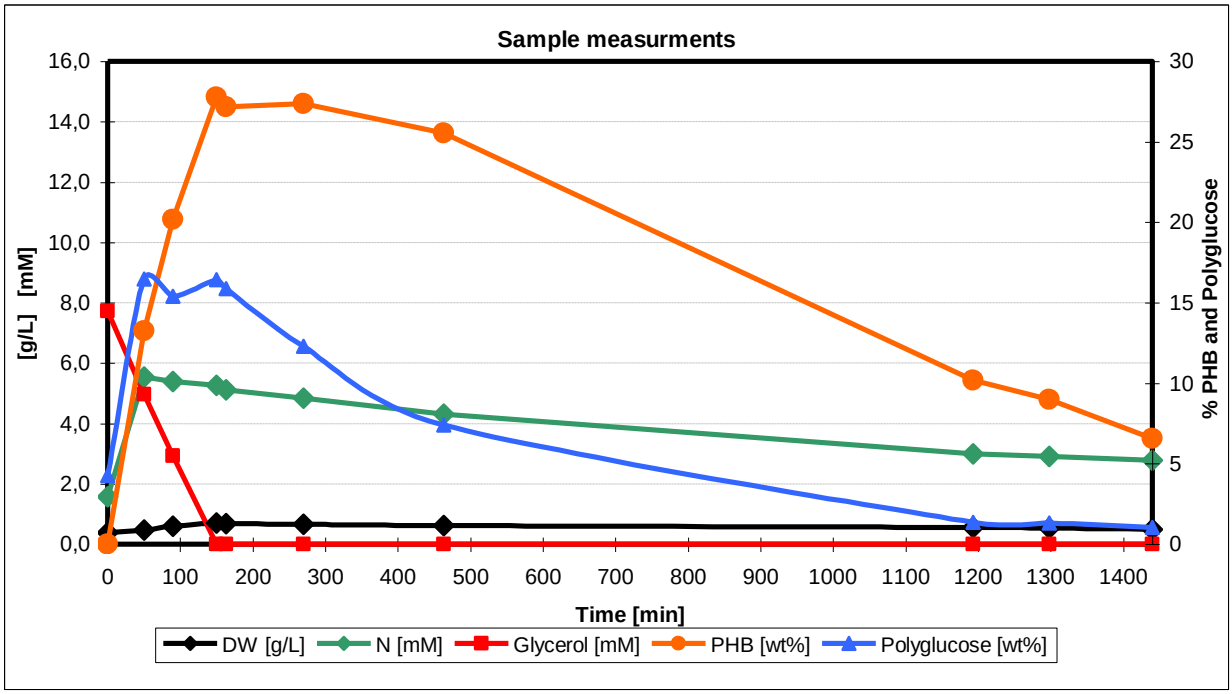

Figure 20 – Samples evolution throughout the cycle experiment done on the 3rd of May

It is possible to see in figure 20 that the end of the feast phase matches with the ending of the glycerol present in the reactor, step where PHB and polyglucose reach a maximum storage percentage. The maximum PHB stored is of approximately 27 % and the polyglucose maximum is about 15 % of the bacteria dry weight. The polyglucose curve seems to keep a stable maximum during 3 of the samples and only when the famine phase starts, does the polyglucose starts to decrease. The ammonium curve is quite important as during the feast phase practically no ammonium is uptaken by the bacteria, which means that they are storing instead of growing. In the famine phase, all the variables decrease fast except for the dry weight (DW). Although the stored polymers are being consumed, the DW curve increases and the ammonium started being consumed at a higher rate, showing that growth is happening.

The next experiment was done on the 17th of May. To make sure that there was dissolved oxygen limitation, the stirring speed was set to 400 rpm. The operating conditions were of 30° Celsius, pH 7, 1,037 L/min total air flow with 0,857 L/min recycled air and 950 g of biomass used. The following figure shows the DO curve:

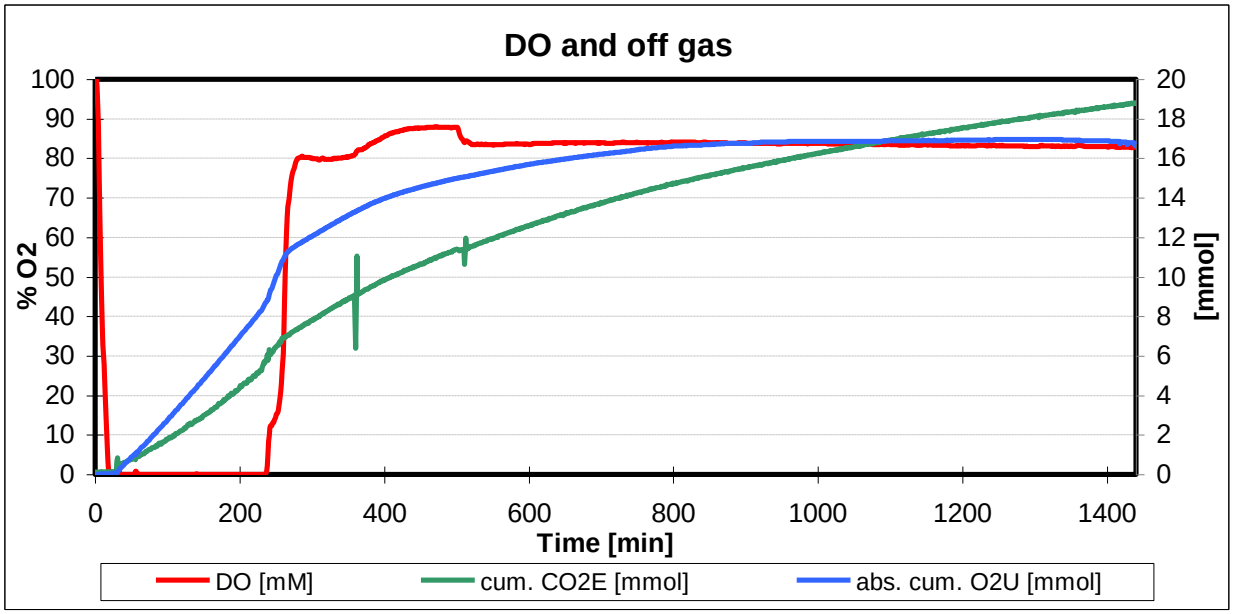

Figure 21 – DO and off gas curves of the cycle experiment done on the 17th of May

It is possible to see in figure 21 that the feast phase has dissolved oxygen limitation. The off gas curves indicate the end of the feast phase when the rate of production changes. In the next figure, it is possible to visualize the evolution of the different variables of the system:

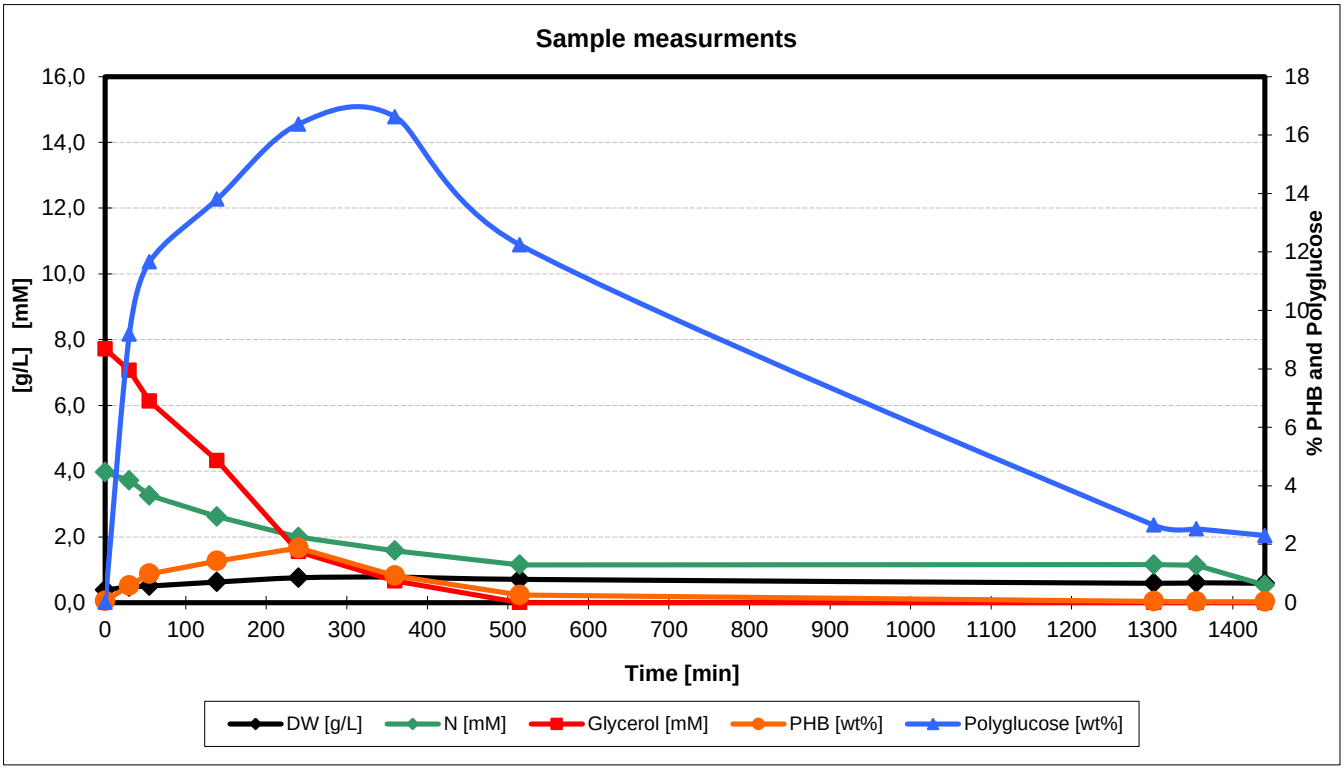

Figure 22 - Samples evolution throughout the whole experiment done on the 17th of May

Observing figure 22, one can see that the maximum PHB stored was 1,87 % and the maximum polyglucose stored was approximately 17 %. Comparing the two previous figures, it is possible to see that, although in the DO curve the feast phase ends around minute 250, the glycerol curve reaches a value of approximately 0 after minute 360. The fact that the PHB curve rises until minute 250 and then starts decreasing and the polyglucose rises until minute 250-360 and starts decreasing leads to conclusion that the glycerol values should not be accepted after minute 250 . Once there is the consumption of the polymers and the DO curve rises, it symbolizes the end of the feast phase and the beginning of the famine phase. Looking at the ammonium curve, it keeps a normal consumption rate until minute 500, meaning that the bacteria are growing during the entire feast phase and there is small growth percentage in the famine phase. The DW curve increases until minute 250 and after it slightly continues to increase until minute 500 where the values finally stabilize.

Comparing the two cycle experiments, it is possible to see the clear difference in the storage compounds:

Table 10 – Polymer storage results for the cycle experiments

	PHB (%)	Polyglucose (%)
No dissolved oxygen limitation	27	15
Dissolved oxygen limitation	1,87	17

Clearly the limitation of oxygen affects the bacteria storage system. Regarding the PHB values, once there is a dissolved oxygen limitation, the bacteria prefer to store polyglucose for its need of less oxygen moles to oxidize the $NADH_2$ to NAD+, so, the PHB storage is significantly lower when no oxygen is available. Regarding the polyglucose, in the first cycle there is a stable maximum value that is similar to the maximum in the second cycle, therefore, it is possible to say that the maximum percentage of stored polyglucose by these bacteria during a cycle experiment is around 17 %. Taking a look at all the rates and yields in the feast phase, it is possible to compare the differences between the two cycle experiments:

Table 11 – Total conversions in the feast phase of the materials used and produced in the cycle experiments

Total conversions				
Experiment	**Glycerol [Cmmol]**	**PHB [Cmmol]**	**X_{active} [Cmmol]**	**Polyglucose [Cmmol]**
No dissolved oxygen limitation	-45,142	18,696	6,186	7,613
Dissolved oxygen limitation	-45,144	1,373	22,720	9,720

Table 12 - Total conversions in the feast phase of the materials used and produced in the cycle experiments

Total conversions			
Experiment	**NH_4^+ [mmol]**	**CO_2 [Cmmol]**	**O_2 [mmol]**
No dissolved oxygen limitation	0,544	8,605	-14,287
Dissolved oxygen limitation	2,703	5,925	-8,943

Table 13 - Observed yields in the feast fase relative to glycerol

Observed yields				
Experiment	**$Y_{PHB/Glycerol}$ [Cmmol/Cmmol]**	**$Y_{Xactive/Glycerol}$ [Cmmol/Cmmol]**	**$Y_{CO2/Glycerol}$ [Cmmol/Cmmol]**	**$Y_{Polyglucose/Glycerol}$ [Cmmol/Cmmol]**
No dissolved oxygen limitation	0,414	0,137	0,191	0,169
Dissolved oxygen limitation	0,0304	0,503	0,1313	0,215

Table 14 - Observed yields in the feast phase

Observed yields					
Experiment	**$Y_{Xactive/Polyglucose}$ [Cmmol/Cmmol]**	**$Y_{Xactive/PHB}$ [Cmmol/Cmmol]**	**$Y_{CO2/PHB}$ [Cmmol/Cmmol]**	**$Y_{CO2/O2}$ [Cmmol/Cmmol]**	**$Y_{CO2/Polyglucose}$ [Cmmol/Cmmol]**
No dissolved oxygen limitation	0,813	0,331	0,460	-0,602	1,130
Dissolved oxygen limitation	2,3374	16,5435	4,3145	-0,6625	0,6096

Table 15 – Average biomass specific rates in the feast phase

Average biomass specific rates			
Experiment	**$q_{Glycerol}$ [Cmmol/Cmmol/h]**	**q_{PHB} [Cmmol/Cmmol/h]**	**$q_{Polyglucose}$ [Cmmol/Cmmol/h]**
No dissolved oxygen limitation	-0,510	0,2110	0,0859
Dissolved oxygen limitation	0,241	0,00734	0,0519

Table 16 – Average biomass specific rates in the feast phase

Average biomass specific rates			
Experiment	**q_{CO2} [Cmmol/Cmmol/h]**	**q_{O2} [mmol/Cmmol/h]**	**q_{NH4+} [mmol/Cmmol/h]**
No dissolved oxygen limitation	0,0971	-0,1613	0,0061
Dissolved oxygen limitation	0,0317	-0,0478	0,0144

Observing tables 11 and 12, one can observe that the PHB converted value is a lot higher in the non limited experiment and that the polyglucose converted value is higher on the limited experiment, as expected. Since the same amount of glycerol was dosed to both experiments and taking a look at the active biomass (X_{active}) converted values and the ammonium converted values, one can see that the values are higher in the limited experiment which symbolizes

higher growth. This is not what was expected as the growth pathways uses more ATP and $NADH_2$. Now analyzing table 13, in the limited cycle experiment, one can see that the active biomass yield related to glycerol is higher than sum of the yields of PHB and polyglucose related to glycerol, which means that in this experiment, the growth percentage is higher than the storage, in the feast phase. In table 12 it is possible to see that the consumed oxygen values are higher in the non limited system, which means that the presence of oxygen is the key to have higher storage values. In the limited system less CO_2 is produced, meaning that more polyglucose is stored compared to PHB. The biomass growth on the non limited system only happens on the famine phase.

5.3.2 PHA accumulation

The first PHA accumulation experiment was performed on the 18th of May. This experiment was done with no dissolved oxygen limitation, meaning that the air was sparged into the reactor. To make sure that the reactor had enough oxygen, the Kla was measured. The Kla value was determined for 420 rpm and its value was 15,765 h^{-1}, which comparing with the non-sparged system, it is almost two times bigger. The operating conditions of this experiment were 30° Celsius, pH 7, 420 rpm, 940 grams of biomass and 1,010 L/min of total air flow with 0,818 L/min recycled air.

The DO curve is presented in the following figure:

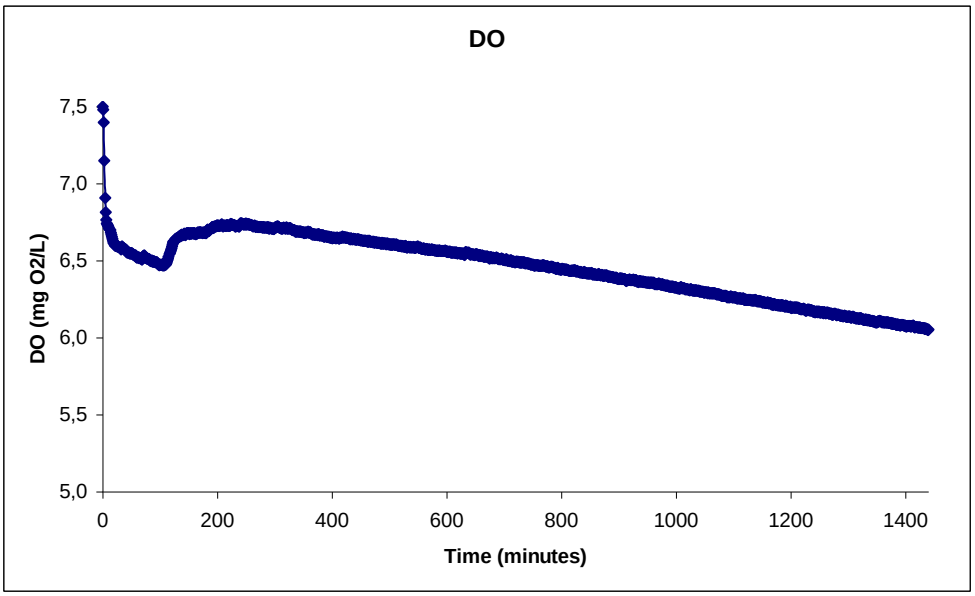

Figure 23 – DO curve for an accumulation experiment done on the 18th of May

It is clear that this experiment had no oxygen limitation just by looking at the lower value of the DO curve. Since the Kla is so high, there is an excess of oxygen being supplied to the reactor and only a small part is uptake by the bacteria. When in the presence of an excess of carbon source, the bacteria will uptake it until the end of the experiment, which is the reason there isn't a feast and famine regime. In the next figure, it is possible to see the evolution of the different variables of the system:

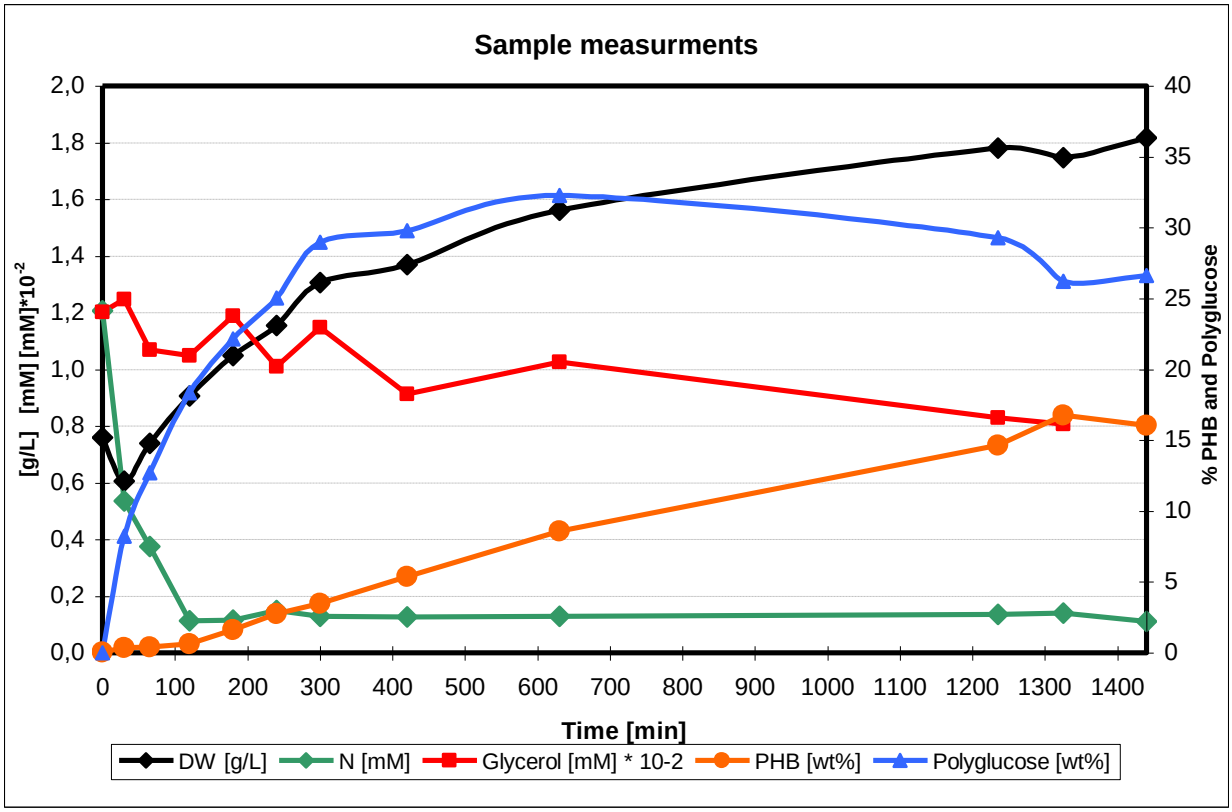

Figure 24 – Samples evolution throughout the whole experiment done on the 18th of May

The maximum polyglucose stored was around 30 % and the maximum PHB stored was around 17 %. The glycerol curve has several oscillations during the whole cycle. The reason why that happens is unknown, but it was expected for the values to decrease in every time point. The starting value of ammonium is the value of the end of the famine phase of the reactor from which the biomass was taken, fermentor 7. Since the bacteria have limited amount of ammonium, there is an immediate consumption on the early stages of the experiment. The bacteria uptake almost all present ammonium while storing polymers.

Working in a system with limited dissolved oxygen created a routine storage system for the bacteria. Since there is not enough oxygen, the polyglucose pathway is preferred due to its lower oxygen demand. Therefore, when placed in a non-limited environment, the bacteria

store the polyglucose at a higher rate than PHB. Although there was an increase of PHB in the initial values, those values were only of around 0,2 %. Only after polyglucose reached around 23 % did the PHB values started increasing at a higher rate. The DW values follow the evolution of the other variables. When both polyglucose and PHB are increasing, the DW values are also increasing and when the stored polymers are at its maximum, the DW value is maximum.

The next accumulation experiment was performed on the 25th of May. This experiment was done under dissolved oxygen limitation using the addiction of air into the reactor's headspace. The operating conditions were of 30° Celsius, 400 rpm, pH 7, 970 g of biomass, total air flow of 1,069 L/min with 0,940 L/min recycled air. The DO curve is presented in the following figure:

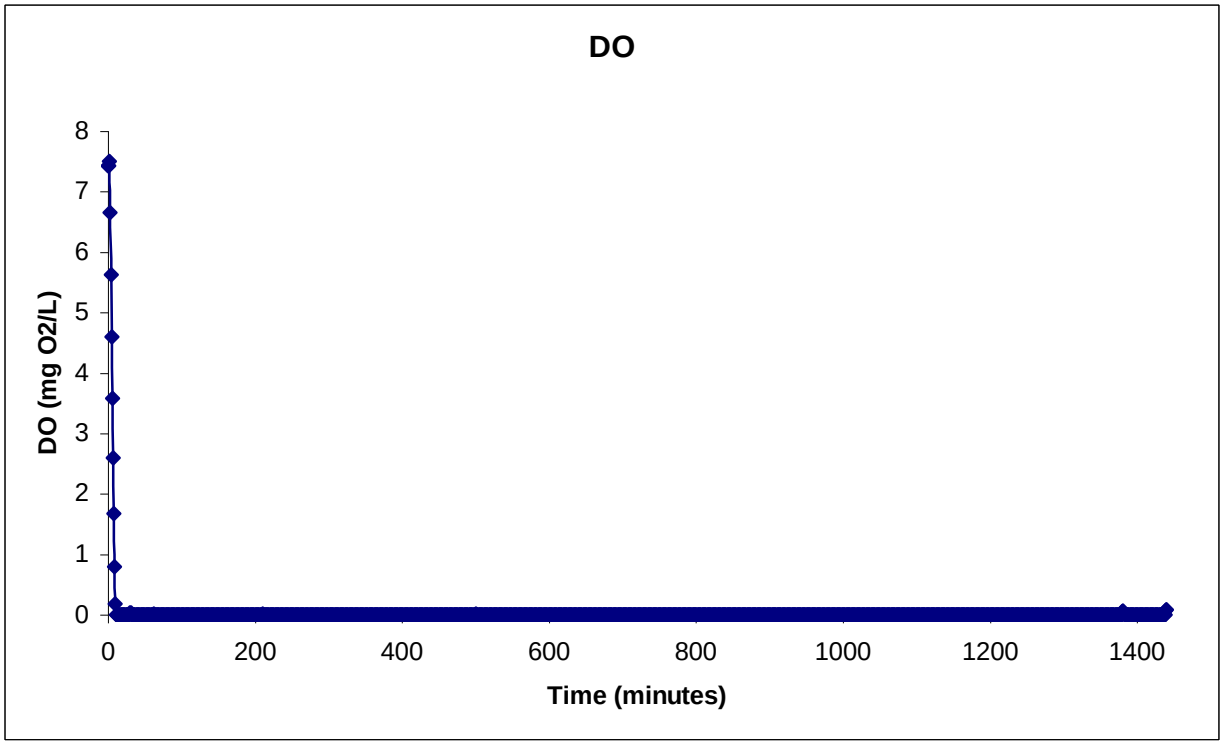

Figure 25 – DO curve for an accumulation experiment done on the 25th of May

The whole experiment is performed under dissolved oxygen as it is possible to see in the previous figure. The DO curve immediately goes to value 0 once the carbon source and medium are added and it remains as value 0 during the entire experiment since there is biomass activity during the whole experiment. The next figure represents the evolution of the different variables throughout the experiment:

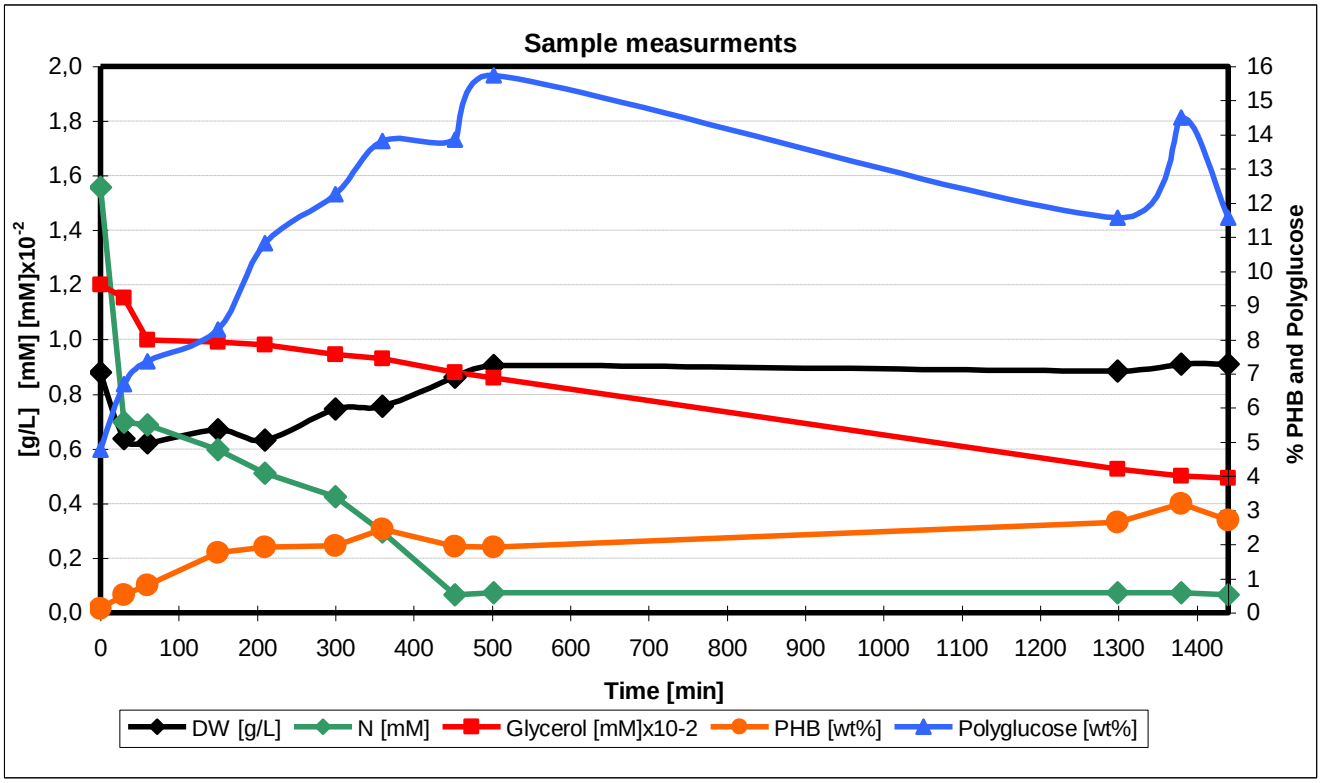

Figure 26 -Samples evolution throughout the whole experiment done on the 18th of May

By looking at the previous figure it is possible to see that the maximum PHB accumulated was around 3,2 % and the maximum polyglucose stored was around 16 %. There is a very fast consumption rate of the ammonium from the first to the second sample but after this, the rate decreases until it the ammonium value reaches approximately 0.

Comparing both accumulation experiments, the biggest differences would be in the storing values:

Table 17 – Polymer storage results for the accumulation experiments

	PHB (%)	Polyglucose (%)
No dissolved oxygen limitation	17	30
Dissolved oxygen limitation	3,2	17

It is clear that the presence of oxygen increases the storage system also in the accumulation experiments. The fact that oxygen is present on the non limited accumulation experiment means more oxidizing options, therefore, more PHB and polyglucose storage. Polyglucose remains the most stored polymer on these experiments by these bacteria. In the next tables the yields and rates of both experiments will be presented:

Table 18 – Total conversions of the materials used and produced in the accumulation experiments

Total conversions				
Experiment	**Glycerol [Cmmol]**	**PHB [Cmmol]**	**X_{active} [Cmmol]**	**Polyglucose [Cmmol]**
No dissolved oxygen limitation	229,396	26,284	51,086	34,742
Dissolved oxygen limitation	389,886	2,929	47,640	10,994

Table 19 - Total conversions of the materials used and produced in the accumulation experiments

Total conversions	
Experiment	**NH_4^+ [mmol]**
No dissolved oxygen limitation	2,121
Dissolved oxygen limitation	3,094

Table 20 – Observed yields relative to glycerol

Observed yields				
Experiment	**$Y_{PHB/Glycerol}$ [Cmmol/Cmmol]**	**$Y_{Xactive/Glycerol}$ [Cmmol/Cmmol]**	**$Y_{CO2/Glycerol}$ [Cmmol/Cmmol]**	**$Y_{Polyglucose/Glycerol}$ [Cmmol/Cmmol]**
No dissolved oxygen limitation	0,1146	0,2227	0,1852	0,1515
Dissolved oxygen limitation	0,00751	0,12219	0,0336	0,02820

Table 21 – Observed yields

Experiment	$Y_{Xactive/Polyglucose}$ [Cmmol/Cmmol]	$Y_{Xactive/PHB}$ [Cmmol/Cmmol]	$Y_{CO2/PHB}$ [Cmmol/Cmmol]	$Y_{CO2/Polyglucose}$ [Cmmol/Cmmol]
No dissolved oxygen limitation	1,4704	1,9436	1,6162	1,193
Dissolved oxygen limitation	4,333	16,266	4,479	1,193

Table 22 – Average biomass specific rates

Average biomass specific rates			
Experiment	**$q_{Glycerol}$ [Cmmol/Cmmol/h]**	**q_{PHB} [Cmmol/Cmmol/h]**	**$q_{Polyglucose}$ [Cmmol/Cmmol/h]**
No dissolved oxygen limitation	-0,1536	0,0402	0,0532
Dissolved oxygen limitation	-0,2672	0,0020	0,0216

Table 23 – Average biomass specific rates

Experiment	q_{CO2} [Cmmol/Cmmol/h]	q_{NH4+} [mmol/Cmmol/h]
No dissolved oxygen limitation	0,0650	0,0032
Dissolved oxygen limitation	0,0090	0,006560

Observing tables 18 and 19, one can observe that the total conversions of the stored polymers are higher in the non limited experiment, especially PHB. By analyzing the active biomass (X_{active}) converted values and the ammonium converted values, one can see that the values are similar since there is the same amount of ammonium present in the reactor. Now analyzing table 20, in the limited cycle experiment, one can see that the active biomass yield related to glycerol is higher than sum of the yields of PHB and polyglucose related to glycerol, which means that in this experiment, the storage percentage is lower than the growth. The non limited system has higher storage but the same amount of active biomass. In table 20 it is possible to see that the absolute value of the carbon dioxide relative to glycerol yield is higher in the non limited experiment, which means that more oxygen was consumed and therefore, higher values of stored polymers.

5.3.3 General discussion

By observing the results in the previous chapters, one can make some assumptions about the behaviour of these bacteria. The similarity of some results is quite clear. In the following table, the different parameters of each experiment will be compared and evaluated:

Table 24 – Polymer storage results from both cycle and accumulation experiments

Conditions	**PHB (%)**		**Polyglucose (%)**	
	Cycle	Accumulation	Cycle	Accumulation
No dissolved oxygen limitation	27	17	15	30
Dissolved oxygen limitation	1,87	3,2	17	17

Several conclusions can be made by looking at the previous table. Taking into account only the oxygen limitation factor, it is clear that an oxygen limited system is unfavourable for PHB storage in both cycle and accumulation experiments. The polyglucose results show that, in the cycle experiments, the oxygen limitation has no effect on the bacteria storing capacity but, in the accumulation experiments, oxygen limitation inhibits the polyglucose storage.

Using the previous conclusions and now focusing on the fact that accumulation experiments use an excess carbon source, different conditions seem to have a good influence on the storage effect in cycle and accumulation experiments. The polyglucose results are interesting since there is almost no change in the maximum percentage of polyglucose stored in the limited system, but once the system has no oxygen restrictions, the maximum value stored doubled comparing the cycle and the accumulation experiments. Once more, oxygen unavailability limits the bacteria's storage system. The PHB results show that, in the limited system, the maximum value doubled comparing the cycle and the accumulation experiments. This could be explained by the fact that there is more carbon source available, so once the maximum polyglucose is almost reached, a bigger portion of carbon source is used for the PHB production. Contrary to what was expected, the values from the non-limited system decrease from the cycle to the accumulation experiments. Once having more carbon source and oxygen available, one would assume more PHB storage, but as the results show, that is not what happens. Some of the explanations that could explain this unexpected result are the fact that the culture was unstable a few cycles before the experiment or that the oxygen availability made bacteria store more PHB than usual. Comparing these last results with the cycle experiment done in fermentor 5, it is possible to see that the stored values are very similar, so this experiment, although the *Kla* was clearly lower, had the same storage values.

Comparing the yields and rates of the cycle experiments it is possible to observe that in the limited system, the growth yield, active biomass relative to glycerol, is higher than the stored polymers yields. Although the same amount of ammonium is added, it was expected that consumption to be inferior in the limited system due to its need of oxygen. In the cycle experiment with no limitation, growth happens in the famine phase. Looking at figure 5 and at table 1, one can see that the polyglucose path uses less NAD^+ than all the following pathways, therefore, the higher percentage of the bacteria's activity should be on polyglucose storage. As we can see in the results, growth is the one taking a higher role in the biomass activity. These results are quite interesting since the biomass production also involves the use of oxygen and more NAD^+ than the polyglucose storage.

In the accumulation experiments, although the ammonium consumption and the active biomass conversions are the similar, what was expected were lower values in the limited system due to the lack of oxygen.

6 Conclusions

After all the experiments performed in this thesis, the main conclusion in this thesis is that dissolved oxygen limitation influences the bacteria storing system. By adding air into the reactor's headspace, one is decreasing the mass transfer coefficient, therefore, less oxygen is being transferred into the system. The lack of oxygen makes the bacteria store more polyglucose than PHB since the polyglucose storage pathway requires less oxygen. In a non limited system, the ratio PHB/polyglucose would be preferred but due to the oxygen limitation studied in this thesis, the polyglucose/PHB ratio is higher.

After analyzing both cycle and accumulation experiments, it is possible to conclude that in a limited system, the excess of carbon source only affects positively the PHB storage since it doesn't have any alterations in the maximum polyglucose stored. In a non limited system, the excess of carbon source results in an inhibition of stored PHB while the polyglucose storage is enhanced. The maximum polyglucose and PHB stored in an experiment with dissolved oxygen limitation was 17 % and 3,2 % of the bacteria's dry weight.

Using the yields and conversions values of all the experiments, one can conclude that the presence of oxygen leads to storage and the lack of oxygen leads to growth. In the experiments with oxygen limitations, the active biomass yield was higher than the polymers yields. The lack of oxygen would give the idea tha polyglucose should be the main pathway for the bacteria's activity but growth also happened aong side with PHB storage. For the cycle experiments with no oxygen limitation, although growth happens during the feast phase, the higher consumption of ammonium in the famine phase leads to a higher growth percentage in the famine phase.

One can conclude that oxygen is the key factor in the PHA production by bacteria. In this thesis it was proven that the limitation of this molecule influences negatively the storage of PHAs and favours the bacteria's growth.

7 Review of the performed work

7.1 Accomplished objectives

Lowering the stirring speed and adding air into the reactor's air flow has proven to be successful in achieving an oxygen limitation system.

Oxygen is a key component on the bacteria storage system, especially for PHB storage. The lack of oxygen leads to a big decrease in the PHB storage. When performing a cycle or accumulation experiment, the presence of extra carbon source has positive effects on the polyglucose storage and negative effects on PHB storage.

Dissolved oxygen limitation has proven to enhance the polyglucose/PHB ratio but it also enhances the bacteria growth. In both cycle and accumulation experiments performing in limited conditions, the growth yields were higher than the sum of the polymers yields. Glycerol was not only uptake for storage but also for growth.

7.2 Bottlenecks and future work

The biggest bottleneck in this thesis is time. Every experiment takes a lot of time until all samples are prepared and analyzed. Also, any unforeseen mistake or change in the reactor's conditions affects the outcome of the whole experiment and forces the repetition and more time spent. The unpredictability of the stability of the biomass in the enrichment culture reactor also conditioned the timing of the future experiments, delaying some scheduled experiments.

For future work, more experiments are needed to have more matching results. Also using another electron receiver with lower oxidizing power would be a good idea. Limiting one of the medium components to prevent growth could be an idea for the future. Growth has been studied by limitation of ammonium but after analyzing the work performed in this thesis, oxygen limitation enhances growth so different oxygen limiting conditions should be studied to see the impact on the biomass growth.

7.3 Final remark

Using the conditions studied on this thesis, an industrial scale production wouldn't have a lot of interest since there is more growth than stored polymers. Glycerol is a good substrate since this process could be integrated as an end-of-line process in the soap production industry and also in a biodiesel industry that produces glycerol as by-product. In fact, its contribution is higher that the soap industry.

8 Appendix

8.1 **Appendix A –** Medium content

Table 25 – Medium for culture enrichment during 6 weeks

	Compound	Carbon source	Nutrients	Demi Water
Carbon source	Glycerol	70,950 g/5L		
Nutrients	NH_4CL KH_2PO_4 $MgSO_4.7H2O$ KCL Trace elements solution ATU (33g/L) after autokl.!		18,050 g/5L 16,950 g/5L 6,850 g/5L 2,650 g/5L 75 mL 75 mL	
Dilution water	Demi water			To 5 L

Table 26 - Trace Elements Composition[#] (Vishniac and Santer 1957)

Compound	Concentration (g/L)
EDTA Titriplex III	63,69
$ZnSO_4.7H_2O$	22
$CoCl_2.6H_2O$	1,61
$MnCl_2.4H_2O$	5,06
$CuSO_4.5H_2O$	1,51
$FeSO_4.7H_2O$	2,99
$(NH_4)_6Mo_7O_{24}.4H_2O$	1,10
$CaCl_2.H_2O^+$	7,34

[#] pH adjusted to 6 with KOH

[+]Mw = 111.1 g/mol

Table 27 – Medium for cycle experiment

	Compound	Carbon source	Nutrients	Demi Water
Carbon source	Glycerol	1,419 g/5L		
Nutrients	NH_4CL KH_2PO_4 $MgSO_4.7H2O$ KCL Trace elements solution ATU (33g/L) after autokl.!		0,361 g/5L 0,339 g/5L 0,137 g/5L 0,053 g/5L 1,5 mL 0,15 mL	
Dilution water	Demi water			To 1 L

For the accumulation experiment, the nutrients feedstock composition has the same composition except that **no ammonium chloride is added**. The carbon source composition is 10 g/L

8.2 Appendix B – Solid retention time (SRT)

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

$$SRT = \frac{TSS^R\left(t_{end}\right).V_L^R}{TSS^{Sludge}.V^{Sludge} + TSS^{Effl}.V^{Effl}} t_{cycle} \quad (8.1)$$

$$TSS^R(t_{end}) = TSS^{sludge} = TSS^{Effl} \quad \text{and} \quad V^{Sludge} = 0 \quad (8.2)$$

8.3 **Appendix C –** Dilution factor

For all the experiments, the dilution factor was used on all the concentrations to correct the dilution factor. To correct them, all concentrations were multiplied by $f_{L,i}$.

$$f_{L,t(i)} = 1 + \frac{((V_{acid,t(i)} - V_{acid,t(i-1)}) + (V_{cum\,dil,t(i)} - V_{cum\,dil,t(i-1)}) + (V_{base,t(i)} - V_{base,t(i-1)}))}{(V^{R}_{L\,true,t(i)} - V_{st,t(i)})} \quad (8.3)$$

For the initial dilution factors, the following formula was used:

$$f_{L,i} = \frac{V^{R}_{L\,true,t(i)}}{(V^{R}_{L\,true,t(i)} - V_{acid,t(i)} - V_{cum\,dil,t(i)} - V_{base,t(i)})} \quad (8.4)$$

After minute 3, the dilution factor formula was changed to the following:

$$f_{L,i} = f_{L,i-1}.f_{L,t(i)} \quad (8.5)$$

8.4 **Appendix D –** Yields and rates

The yield formula for component A related to component B is:

$$Y_{A/B} = \frac{\text{Total Conversion}_A}{\text{Total Conversion}_B} \tag{8.6}$$

The rates formula for the cycle experiments is the following:

$$q_{component} = \frac{\text{Total conversion}_A}{\frac{\text{Average biomass}}{t_{feast\ phase}}} \tag{8.7}$$

The rates formula for the accumulation experiments is the following:

$$q_{component} = \frac{\text{Total conversion}_A}{\frac{\text{Average biomass}}{t}} \tag{8.8}$$

9 References

- Belo, I. and M. Mota (1998). “Batch and fed-batch cultures of E. coli TB1 at different oxygen transfer rates: Effect of stirring and oxygen partial pressures on cell growth and cytochrome b5 production " Bioprocess and Biosystems Engineering **18**(6): 451-455.

- Beun, J. J., K. Dircks, et al. (2002). "Poly-beta-hydroxybutyrate metabolism in dynamically fed mixed microbial cultures." Water Research **36**(5): 1167-1180.

- Dias, J., P. Lemos, et al. (2006). "Recent Advances in Polyhydroxyalkanoate Production by Mixed Aerobic Cultures: From the Substrate to the Final Product." **6**(11): 885-906.

- Garcia-Ochoa, F. and E. Gomez (2009). "Bioreactor scale-up and oxygen transfer rate in microbial processes: An overview." Biotechnology Advances **27**(2): 153-176.

- Henderson, R. A. and C. W. Jones (1997). Physiology of poly-3-hydroxybutyrate (PHB) production by Alcaligenes eutrophus growing in continuous culture. **143:** 2361-2371.

- Johnson, K. (2010). PHA Production in Aerobic Mixed Microbial Cultures. Biotechnology. Delft, TU Delft. **PhD**.

- Katie, A. T., N. Mark, et al. (2003). The effect of dissolved oxygen on PHB accumulation in activated sludge cultures. **82:** 238-250.

- Kim, B. S. (2000). "Production of poly(3-hydroxybutyrate) from inexpensive substrates." Enzyme and Microbial Technology **27**(10): 774-777.

- Kleerebezem, R. and M. C. M. van Loosdrecht (2007). "Mixed culture biotechnology for bioenergy production." Current Opinion in Biotechnology **18**(3): 207-212.

- Kumar, A., P. Ghosh, et al. (1989). "Biosynthesis of Bacterial Glycogen." The Journal of Biological Chemestry **264**(18): 10464-10471.

- Lemoigne, M. (1926). "Produits de dehydration et de polymerisation de l’acide ß-oxobutyrique." Bull Soc Chim Biol 8: 770–782.

- Patnaik, P. R. (2009). "Cognitive optimization of microbial PHB production in an optimally dispersed bioreactor by single and mixed cultures " Bioprocess and Biosystems Engineering **32**(4): 557-568.

- Puthli, M. S., V. K. Rathod, et al. (2005). "Gas-liquid mass transfer studies with triple impeller system on a laboratory scale bioreactor." Biochemical Engineering Journal **23**(1): 25-30.

- Radtke, D. B., A. F. White, et al. (1998). "Dissolved Oxygen." (U.S. Geological Survey TWRI).

- Slater, S., W. H. Voige, et al. (1988). "Cloning and expression in Escherichia coli of the Alcaligenes eutrophus H16 poly-beta-hydroxybutyrate biosynthetic pathway." Journal of Bacteriology **170**(12): 4431-4436.

- Steinbüchel, A. (2001). "Perspectives for biotechnological production and utilization of biopolymers: Metabolic engineering of polyhydroxyalkanoate biosynthesis pathways as a successful example." Macromolecular Bioscience **1**(1): 1-24.

- Strange, R. E. (1968). "Bacterial "Glycogen" and Survival." Nature **220**(5167): 606-607.

- Taras, M. J., A. E. Greenberg, et al. (1971). "Standard Methods for the Examination of Water and Wastewater." New York: American Public Health Association.

- Verlinden, R. A. J., D. J. Hill, et al. (2007). Bacterial synthesis of biodegradable polyhydroxyalkanoates. **102:** 1437-1449.

- Vishniac, W. and M. Santer (1957). "The thiobacilli." Bacteriological Reviews **21**(3): 195-213.

- Wältermann, M. and A. Steinbüchel (2005). "Neutral lipid bodies in prokaryotes: recent insights into structure, formation, and relationship to eukaryotic lipid depots." Journal of Bacteriology **98**(8): 1690-1693.